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Plant Peptide Hormones and Growth Factors

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Plant Peptide Hormones and Growth Factors

Edited by

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Preface

In addition to classical phytohormones, peptides are now recognized as a novel class of signaling molecules that play important roles as peptide hormones and growth factors in plant development, as well as in plant interactions with their biotic and abiotic environment. There has been tremendous progress in this field in recent years, leading to the identification of numerous new signaling peptides and to the elucidation of peptide perception and signal transduction mechanisms. These advancements were made possible through the development of methods suitable for peptide identification and characterization. A collection of these protocols, written by leading experts in the field, is presented in this volume of the Methods in Molecular Biology series on *Plant Peptide Hormones and Growth Factors.*

Part I of this book presents protocols for computational identification of novel signaling peptides and phytocytokines, for analyzing post-translational peptide maturation events, and for isolation of mature peptides from plant tissues. Part II focuses on peptide bioactivity, providing a collection of in vivo bioassays, as well as protocols for in vitro analysis of peptide signaling. Part III concentrates on peptide-receptor interactions, including protocols for identifying unknown binding partners and for quantitative binding analysis of peptide ligands to cognate receptors.

This book describes state-of-the-art approaches for identification and functional characterization of plant peptide hormones and growth factors. It is intended as a reference source that will be instrumental for the continued development of the highly dynamic plant peptide signaling field.

Stuttgart, Germany *Andreas Schaller*

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Part I

Peptide Prediction, Formation and Isolation



Chapter 1

Bioinformatics Methods for Prediction of Gene Families Encoding Extracellular Peptides

Loup Tran Van Canh and Sébastien Aubourg

Abstract

Genes encoding small secreted peptides are widely distributed among plant genomes but their detection and annotation remains challenging. The bioinformatics protocol described here aims to identify as exhaustively as possible secreted peptide precursors belonging to a family of interest. First, homology searches are performed at the protein and genome levels. Next, multiple sequence alignments and predictions of a secretion signal are used to define a set of homologous proteins sharing features of secreted peptide precursors. These protein sequences are then used as input of motif detection and profile-based tools to build representative matrices and profiles that are used iteratively as guides to scan again the proteome and genome until family completion.

Key words Phytocytokine, Conserved motif, Secretion signal, Data mining, SCOOP, PIP

1 Introduction

Small secreted peptides (SSPs) are important players in the extracellular space of plants and are known to regulate a large diversity of biological processes. In the last decade, the increasing number of publications describing such peptides has revealed an unexpected diversity of structures and biological functions. Their actions range from antimicrobial to signaling properties, controlling development, growth, reproduction, and defense against biotic and abiotic stresses [1]. In most cases the precursors of these extracellular peptides, named prepropeptides, possess a signal sequence in their N-terminal part, directing them to the endoplasmic reticulum for vesicle-based transport out of the cell into the apoplast. The secreted peptides include phytocytokines that are recognized by transmembrane receptors to trigger signal transduction and immune responses [2, 3]. On the basis of structural features and their mode of maturation, SSPs have been classified into two main groups by Matsubayashi [4]: The post-translationally modified

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peptides (PTMPs) and the cysteine-rich peptides (CRPs). The maturation of PTMPs involves frequent proline hydroxylation (often followed by arabinosylation) and tyrosine sulfation, as well as the proteolytic action of one or several proteases to release the final short functional peptides [5]. A large majority of phytocytokines belong to this class. The CRPs are characterized by an even number of cysteines involved in disulfide bridges defining the final structure of the bioactive peptides.

Bioinformatics approaches for structural annotation of genes encoding precursors of SSPs are limited by their small size and the low level of sequence conservation of the coding regions, thus impairing their detection along genomic sequences by hidden Markov models and similarity searches. Furthermore, functional annotation of SSPs, mainly based on function inference by similarity, also suffers from very low conservation levels, especially for PTMPs for which the conserved sequences are restricted to the short region encoding the mature peptide. These particular features and the limited sensitivity of classical detection methods have slowed down the correct prediction of genes encoding secreted peptides and their correct clustering and classification into gene families [6]. Even within well-known peptide families in species with frequently updated whole genome annotation, new members are still being discovered, and characterizing the full scope of the families requires careful analyses and expert assessments [7, 8].

The only common features that can be used for the prediction of genes encoding extracellular peptides are small size (usually less than 200 amino acids (aa) for the encoded protein) and the presence of a sequence coding for an N-terminal secretion signal [9, 10]. Beyond these simple filters, the annotation of PTMP precursors requires detection and definition of the protein motif corresponding to the mature functional peptide. This key step relies on the sequence conservation of such motifs and, therefore, on the identification of homologous proteins. The protocol that we propose aims to detect potentially related precursors of secreted peptides starting from a single candidate sequence using a highly supervised strategy. Proceeding step by step, through detection of homologs, prediction of secretion signal, and search of shared motifs, the iterative process aims to retrieve lowly conserved SSP sequences until family completion. We also examine some atypical situations that require special expertise to correct automatic annotation errors or ambiguous detection of signal peptides and provide guidelines to predict shared motifs.

To illustrate this protocol, we applied it in two different contexts: the identification of the PAMP-Induced secreted Peptide (PIP) family in *Solanum lycopersicum*, and the extension of the Serine-riCh endOgenOus Peptides (SCOOP) family in *Arabidopsis thaliana*. These two PTMP families represent distinct situations with medium and low levels of sequence conservation between

homologs, respectively. The PIP/PIP-like family is well described and comprises 11 genes in *A. thaliana* [11]. Our protocol identified 19 putative homologs in *S. lycopersicum*, only four of which were previously reported [12]. The *Brassicaceae*-specific SCOOP family has first been described as a 14-membered gene family in *A. thaliana* [13]. However, new sequence analyses with lower stringencies have recently extended and questioned the scope of the family [14, 15]. The exploration of the Arabidopsis genome and proteome using the protocol described here highlights 48 putative members. This includes seven genes annotated as non-coding RNA genes in Araport11, two other genes for which the position of the start codon had to be corrected, and two previously unpredicted genes. The results support our belief that the plurality of tools combined with a thorough curated analysis of each detected sequence make our approach sensitive and reliable.

2 Materials

2.1 Omic Datasets

This protocol requires genome and proteome datasets for each investigated species. For the proposed examples, datasets of the latest *A. thaliana* (Col-0) and *S. lycopersicum* (cv. Heinz 1706) genomes and their gene/protein annotations (FASTA and GFF files) were downloaded from TAIR (https://www.arabidopsis.org; Araport11) and Phytozome (https://phytozome-next.jgi.doe.gov; ITAG4.0), respectively. In theory, a file containing the protein sequences deduced from the structural annotation process of the whole genome would be sufficient to detect genes encoding secreted peptides. However, jointly exploring the whole genome sequence allows us to ease our dependence on the gene prediction process that is known to be poorly effective for these types of genes. In this way, we can detect candidate genes which are underpredicted or erroneously annotated as either pseudogenes or non-coding RNA genes. As an alternative method, if the genomic sequence is partial or of poor quality (low sequence coverage), a de novo transcriptome assembly can be used as nucleic acid sequence input.

2.2 Starting Sequences

At least one protein sequence of a putative secreted peptide precursor is required as input to start the workflow. As starting sequences, we used two datasets, the first composed of the *A. thaliana* precursors of three PIP and eight PIPL (PIP-like) proteins according to Vie et al. [11], and the second being the *A. thaliana* PROSCOOP12 protein, precursor of the predicted SCOOP12 peptide [13]. Beyond these examples, any small protein exhibiting an N-terminal signal peptide may be chosen as an interesting candidate to start the proposed workflow (*see* Subheading 3.2.2 for prediction of such candidates). With a cut-off size of 300 aa, the

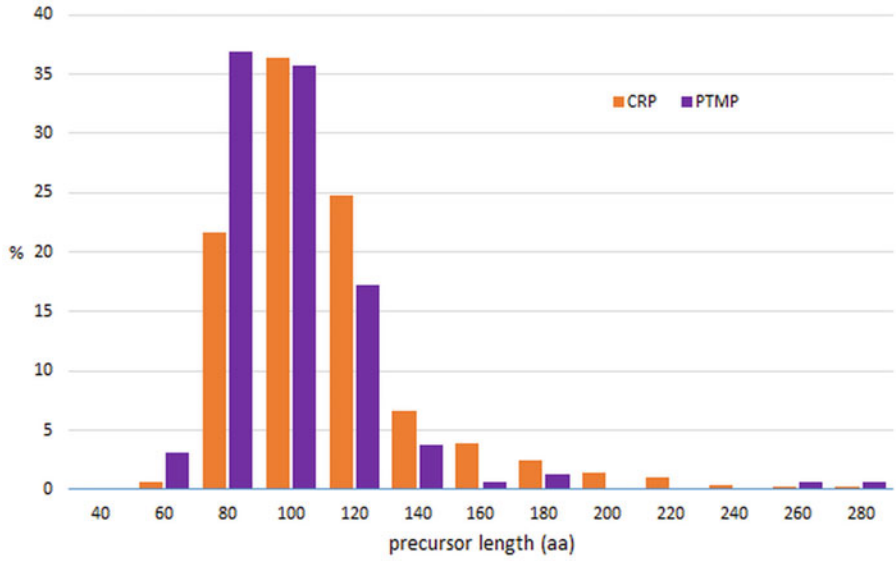


Fig. 1 Distribution of SSP precursor lengths (in aa). Data are based on 672 CRP (Cystein-Rich Peptide) and 157 PTMP (Post-Translationally Modified Peptide) precursors previously described in *A. thaliana*. Their average size is 103 and 90 aa, respectively

diversity of currently known peptide precursors characteristics is covered (Fig. 1).

2.3 Operating System, Hardware, and Software Requirements

All the software used in this protocol are run through a web browser or shell commands on a Debian GNU/Linux 11 bullseye (×86–64) Operating System, but should work under other Unix systems (e.g., MacOs) as well. All software must be available in the executive path. When available, web-based alternative versions are mentioned.

Hardware requirements depend on the size of the dataset. Here we used an Intel© Xeon© CPU E3–1240 v5 @ 3.50GHz × 4; 15.6 Go RAM.

All the tools used to investigate secreted peptide families in this protocol are freely available. The *BLAST+ v2.11.0* package (https://ftp.ncbi.nlm.nih.gov/blast/executables/LATEST/) comprising *makeblastdb*, *blastp*, and *tblastn* is used to retrieve sequences presenting local similarities. Motif predictions and iterative searches are performed using *HMMER v3.3.2* (https://github.com/EddyRivasLab/hmmer) tools (*HMMbuild*, *HMMsearch*, *jackhmmer*) and *MEME suite 5.4.1* (https://meme-suite.org/meme/index.html) including *MEME*, *MAST*, *GLAM2*, and *GLAM2SCAN*. Multiple alignments are performed using *MUSCLE v3.8.1551* (https://github.com/rcedgar/muscle) and displayed using *AliView v1.28* (https://github.com/AliView/AliView). Predictions of signal peptides, topological features, and cellular localization of proteins are performed using *SignalP v5.0*

(https://services.healthtech.dtu.dk/service.php?SignalP-5.0), *DeepLoc v2.0* (https://services.healthtech.dtu.dk/service.php?DeepLoc-2.0), *DeepTMHMM v1.0.12* (https://dtu.biolib.com/DeepTMHMM), and *Predotar v1.04* (https://urgi.versailles.inra.fr/predotar/). The manual annotation step is facilitated by the use of *ORFfinder v0.4.3* (https://ftp.ncbi.nlm.nih.gov/genomes/TOOLS/ORFfinder/linux-i64/), *Netgene2 v2.42* (https://services.healthtech.dtu.dk/service.php?NetGene2-2.42), and *Artemis v18.0.0* (http://sanger-pathogens.github.io/Artemis/Artemis/).

3 Methods

The protocol is divided into three main parts (Fig. 2). The first part (Subheading 3.1) aims to detect sequences similar to the starting sequence(s); the second part (Subheading 3.2) integrates sequence analyses for inspection and selection of candidate peptide precursors; the last part (Subheading 3.3) consists in building position weight matrix (PWM) and/or hidden Markov models (HMM) as signature sequences for peptide families that represent the variability of the selected sequences and allow to re-screen sequence libraries through an iterative process. This workflow is reinforced by control and inspection steps ensuring the quality of the results of each part. In our examples, we apply it in order to explore (i) the *S. lycopersicum* genome to identify the PIP/PIPL gene family from the known Arabidopsis members, and (ii) the Arabidopsis genome to identify highly divergent PROSCOOP12 homologs.

3.1 Homolog Search

This protocol aims to detect and retrieve a maximum of sequences similar to the starting sequence(s) of interest (i.e., putative homologs). Since the secreted peptide genes are relatively short and poorly conserved, this search targets not only the annotated protein database but also genome sequences to bypass annotation errors. Similarities are observed at the protein level for higher sensitivity. Therefore, the tools blastp and jackhmmer are used to scan the proteome, and tblastn is applied to scan nucleic sequences (genome if available, transcriptome assembly if not). Albeit slower, jackhmmer has the advantage to run efficient iterative searches [16].

Hereafter, <protein_query_file> is the file containing the starting protein sequence of interest, <proteome_file> contains all the protein sequences deduced from the whole genome annotation, and < genome_file> contains the genomic sequences (Araport11 or ITAG4.0 in our study case). All these files must be in FASTA format.

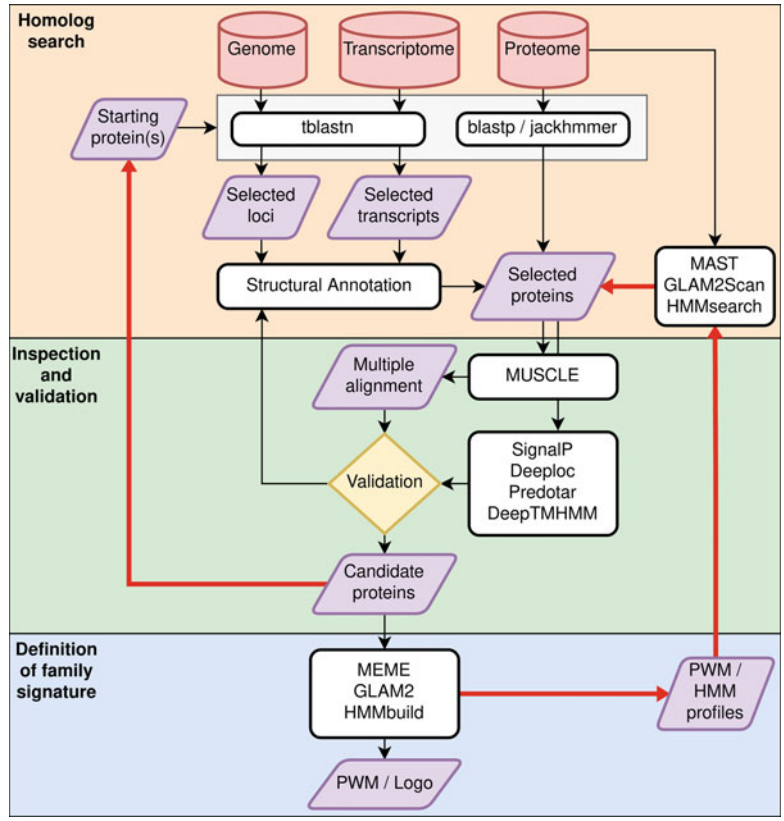


Fig. 2 Protocol for the definition of gene families encoding putative secreted peptide precursors. Software and files are represented by white and purple shapes, respectively. Red arrows illustrate the iterative parts of the method

3.1.1 Commands and Parameters

Strictly following the proposed parameters ensures a search of high sensitivity but reduces its specificity, thus requiring more hands-on expertise to eliminate false positives, especially in the first and second parts of the workflow. Parameters should be adapted to each situation accordingly.

Prior to the use of blastp and tblastn (BLAST+ package) [17], set up a database using makeblastdb:

```
> makeblastdb -dbtype prot -in <proteome_file> -out <proteo-
me_db>
```

where <proteome_db > is the name of the protein database defined by the user.

```
> makeblastdb -dbtype nucl -in <genome_file> -out <genome_db>
```

where <genome_db > is the name of the nucleic database defined by the user.

Run blastp with the following command:

```
> blastp -query <protein_query_file> -db <proteome_db> -evalue
10 -outfmt 0 -out <output_file>
```

The local alignments of the proteins to the proteome are displayed in the defined output file. Start by setting the *-evalue* at 10 to ensure a low selectivity, then, after inspection of the results, lower it to increase the stringency and reduce non-significant alignments in the next runs. Use the *-outfmt 0* option to format results as detailed pairwise sequence alignments to facilitate examination and eventually to remove false positives.

Run tblastn with the following command:

```
> tblastn -query <protein_query_file> -db <genome_db> -evalue
10 -outfmt 0 -out <output_file>
```

The local alignments of the proteins to the 6 frames-translated genome are displayed in the defined output file (*see* **Note 1**).

To perform an iterative protein search, use jackhammer, either with the web-based version (https://www.ebi.ac.uk/Tools/hmmer/search/jackhmmer), or with the following command (the order of arguments must be respected):

```
>jackhmmer -o <output_file> <protein_query_file> <proteome_-
file>
```

The local alignments with the detected similar proteins are displayed in the output file for each iteration. The advantage of this method is that jackhmmer builds a HMM profile after each iteration to improve the following one. We recommend to start with default settings (low threshold and a maximum of 5 iterations) to avoid getting excessively noisy results.

3.1.2 Result Integration and Gene (Re)annotation

The homolog search provides a list of predicted proteins similar to the starting sequences (blastp and jackhmmer results). This list must be completed by adding the results of tblastn which provides hit positions relative to genome sequences tagging candidate regions. For this purpose, it is necessary to identify only the genomic regions for which no gene/protein has been predicted (comparison of hit positions and GFF files describing the position of all annotated genes, *see* **Note 2**). These selected regions probably contain genes of interest that were missed or considered as non-coding RNA genes by gene predictors (false negatives of the whole genome automatic annotation) and should be analyzed manually (*see* **Note 3**).

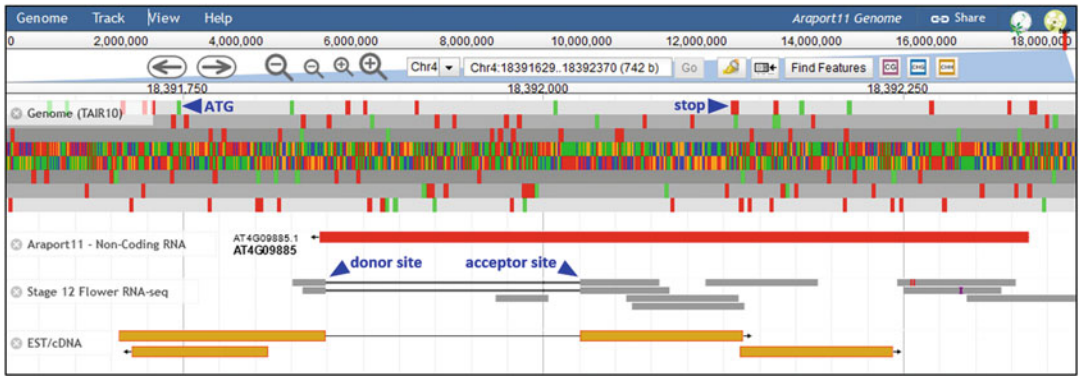


Fig. 3 Screenshot of a genome browser showing the integration of transcript sequences for manual (re)-annotation of a locus of interest. In this example (JBrowse at TAIR, https:/www.arabidopsis.org/), PROSCOOP similarities detected with tblastn partially overlap a non-coding RNA gene predicted in Araport11 (AT4G09885, red track). The display of mapped transcript sequences (RNA-seq reads in grey and EST/cDNA in orange) highlights the presence of an intron. The joint consideration of the 6-frames translated genomic sequences allows the selection of a start codon compatible with both the intron position and the conserved ORF. If several ATG codons have the right properties, signal peptide prediction for each possible N-terminal sequence can be used to select the most likely ATG (*see* Subheading 3.2.2). Start/stop codons and splicing sites selected to predict the final gene/CDS structure are indicated in blue in this example

This (re)annotation aims to predict the correct intron-exon structure and the coding region (CDS) of the gene. To do this correctly, take advantage of available RNA-seq resources. Genome browsers that aggregate and display such resources (i.e., JBrowse at TAIR/Phytozome, Ensembl Plants, or Artemis) are powerful for manual annotation (Fig. 3). If no transcript data are available to guide intron-exon structure annotation, de novo splice sites prediction of the concerned regions can be achieved using software such as NetGene2 [18]. Once the predicted transcript/CDS is recovered (after in silico intron splicing), use ORFfinder to check the integrity of the Open Reading Frame (ORF) and obtain the corresponding translated protein (*see* **Note 4**). Use ORFfinder online (https://www.ncbi.nlm.nih.gov/orffinder/) or with the following command:

```
> ORFfinder -in <predicted_transcript_file.fasta> -strand both
-out <output_file>
```

The protein sequences deduced from each ORF are generated in the defined output file. Select those containing the conserved region(s) previously detected by tblastn.

3.2 Inspection and Validation of the Gene Family

The second part of this protocol uses a FASTA file containing all previously selected homologous proteins (named <selected_proteins_file> hereafter). It contains proteins tagged by blastp and jackhmmer (after removing redundancy) in the whole annotated proteome and those resulting from the (re)annotation tasks.

3.2.1 Multiple Sequence Alignment

The visualization of all the aligned proteins helps to decide which proteins are relevant and which are not. Indeed, the multiple sequence alignment allows the user to consider similarities at the gene family scale and not only locally between two sequences, facilitating the examination of the selected proteins. If false-positive proteins were selected during the search for homologs (Subheading 3.1), they will appear as aberrant in the result of the multiple sequence alignment and should be removed.

For the multiple sequence alignment, run MUSCLE [19] using this command:

```
> muscle -in <selected_proteins_file> -out <alignment_file>
```

where <alignment_file> contains the multiple sequence alignment in aln format.

Because the SSP precursors are often poorly conserved, the multiple alignment may need to be improved locally and manually [20]. Graphical application such as AliView [21] can be used to visualize and edit the multiple alignment file using the following command:

```
> aliview <alignment_file>
```

The multiple sequence alignment provides a first visual overview of the conserved regions(s) shared by the selected proteins. The analysis of this result allows to detect and to remove dissimilar and too divergent proteins wrongly selected at the previous stage. In addition to doubtful similarities, protein length greater than 300 aa (Fig. 1) can be a filtering criterion but it should be employed with caution. Indeed, an unusual protein size (compared to the homologs) can result from errors in the genome annotation pipeline (e.g., erroneous gene structure, gene merging...). Therefore, manual (re)annotation of the respective locus (Subheading 3.1.2) is recommended before eliminating the sequence.

Any modification of the selected protein list requires a new multiple sequence alignment using MUSCLE.

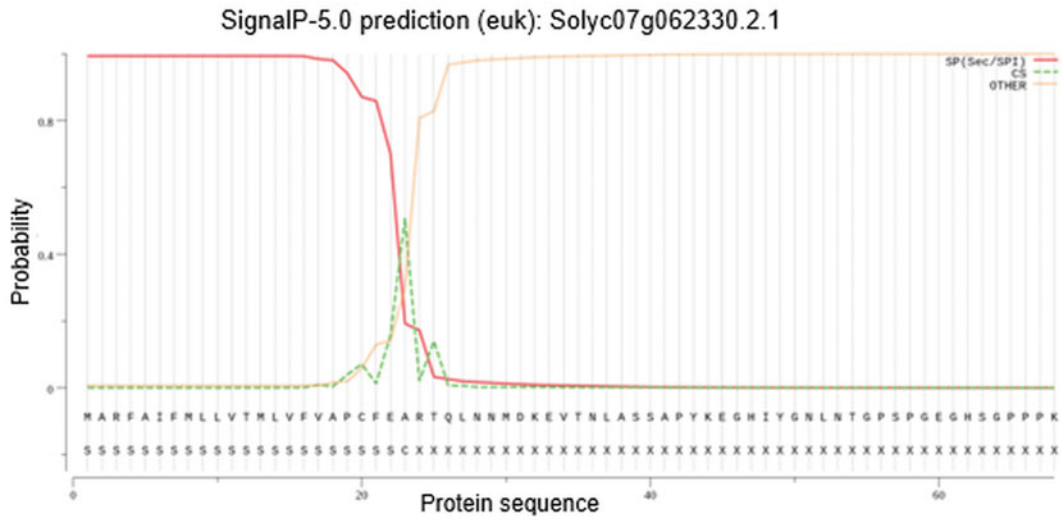
3.2.2 Signal Peptide Prediction

The main feature shared by all SSP precursors, with only rare exceptions such as the PEP family [22], is the presence of an N-terminal signal peptide (SP) addressing them to the endoplasmic reticulum for secretion into the extracellular space. Several

a

```
# SignalP-5.0 Organism: euk
# ID                      Prediction   SP(Sec/SPI)  OTHER     CS Position
Solyc02g090610.1.1        SP(Sec/SPI)  0.763568     0.236432  27-28 IEG-RH
Solyc07g062330.2.1        SP(Sec/SPI)  0.993543     0.006457  23-24 FEA-RT
Solyc02g090590.1.1        SP(Sec/SPI)  0.947082     0.052918  23-24 SQG-RN
Solyc03g044530.1.1        OTHER        0.433999     0.566001
Solyc02g090600.2.1        OTHER        0.010717     0.989283
Solyc02g090600.2.1_newATG SP(Sec/SPI)  0.984710     0.015290  24-25 AEG-RQ
```

b



c

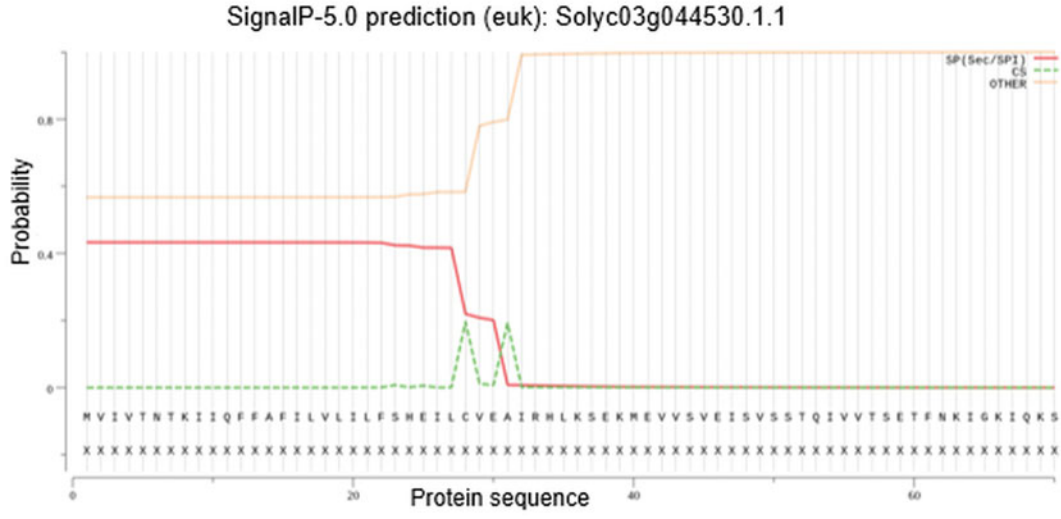


Fig. 4 Example of SignalP5.0 results obtained for *S. lycopersicum* proteins similar to *A. thaliana* PIP/PIPL precursors. (**a**) Tabulated results describing the SIGNALP5.0 predictions. Input sequence names are listed in the first column, associated prediction is indicated in the second column, "SP" corresponds to secreted peptide, whereas "OTHER" indicates that the peptide is not secreted; associated probabilities are displayed in the third and fourth columns, respectively; the fifth column indicates the predicted cleavage site position, its

complementary tools are used to predict the secretion signals of the considered protein sequences.

SignalP5.0 is a reference [23] and should be used first, via its web-based version (https://services.healthtech.dtu.dk/service.php?SignalP-5.0). Alternatively, run it locally with the following command:

```
> signalp -fasta <selected_proteins_file> -format long -mature
```

The optional parameter *-mature* produces a FASTA file containing exclusively the protein sequences lacking the predicted SPs. The option *-format long* generates graphs in png format relevant to assess the predictions (Fig. 4).

To finalize this step, submit the protein sequences for which SignalP5.0 gave unclear results to alternative tools. We propose to use DeepLoc2.0 [24], DeepTMHMM [25], and Predotar [26] that differ in sensitivity. Use DeepLoc2.0 through the web-based application (https://services.healthtech.dtu.dk/service.php?DeepLoc-2.0) or call it using the following command:

```
> deeploc2 -f <selected_proteins_file> -o <output_file> -p -m
Accurate
```

Results are summarized in the defined output file and are completed with graphs if the option *-p* is used. The argument *-m Accurate* uses a high-quality model instead of the default fast high-throughput model.

DeepTMHMM is available online at https://dtu.biolib.com/DeepTMHMM where the <selected_proteins_file> can be submitted as input. Results detail the position of SP and transmembrane segments (*see* **Note 5**). Predotar is available on a web server at https://urgi.versailles.inra.fr/predotar/ for the prediction of subcellular localization. These tools have similar objectives but differ in their algorithm, settings, and training set. In some situations, they give slightly different results and are therefore complementary (see for example Fig. 4c).

The absence of an expected predicted SP should primarily question the protein annotation quality. Indeed, the selection of a wrong start codon can mask the presence of an SP. Therefore, it is necessary to check alternative upstream or downstream start codon

Fig. 4 (continued) 3 upstream and 2 downstream residues, and its associated probability. (**b**) Graphical output corresponding to the N-terminus of the protein Solyc07g062330 for which a clear SP (score 0.99, position 1–23) has been predicted. (**c**) SignalP5.0 concludes that there is no SP in Solyc03g044530 but the graphical output relativizes this conclusion as its N-terminus has SP properties with unclear cleavage site around the 30th aa. For this protein, DeepLoc2.0 predicts extracellular localization, DeepTMHMM predicts an SP (region 1–31), and Predotar localization in the endoplasmic reticulum

(s) in the same reading frame and if present, to test again the SP prediction with the modified protein sequence(s). The protein Solyc02g090600 (putative PIPL) illustrates this situation (Fig. 4a): no peptide signal is detected with the initial protein (ITAG4.0, 173 aa) but the selection of a downstream start codon results in the identification of a new shorter protein of 145 aa with a clear SP (SignalP5.0 score of 0.98).

Finally, proteins for which the absence of an SP is confirmed and for which similarities with the starting protein are doubtful should be removed from the selection (*see* **Notes 5** and **6**).

3.3 Definition of a Family Signature

The third part of this protocol focuses on the characterization of a signature sequence specific to the studied SSP precursor family. Because all SP sequences have similar features (stretch of hydrophobic residues, mainly Leucine) shared by almost all the secreted proteins, we strongly advise you to generate a new file (in FASTA format) containing the sequences of the previously selected homologous proteins excluding the predicted signal peptides (Subheading 3.2.2). This file is named <selected_proteins_withoutSP_file> in the following steps.

3.3.1 Motif and Logo Construction

The definition of conserved motifs, which may correspond to the mature secreted peptides, in a set of sequences can be performed with the MEME tool from the MEME suite v5.4.1 [27]. MEME has the advantage of searching motifs on unaligned sequences and therefore of detecting a variable number of motifs on each input sequence. This may be of interest since precursor proteins may be processed into different SSPs, as described for some members of PTMP families [9, 11, 28, 29]. Use MEME as a web-based application (https://meme-suite.org/meme/tools/meme) or locally with the following command:

```
> meme <selected_proteins_withoutSP_file> -o <output_folder>
-minw <min> -maxw <max> -nmotifs <nmotifs>
```

All results (XML, html, png, and txt files) are saved in the output folder defined by the user. By default, the size of the searched motif is between 8 and 50 aa, but you can adjust it with the *-minw* and *-maxw* parameters according to the first results and the previous multiple sequence alignment (Subheading 3.2.1). If short conserved regions are expected, especially for PTMPs, the motifs can be sized from 5 to 25 aa. The number of searched motifs (1 per default) can also be changed with the *-nmotifs* option if relevant (secondary motifs defining subgroups of proteins can be found). MEME describes the detected motifs with Position Weight Matrix (PWM), also called Position-Specific Scoring Matrix (PSSM) as well as their representative sequence logo. The html file displays interactive graphical results with logo, sequence

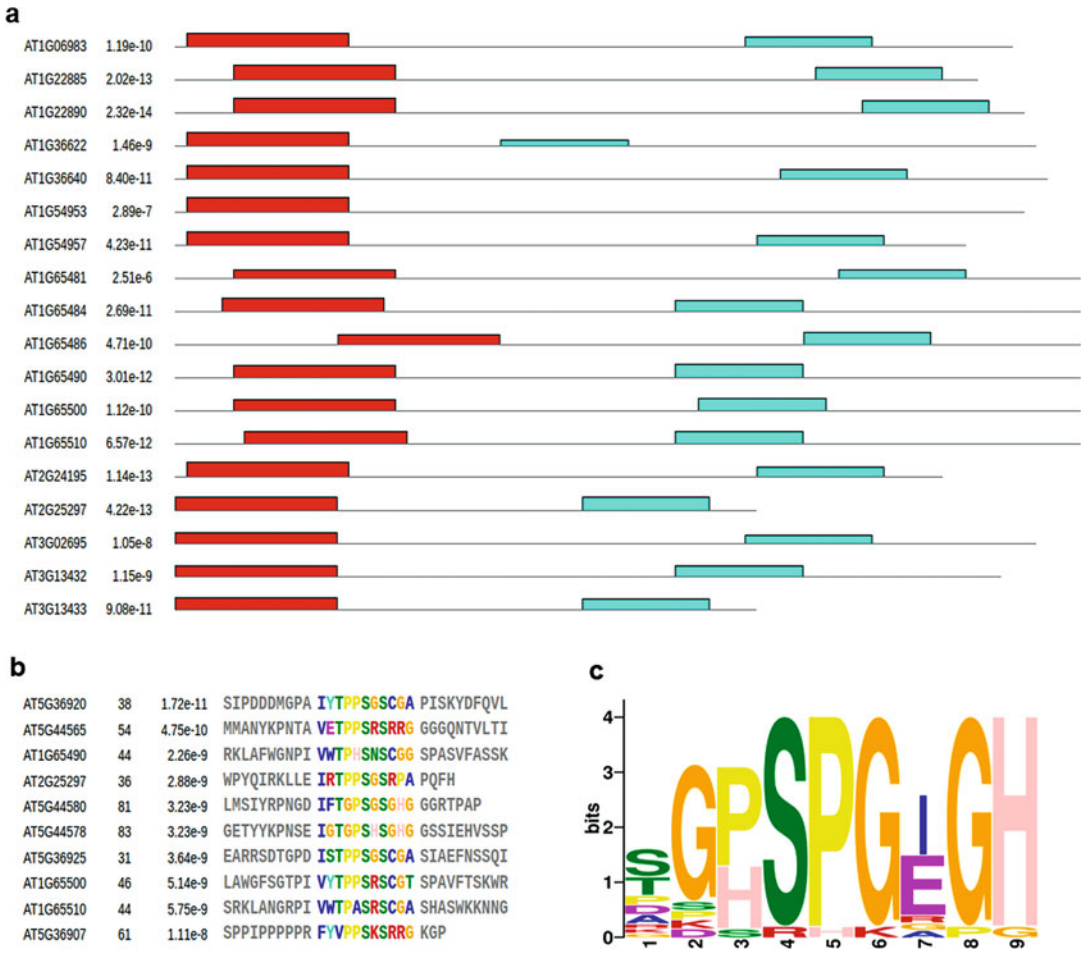


Fig. 5 Illustration of MEME html outputs for motif detection. (**a**) This result has been obtained with two searched motifs ranging from 8 to 50 aa and Arabidopsis candidate PROSCOOP proteins as input. The predicted signal peptides have not been removed before MEME analysis to highlight their biased composition. Motif 1 (red box) overlaps with the SP, and Motif 2 (cyan box) matches with the active SCOOP peptide [13]. (**b**) Sequence alignment provided for each detected motif (here the SCOOP motif) with start positions and p-values. (**c**) Sequence logo representing the sequence signature of the detected motif, here a result obtained with one searched motif ranging from 5 to 9 aa with the 19*S. lycopersicum* candidate PIP/PIP-like proteins

alignment, and motif locations relative to the protein sequences (Fig. 5). Input sequences in which the conserved motif would not be detected deserve to be checked for the robustness of their selection.

The MEME suite proposes an alternative tool named GLAM2 [30] allowing for insertions and/or deletions in the search motifs. Run GLAM2 online (https://meme-suite.org/meme/tools/glam2) or locally using the following command:

```
> glam2 p <selected_proteins_withoutSP_file> -o <output_-
```

```
folder>
```

The output folder defined by the user contains result files (html, png, and txt) including sequence motif alignment, motif description in logo, regular expression, and PSSM matrix.

Another powerful way to describe a protein family is to use a Hidden Markov Model (HMM). The package HMMER3 contains the HMMbuild tool [31] which uses a multiple sequence alignment as input. To generate this alignment file in the required aln format, use MUSCLE and AliView for visualization and optimization, if necessary:

```
> muscle -in <selected_proteins_withoutSP_file> -out <align-
ment_file>
> hmmbuild --amino <output_file> <alignment_file>
```

The output of hmmbuild is a text file corresponding to the HMM profile.

3.3.2 Iterative Search with PWM and HMM Profile

This last step of the protocol exploits the previously generated PWM and HMM profiles to re-scan the entire proteome with greater sensitivity. Indeed, this new proteome screening takes into account the sequence degeneracy observed and tolerated within the signature motif. For this purpose, the results from MEME, GLAM2, and HMMbuild are used as inputs for the tools MAST [32], GLAM2Scan [30], and HMMsearch [31], respectively.

Run MAST and GLAM2Scan online (https://meme-suite.org/meme/tools/mast and https://meme-suite.org/meme/tools/glam2scan) or locally using the following commands:

```
> mast <MEME_output> <proteome_file> -o <output_folder>
> glam2scan p <GLAM2_output> <proteome_file> -o <output_-
folder>
```

The inputs <MEME_output> (xml format) and < GLAM2_output> (txt format) are the files describing the motif (s) previously obtained with MEME and GLAM2 respectively. For both tools, the results are presented in html files listing the proteins in which the motif has been detected with its relative position. Files are generated in the output folders defined by the user.

Run HMMsearch using the following command:

```
> hmmsearch --incE 10 --max -o <output_file> <HMMbuild_output>
<proteome_file>
```

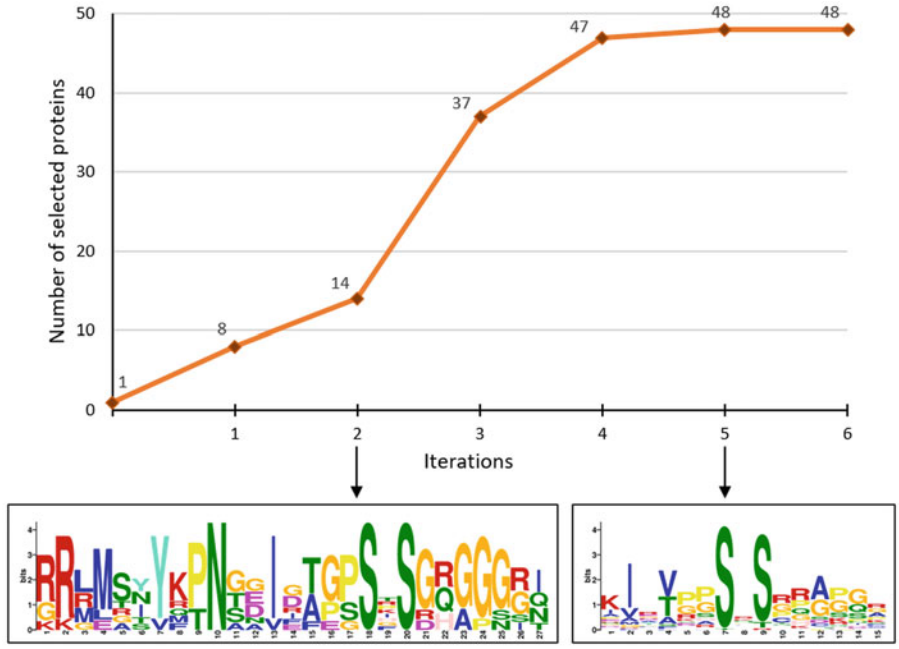


Fig. 6 Progress of the number of selected homologous proteins according to the protocol iterations. The PROSCOOP12 protein is used as starting sequence. The conserved motif (PWM/logo) defined by the MEME tool with default parameters is shown after iterations 2 and 5

The file <HMMbuild_output> contains the HMM profile describing the protein family generated by HMMbuild. The defined output is a txt file containing local alignments between profile and tagged proteins. The optional argument *--incE* is set at 10 to retrieve more proteins than the default settings, but it can be lowered down to gain stringency according to the first results. The optional argument *--max* produces better results at the expense of speed.

The results of MAST, GLAM2Scan, and HMMsearch allow the identification of new proteins that probably belong to the studied family. To verify this, these new sequences should be added to the <selected_proteins_file> file for inspection in comparison with the previously selected proteins (part 2 of the protocol, Subheading 3.2). After validation, these additional sequences will allow the definition of new and more relevant matrices and profiles that can be used again to scan the proteome in an iterative way (red arrows in Fig. 2). For completeness, the newly identified proteins should also be used as new input of jackhmmer and tblastn to re-scan the proteome and the genome and identify new candidates (Subheading 3.1.2). New iterations have to be performed until no more candidates are detected (Fig. 6). The PWM obtained after the last iteration, the final result of the proposed protocol, is an informative

signature sequence, limited to the most highly conserved residues, diagnostic of the SSP family studied.

As a final guideline, gradually expanding the omics dataset to other species can also help to construct more representative and pondered PWM and HMM profiles that can recursively feed the workflow to strengthen its efficiency. Although the notion of homology remains questionable with such low similarities, the conserved motif finally defined is a robust prediction of what the functional mature peptide may be. Of course, experimental tests (e.g., with synthetic peptides) remain necessary to confirm the identification of these extracellular peptides.

4 Notes

1. If no whole genome sequence is available for the species of interest, RNA-seq data can also be used as omic resource. In such case, tblastn can be used in the same way by replacing the genome sequence file with transcriptome de novo assembly (FASTA format):

```
makeblastdb -dbtype nucl -in <RNAseq_assembly_file> -out
<RNAseq_assembly_db>

tblastn -query <protein_query_file> -db <RNAseq_assembly_db>
-evalue 10 -outfmt 0 -out <output_file>
```

2. To avoid the subtraction of loci tagged with tblastn hits with those corresponding to annotated genes (and also tagged at the protein level), you may want to apply tblastn only against all intergenic regions (instead of the whole genome). Such a file can be generated from the genome sequence and gene feature annotations (GFF file).
3. This manual curation and reannotation is time-consuming, but it should be required only for a limited number of loci. In situations where the number of unannotated loci is too high, automatic gene prediction pipelines could be considered with specific software such as SPADA [33]. However, previously wrongly annotated regions risk to be wrongly annotated again unless additional RNA-seq libraries are supplied to the pipeline.
4. In the same way, transcript sequences tagged as similar to the query sequence by tblastn in a transcriptome assembly (gathered in <selected_transcript_file> in FASTA format) can be analyzed by ORFfinder to retrieve the protein sequences.

```
ORFfinder -in <selected_transcript_file> -strand both -out
```

```
<output_file>
```

Depending on the quality of the assembly, eventual frameshifts (short indels) have to be considered by comparison with the tblastn results.

5. There are certain sequence features that may cast doubt on the secretion of the candidate SSP precursor and therefore justify their exclusion: (i) the presence of transmembrane segment (outside the SP which has similar properties) predicted with DeepTMHMM; (ii) the presence of a C-terminal endoplasmic reticulum-retention signal that can be suspected if a positive match with the motif PS00014 is obtained using ScanProsite (https://prosite.expasy.org/scanprosite/) [34]; (iii) the presence of Glycosylphosphatidylinositol (GPI)-anchor that can be predicted online with PredGPI (http://gpcr.biocomp.unibo.it/predgpi) [35].
6. The spreading of false positives through iterative homolog searches is a concern inherent to the procedure. We advise users to carefully select their protein candidates. Proteins that do not fulfill requirements (e.g., relative position of the conserved regions along the sequence, presence of large insertion, atypical N- or C-termini, and/or even rare intron/exon structure) should be discarded and stored independently until more insights about the family have been obtained. Note that false positives will tend to exclude themselves during the multiple alignment process, producing visual subgroups separating them from the correctly discovered candidates.

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Chapter 2

Identification of Bioactive Phytocytokines Using Transcriptomic Data and Plant Bioassays

Jack Rhodes and Cyril Zipfel

Abstract

Plant genomes contain thousands of short open reading frames that encode putative peptides. Some of these peptides play important signaling roles in response to environmental stress. Here we describe a pipeline used to identify the CTNIP/SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS (SCREW) family of phytocytokines, based upon their transcriptional upregulation during biotic stress. Moreover, we describe approaches to assay their activity *in planta* by measuring increases in cytoplasmic calcium concentration, reactive oxygen species production, and mitogen-activated protein kinase phosphorylation.

Key words Peptide signaling, Phytocytokine, Immunity, Transcriptomics, Reactive oxygen species, Mitogen-activated protein kinase, Calcium influx

1 Introduction

In response to stress, plants produce and secrete a range of signaling peptides [1, 2]. These peptides can be perceived by cells expressing cognate receptors in either an autocrine, paracrine, or even systemic manner, modulating subsequent stress responses. Many of these responses overlap with those triggered by immune elicitors or modulate immune signaling. Such peptides have thus been termed phytocytokines, in analogy with metazoan cytokines [3]. Distinct phytocytokines can potentiate or attenuate immune responses, and may also mediate longer term developmental responses to the biotic environment [1, 2]. Similar to most signaling peptides, phytocytokines are generally produced as short, secreted proteins with an N-terminally encoded signal peptide, variable pro-domain, and C-terminally encoded active peptide [4, 5]. The apoplast-localized peptide is then able to bind a cognate receptor complex and induce downstream responses.

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While some phytocytokines are regulated at the post-translational level, for example, through proteolytic processing or modulation of peptide-receptor binding [6, 7], the majority of phytocytokines identified to date are regulated at the transcriptional level, being induced by biotic stress [1, 2]. Consequently, analysis of stress-induced transcriptional responses provides a useful tool to identify novel phytocytokines among the thousands of predicted short open reading frames [8].

Here, we describe the approach taken to identify the CTNIP peptide family [9], also independently identified as SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS (SCREWs) [10], from publicly available transcriptomic data. We discuss the methodology and rationale we employed to identify this phytocytokine family. We then outline approaches used to confirm the activity of synthetic CTNIP peptides *in planta*, namely increases in cytoplasmic calcium concentration, apoplastic reactive oxygen species (ROS) production, and mitogen-activated protein kinase (MAPK) phosphorylation. These approaches provide evidence that the peptides are perceived by the plant and may function as phytocytokines. Moreover, these methods can also facilitate preliminary genetic characterization of signal perception. Mutants in receptor-mediated signaling, for example, *bak1–5*, a dominant negative allele of the common co-receptor BRASSINOSTEOID-INSENSITIVE1-ASSOCIATED KINASE 1 [11] can be used to test whether the identified peptides employ typical phytocytokine signaling pathways.

2 Materials

2.1 Equipment

1. Personal computer.
2. Bead beater for tissue homogenization. We used the Geno-grinder 2010 SPEX (Thermo Fisher).
3. Chemiluminescence plate reader. We used the Varioskan® Flash plate reader (Thermo Scientific) to record the calcium burst, and a HIGH-RESOLUTION PHOTON COUNTING SYSTEM (HRPCS) 218 (Photek) equipped with a 20-mm F1.8 EX DG ASPHERICAL RF WIDE LENS (Sigma Corp) for ROS assays.
4. Western blotting equipment: 10–12% polyacrylamide gel, compatible SDS running buffer, gel tank, PVDF membrane, 3MM blotting paper, electrophoretic transfer cell, compatible transfer buffer, and power supply.
5. Western blot imaging systems for chemiluminescence detection.

6. Plant growth chamber.
7. Benchtop centrifuge.

2.2 Consumables and Reagents

1. White 96-well plates.
2. 70% (v/v) ethanol.
3. Kai Biopsy Punch with Plunger – 4 mm diameter.
4. 2 mM coelenterazine, stock solution in methanol.
5. 2 M $CaCl_2$ in 20% (v/v) ethanol.
6. 100 mM luminol, stock solution in DMSO.
7. 10 mg/mL horseradish peroxidase Type VI-A (e.g., Sigma, P6782).
8. Petri dishes.
9. 24-well clear cell culture plates.
10. SDS loading buffer (6× stock): 300 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 6% (w/v) SDS, 0.05% (w/v) bromophenol blue.
11. DTT: Prepare a stock solution of 500 mM dithiothreitol in water; store at −20 °C.
12. Synthetic peptides: flg22 (QRLSTGSRINSAKDDAAGLQIA); Pep1 (ATKVKAKQRGKEKVSSGRPGQHN); elf18 (acetyl-MSKEKFERTKPHVNVGTI); CTNIP1 (AMRPFPTAADEIRFVFQALQRGPVSGSGPNGCTNIPRGTPRCHG); CTNIP2 (AARPLQADSEIRFVFQLLQRGQVIGSGPNGCTNIPGGSGTCRP); CTNIP3 (ATRMLRITFDSDIRFVFESLQKGTVPGSGPNRCSHIPKGSGSCHG); CTNIP4 (AMRPFPDPVDEIRLLFQALQRGPVRGSGRNGCTNIPRGSGRCHN); $CTNIP4^{48–70}$(GPVRGSGRNGCTNIPRGSGRCHN); $CTNIP4^{C58S/C68S}$(GPVRGSGRNGSTNIPRGSGRSHN). Peptides were custom-synthesized at >80% purity and resuspended in water (*see* **Note 1**). Final concentration is between 100 nM and 1 μM.
13. Glass beads (2-mm diameter).
14. 1.5-mL and 2-mL microcentrifuge tubes.
15. Liquid nitrogen.
16. Prestained Protein Ladder.
17. Bovine serum albumin.
18. 10× Tris-buffered saline (TBS): 24.2 g/L Tris, 80 g/L NaCl.
19. TBS + Tween (TBST): 1% (v/v) Tween®20 in 1x TBS.
20. PVDF (polyvinylidene difluoride) western blotting membrane.

21. Enhanced Chemiluminescence (ECL) western blotting detection reagent.
22. Coomassie Brilliant Blue protein staining solution (e.g., SimplyBlue™ SafeStain, Invitrogen).
23. Anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (e.g., Cell Signaling Technology; #9101).
24. Anti-rabbit IgG HRP-conjugate (e.g., Sigma; A0545).

2.3 Media

1. ½ MS medium 1% (w/v) sucrose: adjust pH to 5.8; add 1% (w/v) agar for solid media; sterilize by autoclaving.

2.4 Plant Material

2. Seeds of *Arabidopsis thaliana* including a transgenic line expressing the genetically encoded calcium reporter apoaequorin [12] (*see* **Note 2**).

3 Methods

3.1 Identification of the CTNIP Family of Phytocytokines from Publicly Available Transcriptomics Data

1. Download an up-to-date annotation of your proteome of interest. In this case, we used the Araport11 proteome annotation from *Arabidopsis thaliana* Col-0 [13] (*see* **Note 3**).
2. Sort the proteome based on the predicted length of the amino acid sequence and select all those proteins that are less than 150 amino acids in length (SET<150). Most simply, this can be done in Microsoft Excel using the = LEN(A1) function to quantify the length of the amino acid sequences (hereafter A1 represents the cell containing the amino acid sequence) (*see* **Note 4**).
3. Predict the probability that proteins in SET<150 contain a signal peptide using SignalP 6.0 [14].
4. (Optional) Determine other subcellular localization predictions of SET<150 using available software such as SUBA4.0 or DeepLoc2.0 [15, 16].
5. Determine the number of cysteine residues within amino acid sequences of SET<150 proteins. This can be done using different approaches; the simplest being Microsoft Excel using the function = LEN(A1)-LEN(SUBSTITUTE(A1,"C", "")). This allows the peptides to be classified as "cysteine-rich" if they contain more than two cysteine residues [4].
6. Extract the expression data for SET<150 from a transcriptomic dataset of interest. In this case we use a large elicitor-induced time course experiment [17] (available as Supplementary Table 1 in reference [16]). In Excel, desired data can be extracted using the = VLOOKUP() function.

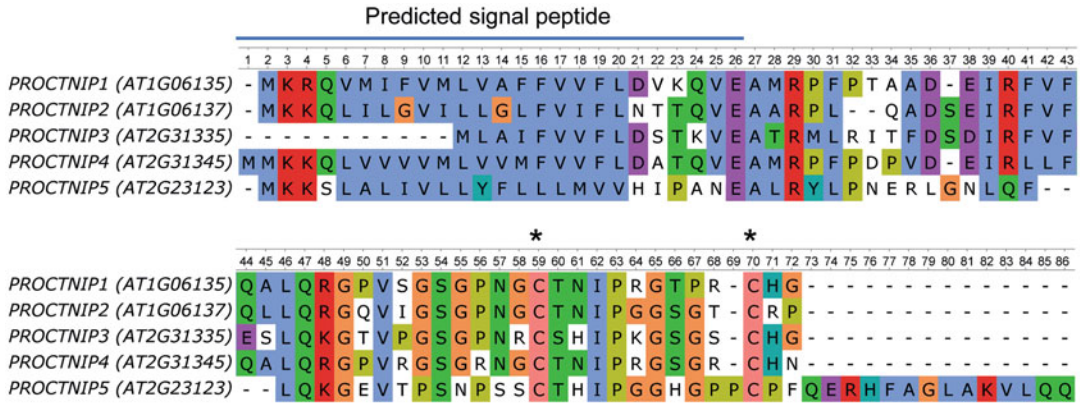


Fig. 1 Alignment of CTNIP peptides from *Arabidopsis thaliana*. Asterisks indicate conserved cysteine residues mutated in Fig. 2c. Signal peptide as predicted by SignalP 6.0

7. Sort the transcripts according to expression level at the time point of interest after elicitor treatment. Here we sorted at 90 min following 100 nM flg22 treatment, the condition with one of the strongest transcriptional responses [17]. Select candidate phytocytokines from the sorted list manually applying the desired selection criteria. CTNIP peptides were selected based on strong transcriptional upregulation, presence of a signal peptide for secretion, and relatively few cysteine residues. We decided to focus on peptides with few cysteine residues to avoid challenges in peptide synthesis and recombinant protein production.
8. (Optional) Perform a BLAST all-versus-all analysis to identify families of peptides. This can either be done manually or by use of an algorithm such as CLUSTER ANALYSIS OF SEQUENCES [18]. Additional members of peptide families can then be identified through BLAST, MAST or HHM searches against proteomes/genomes, as not all members of the peptide family are necessarily transcriptionally upregulated [19–22]. An alignment of the CTNIP family from *Arabidopsis thaliana* is shown in Fig. 1.

Once candidate phytocytokines have been identified, the next step is to demonstrate activity. Phytocytokines induce diverse, sometimes antagonistic, responses so this is not trivial. In the following, we outline several methods that we used to confirm the activity of CTNIP peptides *in planta* namely calcium influx, reactive oxygen species production, and mitogen-activated protein (MAP) kinase phosphorylation. These methods were chosen because they assay typical plant responses to pattern recognition receptor activation, they are highly sensitive, and they may thus be suitable for a wide range of phytocytokines.

3.2 Measurement of Changes in Cytoplasmic Calcium Concentration

1. In order to test for biological activity, have the full-length peptides custom synthesized, excluding the signal peptide (*see* **Note 5**).
2. Sterilize seeds of a transgenic *Arabidopsis* line expressing the genetically encoded calcium reporter apoaequorin [22]. Put the desired amount of seeds into a 1.5-mL microfuge tube, add 1 mL of 70% ethanol, incubate for 10 min at room temperature with gentle agitation. Use a pipet to remove the 70% ethanol and replace with 1 mL 100% ethanol, incubate for 1 min before removing as much ethanol as possible using a pipette and allow the seeds to air dry under sterile conditions.
3. Sow seeds on solid plates containing ½ MS media with 1% sucrose and cold-stratify for at least 2 days at 4 °C in the dark.
4. Transfer to the growth room (22 °C constant temperature, 16-h photoperiod).
5. Three days after transfer to the growth room, move individual seedlings into a 96-well plate with 150 μL liquid MS medium with 1% sucrose. It is best to transfer the seedlings as soon as they are large enough to be handled with forceps. A transparent lid should then be placed upon the plate and sealed with parafilm. It is important that all steps are carried out in a sterile environment.
6. Grow the seedlings in the liquid MS medium for a further 5 days (*see* **Note 6**).
7. Exchange the liquid MS medium with 200 μL of a 10 μM coelenterazine solution in water (diluted from the 2 mM stock solution in methanol) and incubate in the dark overnight. This can be achieved by covering the plate in aluminum foil.
8. On the morning of the reading, replace the coelenterazine solution with 100 μL of water at least 1 h prior to measurement of the sample. It is important to allow the sample to recover from the changing of the solutions and associated stress.
9. Measure light emission in a plate reader (*see* **Note 7**). Luminescence is recorded for 200 milliseconds per well at 30-s intervals. Take background readings for 5 min (10 readings total) prior to the addition of the elicitor peptide.
10. Add 50 μL of 3 times concentrated (3 μM) peptide solution. Start measurements immediately for at least 30 min (60 readings of 200 ms). A final concentration of 1 μM synthetic peptide is recommended and was used here to initially test CTNIP peptides. It is important to also include both negative and positive controls (*see* **Note 8**).
11. (Optional) Discharging can be used to measure maximum luminescence, which may serve as an internal control. To discharge, add 150 μL 2 M $CaCl_2$ in 20% ethanol. If your system

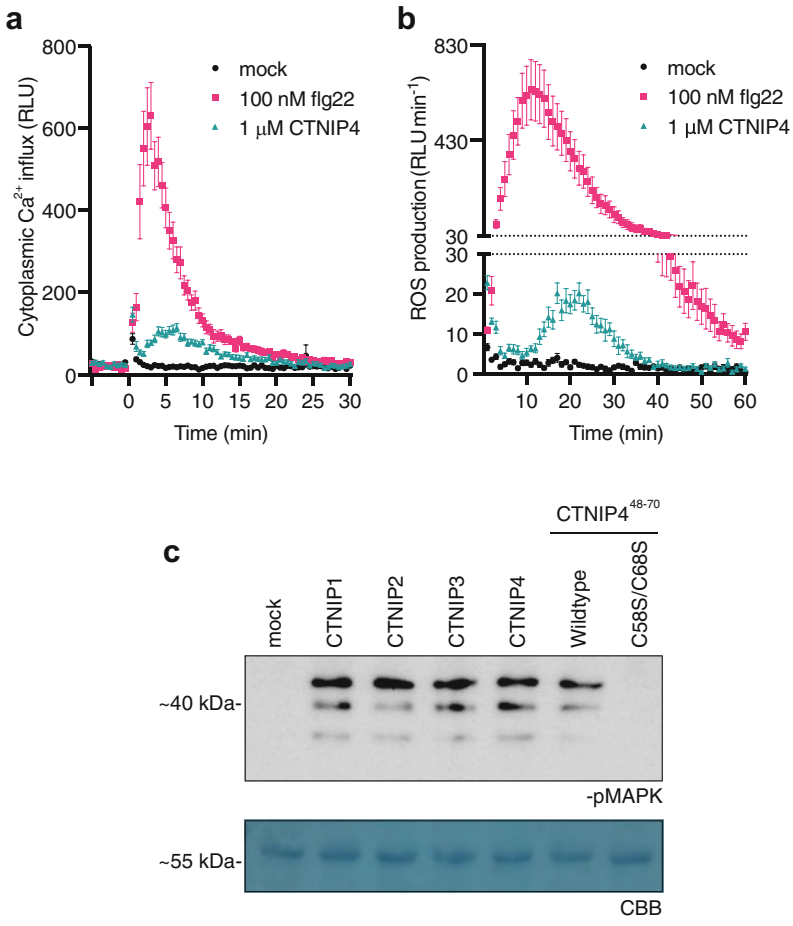


Fig. 2 CTNIPs activate early signaling responses indicative of pattern-triggered immunity (**a**) Cytoplasmic calcium influx measured in Col-0AEQ seedlings after treatment with mock, 100 nM flg22 or 1 μM CTNIP4 ($n = 16$ seedlings). Points represent the mean and error bars represent SEM. (**b**) ROS production in leaf disks collected from 4-week-old Arabidopsis plants induced by mock, 100 nM flg22 or 1 μM CTNIP4 application ($n = 8$). The Y-axis is split to accommodate the different magnitudes of the ROS responses. Points represent the mean and error bars represent SEM. (**c**) Western blot using α-p42/p44-ERK against phosphorylated MAP kinases in seedlings treated with mock or 100 nM CTNIPs for 15 min. The CTNIP4C58S/C68S peptide is mutated in conserved cysteine residues which are required for CTNIP activity and is used as an additional negative control [9, 10]. The membrane was stained with CBB, as a control for equal loading.

has an injector, we recommend using it at this stage, as discharge will begin instantaneously. Measurements of 200 milliseconds should be taken continuously for 1 min.

12. Plot average readings for each treatment as a function of time. Treatment with CTNIP induced a characteristic cytoplasmic calcium burst, showing a rapid increase in cytosolic calcium,

which rapidly returned to near basal levels within 30 min (Fig. 2a).

3.3 Reactive Oxygen Species (ROS) Production Assay

1. Grow A*rabidopsis* plants on soil under short day (10-h photoperiod) conditions for 4–5 weeks. We use 24-cell seedling trays. Grow the plants until the rosettes fill the cell but do not yet touch each other.
2. Using a biopsy punch (4-mm diameter) to collect a leaf disk from each of the two youngest fully expanded leaves per plant avoiding the midrib. Collect between 8 and 24 leaf disks per treatment. Place one leaf disk, adaxial surface down, per well into a 96-well plate containing 100 μL water.
3. Rest the leaf disks overnight on the bench in the plate with a transparent lid or cover on.
4. (Optional) Before starting the assay move the plate into the dark for ~10 min before starting the reading. This will reduce the level of background.
5. Prepare the assay solution of 100 μM luminol, 10 μg/mL horseradish peroxidase and the elicitor peptide of interest. Here we used 1 μM CTNIP4. Also include a negative and positive control (*see* **Note 8**).
6. Remove the water leaf disks that have been floating on overnight.
7. Replace with 100 μL assay solution and immediately begin measurement of light emission in a chemiluminescence plate reader (*see* **Note 7**).
8. Record light emission for 1 h. Depending upon the elicitor used, the ROS production generally peaks at 10–15 min and returns toward baseline levels after 35–40 min. Exemplary results for CTNIP4 are shown in Fig. 2b.

3.4 Mitogen-Activated Protein Kinase (MAPK) Phosphorylation Assay

1. Sow sterilized Arabidopsis seeds (**step 3.3.2**) on ½ MS media containing 1% sucrose and stratify for at least 2 days at 4 °C in the dark.
2. Transfer to the growth room (22 °C constant temperature; 16 h photoperiod).
3. After 3–4 days, transfer two seedlings per well into a clear 24-well culture plate containing 500 μL liquid MS medium with 1% sucrose and seal with parafilm. Work under sterile conditions. Return the seedlings to the growth room for a further 8–9 days.
4. Remove the medium from the plates by inverting onto absorbent paper, seedlings will adhere to the plates. Replace with 500 μL fresh ½ MS medium +1% sucrose. Return to the growth room overnight.

5. The next day perform treatment with the elicitor adding 500 µL of 2× concentrated elicitor solution in ½ MS medium +1% sucrose. Here we used a final concentration of 100 nM.
6. After 15 min, rapidly blot dry and harvest two seedlings per treatment into 2-mL tubes containing two 2-mm diameter glass beads (*see* **Note 9**). Flash freeze samples in liquid nitrogen. The experiment can be interrupted at this stage and samples stored at −80 °C.
7. Grind samples using a bead beater while ensuring samples remain frozen.
8. Prepare 3× SDS loading buffer containing 50 mM DTT from the respective stock solutions. Add 1 mL directly to the frozen sample, vortex and place in a 95 °C heating block for 5 min. Vortex the samples again before returning to the heating block. Take care the lids do not burst open due to pressure build-up associated with the heating.
9. Centrifuge samples at 13,000 × *g* for 5 min in a benchtop centrifuge (or otherwise maximum possible speed) to pellet debris.
10. Load 15 µL of each sample on a 10–12% SDS polyacrylamide gel along with a prestained protein ladder. Run until the 25-kDa band is at the bottom of the gel for optimum resolution of the bands.
11. Transfer samples onto a PVDF membrane and block membrane for 1.5 h in 5% (w/v) bovine serum albumin in Tris-buffered Saline +1% TWEEN20 (TBST).
12. Incubate overnight with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody diluted 1:2000 (or as recommended by the manufacturer, if a different product is used) in 5% (w/v) bovine serum albumin in TBST at 4 °C.
13. Rinse membrane in TBST three times to remove excess primary antibody.
14. Incubate the membrane with anti-rabbit IgGHRP-conjugate, diluted 1:30,000 (or as recommended by the manufacturer, if a different product is used) in 5% (w/v) bovine serum albumin in TBST at room temperature.
15. Wash the membrane three times for 15 min in TBST at room temperature with gentle agitation.
16. Wash the membrane once in Tris-buffered Saline (TBS) for 5 min with gentle agitation to remove detergent.
17. Use a chemiluminescence-based western blotting detection system to image the western blot either using a CCD camera or film as shown in Fig. 2c.

18. Stain the membrane with Coomassie brilliant Blue as a loading control. The RUBISCO band should be comparable between samples indicating equal loading as shown in Fig. 2c [23] (*see* **Note 10**).

4 Notes

1. Very hydrophobic peptides may not be soluble in water. Use DMSO instead.
2. Other genetically encoded calcium reporters are available. Check reference [24] for advantages/disadvantages of available options.
3. The quality of the proteome annotation is paramount to successful application of this method. Many annotation pipelines exclude short open reading frames, especially when not supported by transcriptomic data, which is often the case for phytocytokines under basal conditions [25]. Indeed, for the CTNIP/SCREW family ~40% were unannotated in proteomes, but could be identified in genomes [9].
4. 150 amino acids represent an arbitrary cut-off based upon characterized phytocytokines, which may be biased based on our current knowledge. In addition, as peptides become longer, chemical synthetic can be problematic, and recombinant expression may thus be required.
5. For CTNIPs, we observed biological activity for the full-length peptide. This may not always be the case, as proteolytic processing may be required to release the bioactive peptide [7]. If the full-length peptide lacks biological activity, the mature peptide can either be determined experimentally using mass spectrometry (which can also reveal important post-translational modifications) [26], or predictions can be made from sequence alignments (Fig. 1). The predicted receptor binding sequence should be under purifying selection, relative to the remainder of the peptide precursor. Therefore, the mature peptide sequence may stand out as a conserved region within the more variable precursor. Moreover, proteolytic processing sites may be conserved and may thus be evident from the sequence alignment.
6. Incubation time can be modified, depending on growth conditions. Ideally, the first true leaves should be emerging, and seedlings should not fill the well or grow out of the media. This method can also be used on adult leaf disks (grown and harvested as described in 3.2) which can be floated overnight on 100 μL of 10 μM coelenterazine solution. All subsequent steps of the process remain the same (from 3.2.8).

7. Any CCD photon counting camera or plate reader can be used for this application with modifications being made depending upon the specifications of the system.
8. The negative control should be water (and any additional solvents used for the peptide solubilization) or alternatively a scrambled version of the synthetic peptide. Positive controls could include either flg22 [27], Peps [28], elf18 [29], or SCOOP12 [30]; flg22 has a conserved receptor in Angiosperms and is thus functional in many species while Pep peptides display species-specific activities, and the latter two are *Brassicaceae*-specific [30, 31]. These are all potent elicitors of increases in cytoplasmic calcium concentration and thus can be used at 100 nM final concentrations. Note this step should be carried out rapidly as the calcium response is fast, and care should be taken not to damage or disturb the seedlings as these will induce calcium signaling by itself.
9. MAPK phosphorylation peaks between 15 and 30 min after elicitor addition. If desired a time course can be performed.
10. In this protocol equal loading is assumed based on the amount of input tissue. If you wish to use a genotype which is different in size, or has a different growth rate, then the amount of SDS loading buffer added should be adjusted according to weight, or an alternative extraction technique should be used where protein concentration can be determined using the Bradford assay.

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Chapter 3

Purification of Phytaspases Using a Biotinylated Peptide Inhibitor

Raisa A. Galiullina, Ilya A. Dyugay, Andrey B. Vartapetian, and Nina V. Chichkova

Abstract

Phytaspases are plant subtilisin-like proteases (subtilases) that possess a rarely occurring substrate cleavage specificity. These proteolytic enzymes hydrolyze their substrates strictly after an aspartate residue preceded by a characteristic, though degenerate, tripeptide amino acid motif. Having been initially discovered as proteases involved in the accomplishment of programmed cell death in plants, phytaspases were also demonstrated to be instrumental in specific processing of precursor proteins of several plant peptide hormones, thus generating biologically active peptides. Here we provide a protocol for isolation of active phytaspases from leaves, which was shown to be efficient for a wide range of plant species. The key element of the proposed scheme is the use of a specific and reversible biotinylated peptide aldehyde inhibitor of phytaspases for purification of the enzymes by means of affinity chromatography. We also discuss nuances, pitfalls, and possible alternatives for successful isolation of proteolytically active phytaspases.

Key words Affinity purification, Aspartate cleavage specificity, Biotinylated peptide inhibitor, Phytaspase, Plant subtilisin-like protease, Precursor processing, Proteolytic activity

1 Introduction

Phytaspases belong to the family of plant subtilisin-like serine proteases (subtilases) and stand out against the other family members due to their peculiar and strict Asp cleavage specificity. As phytaspases recognize a stretch of amino acid residues preceding the cleavable Asp bond (usually 2–3 upstream residues) [1], they are highly selective and represent processive, rather than digestive, proteolytic enzymes. This mode of cleavage site recognition is very similar to that of animal apoptotic proteases with Asp cleavage specificity, caspases [2, 3], although phytaspases are structurally very different from caspases [4, 5]. Phytaspases were first identified in a search for functional analogs of caspases, with a presumable participation of such enzymes in the accomplishment of

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programmed cell death in plants. Indeed, phytaspases were shown to be instrumental in plant cell death induced by various biotic and abiotic insults [4]. Furthermore, the exclusive cleavage specificity of phytaspases makes them an ideal tool for processing of precursor proteins of the plant peptide hormones systemin and phytosulfokine to trigger responses to external cues, such as wounding and drought [6, 7].

Identification of phytaspases among a plethora of plant subtilases is a challenging task. Here we describe a basic procedure for isolation of phytaspases from crude extracts of leaf tissue that was successfully employed for various plant species including *Nicotiana tabacum, Oryza sativa* [4], *Solanum lycopersicum* [6], *Capsicum annuum*, etc. In this protocol, the key step for obtaining a pure phytaspase preparation, suitable for further analyses addressing identity and properties of the enzyme, is the use of a specific biotinylated peptide aldehyde inhibitor of phytaspases. Such an inhibitor bears a peptide moiety with an amino acid sequence mimicking the phytaspase cleavage site. For example, the TATD (Thr-Ala-Thr-Asp) motif matching the phytaspase cleavage site in the VirD2 protein of *Agrobacterium tumefaciens* [8] was widely employed. This peptide contains the C-terminal Asp residue with an aldehyde replacing the alpha-carboxylic group to allow covalent but reversible binding to the hydroxy group of the catalytic Ser residue in phytaspases. Also, the peptide is biotinylated at its N-terminus to facilitate affinity chromatography purification of the resultant phytaspase-inhibitor complex using an avidin resin (*see* Fig. 1a for the structure of bio-TATD-CHO inhibitor). Reversibility is an important feature of such inhibitors, as it permits to purify the enzyme-inhibitor complex, with the subsequent slow hydrolysis of the enzyme-inhibitor linkage in aqueous solution resulting in phytaspase re-activation.

However, direct application of the bio-TATD-CHO inhibitor for phytaspase isolation from crude leaf extracts is inefficient, probably because of low phytaspase content, and because of interference of crude extract components with phytaspase binding to the inhibitor. Therefore, a set of preliminary steps of fractionation is required, including ammonium sulfate precipitation (Subheading 3.2), anion exchange (Subheading 3.3), and Blue Sepharose chromatography (Subheading 3.4) prior to the final affinity purification step utilizing the bio-TATD-CHO inhibitor (Subheading 3.5). An outline of the procedure is presented in Fig. 1a.

Nevertheless, since phytaspases are known to possess predominantly apoplastic (extracellular) localization in non-stressed plant tissues [4, 9], direct isolation of these proteolytic enzymes from the apoplastic fluid with the use of the biotinylated inhibitor is possible and often represents a convenient alternative to phytaspase isolation from crude leaf extracts (Subheading 3.5).

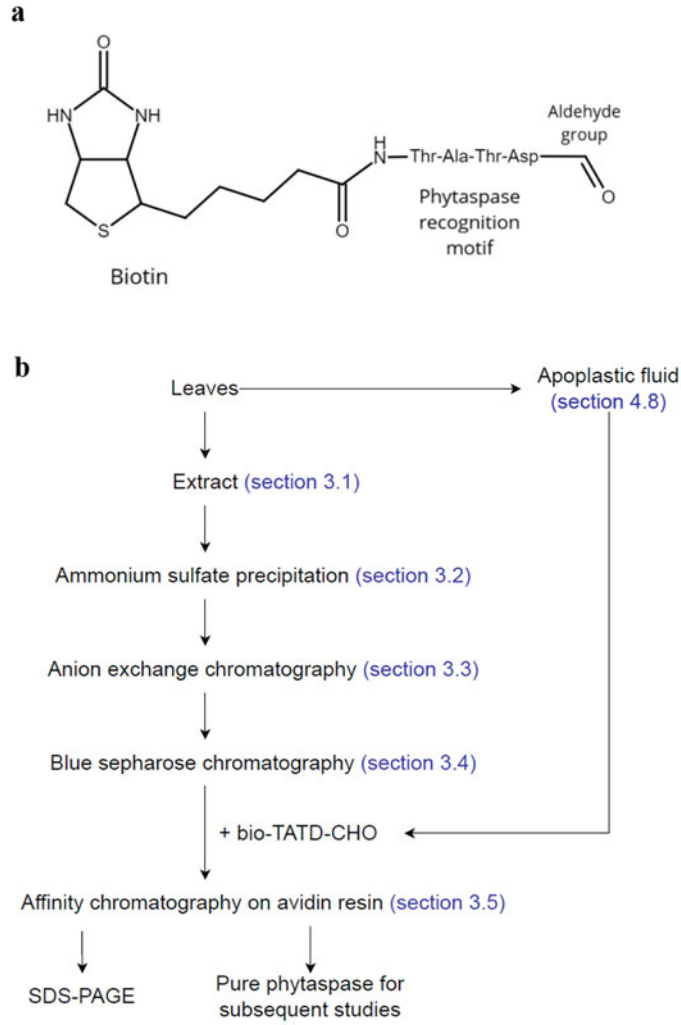


Fig. 1 Peptide inhibitor and outline of the procedure. (**a**) Structure of the custom-synthesized biotinylated peptide aldehyde inhibitor bio-TATD-CHO constructed for reversible inhibition and affinity chromatography purification of phytaspases. Alternatively, for some phytaspases, the commercially available caspase inhibitor bio-YVAD-CHO possessing an analogous structure may be used. (**b**) Schematic outline of the overall protocol depicting the individual procedures for isolation of phytaspases from leaves

2 Materials

2.1 Equipment

1. Blender.
2. Low-speed centrifuge.
3. Incubation shakers for centrifuge bottles and for microfuge tubes.

4. Chromatography equipment (glass columns, tubing and fittings, peristaltic pump, UV detector, fraction collector for low pressure chromatography or, alternatively, a FPLC system).
5. Desiccator with vacuum pump.
6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system.
7. Fluorescence reader (e.g., FLUOstar OPTIMA, BMG Labtech).

2.2 Plant Material

Nicotiana tabacum plants, 6–8weeks old (*see* **Note 1**).

2.3 Buffers, Chromatography Media, and Consumables

1. B1 buffer: 20 mM MES, 2 mM ditiothreitol (DTT), 0.1% (v/v) Tween 20, 5% (v/v) glycerol, pH 5.7.
2. 50 mM NaCl in B1 buffer: prepare B1 buffer as above and add NaCl at 50 mM. Optionally: phenylmethylsulfonyl fluoride (PMSF) can be added up to 1 mM, but *see* **Note 2**.
3. 1 M NaCl in B1 buffer: when preparing B1 buffer as above, add NaCl at 1 M.
4. Apoplast wash buffer: 20 mM MES, pH 5.5, 0.1 M NaCl.
5. Dialysis tubing (e.g., Servapor, MWCO 12000–14,000).
6. DEAE (Diethylaminoethyl) cellulose (e.g., DE53, Whatman). Prepare a column with 20 mL bed volume.
7. Blue Sepharose (e.g., HiTrap Blue HP, GE Healthcare). Prepare a column with 1 mL bed volume.
8. Avidin agarose (e.g., SoftLink Soft Release Avidin Resin, Promega).
9. Sephadex G25 coarse (e.g., GE Healthcare) mini spin columns, or similar, with 10,000 molecular weight cut-off.
10. Centrifugal concentrator (e.g., Amicon Ultra-4 Ultracel-50 k, Millipore), *see* **Note 3**.
11. 100 mM sodium phosphate buffer, pH 8.0. Dissolve 3.4 g $Na_2HPO_4 \cdot 2H_2O$ and 0.17 g $NaH_2PO_4 \cdot 2H_2O$ in 200 mL water.
12. 5 mM biotin in 100 mM sodium phosphate buffer, pH 8.0. Prepare sodium phosphate buffer as above. Add biotin to a final concentration of 5 mM.
13. Silver staining kit for detection of proteins in polyacrylamide gels.

2.4 Synthetic Peptides

1. 20 mM bio-TATD-CHO: biotinylated peptide aldehyde phytaspase inhibitor, custom-synthesized at >90% purity. Prepare a 20 mM stock solution in DMSO.

2. 20 mM bio-DEVD-CHO: biotinylated peptide aldehyde control inhibitor; > 95% purity. Prepare a 20 mM stock solution in DMSO.
3. 1 mM Ac-VEID-AFC: Fluorogenic peptide substrate (Ac, N-terminus acetylated; AFC, 7-amino-4-(trifluoromethyl)-coumarin; > 90% purity). Prepare a stock solution at 1 mM in DMSO.
4. 1 mM Ac-YVAD-AFC. Prepare a stock solution at 1 mM in DMSO.

3 Methods

3.1 Preparation of Crude Leaf Extracts

1. Freeze freshly cut tobacco leaves (approx. 100 g; *see* **Note 1**) in liquid nitrogen and grind in a blender.
2. Suspend the resultant powder in 2 × Vol. (200 mL buffer per 100 g leaf material) of B1 buffer containing 50 mM NaCl in a centrifuge tube, and incubate with shaking for 1 h on an ice water bath for protein extraction. Centrifuge at 14,000 × *g* for 20 min at 4 °C. Resuspend the pellet in 1 × Vol. of the same buffer and repeat the extraction step. Combine the supernatants in a glass beaker to obtain the crude extract. The crude extract can be frozen in liquid nitrogen and stored at −20 °C (*see* **Note 4**).

3.2 Ammonium Sulfate Fractionation of Crude Extracts

1. Put the beaker containing the crude extract on an ice water bath and add dry powder of ammonium sulfate ground in a mortar (22.9 g per 100 mL of extract to achieve 40% ammonium sulfate saturation) in small portions with gentle stirring. After dissolving the last portion, continue incubation on ice for 1 h (or up to overnight), then centrifuge the sample at 14,000 × *g* for 20 min at 4 °C. Transfer the supernatant to a fresh beaker and save the pellet containing proteins that precipitated within the 0–40% interval of ammonium sulfate saturation.
2. Increase ammonium sulfate saturation up to 50% by adding 5.9 g of dry ammonium sulfate per 100 mL of the supernatant and repeat the precipitation and centrifugation steps (**step 3.2.1**) to obtain proteins that precipitate within the 40–50% interval.
3. Repeat **step 3.2.1** by adding sequentially 6.1, 6.3, 6.6, and 6.8 g ammonium sulfate per 100 mL of the newly obtained supernatants, to get proteins that precipitate within the 50–60, 60–70, 70–80 and 80–90% intervals, respectively [10] (*see* **Note 5**).

4. Dissolve the pellets in a small volume of B1 buffer (usually 5 mL) and determine the amount of phytaspase proteolytic activity in 5 μL aliquots of each sample (*see* **Note 6**). For *N. tabacum* phytaspase (*Nt*Phyt), for example, the activity peaks in the 60–70% fraction (Fig. 2a).
5. Collect and pool the peak fractions and dialyze against 0.5 L B1 buffer. Make two changes of the dialysate buffer once in 4 h to remove traces of ammonium sulfate.

3.3 Anion Exchange Chromatography

All the subsequent chromatography steps described in Subheadings 3.3–3.5 should be performed in a cold box or cold room, if time-consuming low-pressure chromatography procedures are used, to avoid protein inactivation and degradation. In case a FPLC system is available (e.g., AKTA Purifier10; GE Healthcare) for rapid protein purification, the procedures can be carried out at room temperature.

1. Apply the sample onto a DE53 column (20 mL) equilibrated with B1 buffer (*see* **Note 7**). Wash the column with 2 × Vol. of the same buffer, and elute the bound proteins with a 0–1.0 M NaCl gradient in the same buffer. Collect 3.5 mL fractions.
2. Determine the amount of phytaspase activity in fractions as described in Subheading 3.2. *Nt*Phyt is eluted within the 100–180 mM NaCl interval (Fig. 2b).
3. Pool the peak fractions and dialyze against B1 buffer.

3.4 Protein Fractionation Using Blue Sepharose

1. Load the sample onto a HiTrap Blue HP column (1 mL) equilibrated with B1 buffer. Wash the column with 2 × Vol. of the same buffer and collect 2 mL fractions (fractions 1–11 in Fig. 2c). Then perform elution with a 0–1.0 M NaCl gradient in the same buffer and collect 0.5 mL fractions (fractions 13–23 in Fig. 2c).
2. Determine phytaspase activity in the collected fractions as described in Subheading 3.2. *Nt*Phyt elutes at 0.25–0.45 M NaCl (Fig. 2c).
3. Pool peak fractions and concentrate using a centrifugal concentrator. Add B1 buffer and repeat the concentration step to reduce the volume to about 150 μL, and the concentration of NaCl down to approximately 50 mM.

3.5 Affinity Chromatography Using the Biotinylated Phytaspase Inhibitor

Start with this step (**3.5.1**) if you use the apoplastic fraction rather than crude extracts obtained from leaves (*see* **Note 8**).

1. Divide the sample (from **step 3.4.3**, or from **Note 8**) into 3 aliquots, 50 μL each. Add 0.4 mM of the bio-TATD-CHO phytaspase inhibitor to the first tube, 0.4 mM bio-DEVD-CHO to the second tube, and an equivalent amount of DMSO to the third tube. Mix and incubate at 27 °C for 1 h.

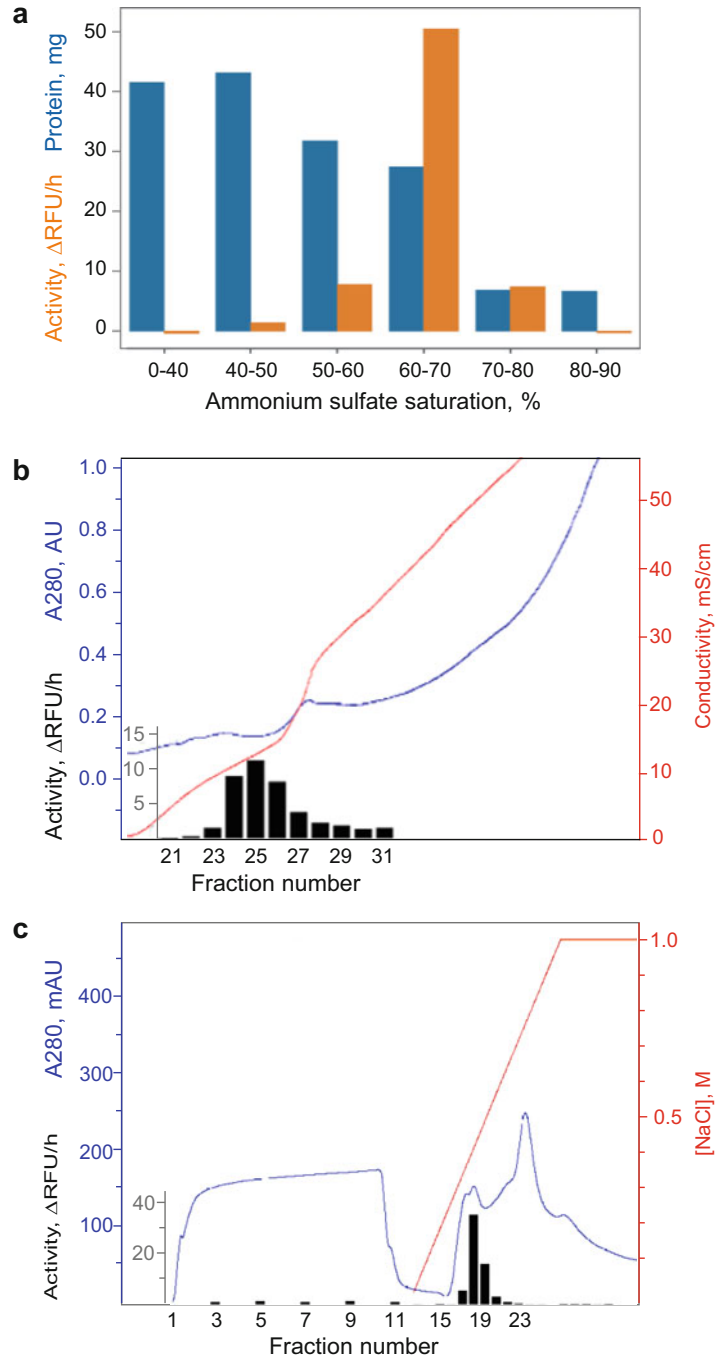


Fig. 2 Purification of tobacco phytaspase. (**a**) Phytaspase activity in fractions obtained by ammonium sulfate precipitation. Phytaspase activity was determined fluorometrically by measuring Ac-VEID-AFC hydrolysis. Protein content was determined with the Biuret test. The determination of protein concentration is optional. It was performed here to illustrate that the bulk of the leaf protein could be separated from tobacco phytaspase activity by fractionated ammonium

2. Use a Sephadex G25 mini spin column equilibrated in B1 buffer containing 50 mM NaCl to remove the excess of unreacted peptide aldehyde according to the manufacturer's instructions.
3. Prepare three 0.5 mL reaction tubes. Add 50 μL SoftLink Soft Release Avidin Resin equilibrated with B1 buffer containing 50 mM NaCl to each of them.
4. Add the three samples from **step 3.5.2** to the three tubes. Incubate at 0 °C for 1 h with shaking.
5. Separate the beads by centrifugation at 2000 × *g* for 3 min; discard the supernatant.
6. Add 250 μL B1 buffer containing 50 mM NaCl, proceed with the previous step.
7. Wash the beads 4 more times with 250 μL B1 buffer containing 50 mM NaCl (**steps 3.5.5** and **3.5.6**).
8. To elute phytaspase from the beads, add 100 μL 100 mM sodium phosphate buffer, pH 8.0, and incubate at 4 °C for 1 h. Optional: use 5 mM biotin in sodium phosphate buffer, pH 8.0, for elution (*see* **Note 9**).
9. Separate the beads by centrifugation at 2000 × *g* for 3 min. Recover the supernatant containing the purified phytaspase into a new tube.
10. Analyze eluates by SDS-PAGE and visualize phytaspase bands by silver staining (Fig. 3a–c) (*see* **Note 10**).
11. Concentrate the eluate and transfer it into B1 buffer containing 50 mM NaCl using a centrifugal concentrator with 50,000 molecular weight cut-off (*see* **Note 11**).
12. Store the purified enzyme at −20° or −80 °C (*see* **Note 12**).

Fig. 2 (continued) sulfate precipitation. (**b**) Anion exchange chromatography. The proteins in the fraction obtained at 60–70% ammonium sulfate saturation were separated by anion exchange chromatography on DEAE cellulose (20 mL bed volume) using a linear gradient of NaCl (red line, conductivity). Protein elution was monitored at 280 nm (A280, blue line). Phytaspase activity was assayed in 3.5 mL fractions by measuring Ac-VEID-AFC hydrolysis. (**c**) Blue Sepharose chromatography. Phytaspase-positive fractions after anion exchange chromatography (fractions 24–26 in panel b) were separated on Blue Sepharose (1 mL bed volume) using a NaCl gradient (0-1 M; same colors and labeling as in panel b). Note that Blue Sepharose turned out to be an extremely efficient medium for phytaspase purification, as the bulk of contaminating plant proteins does not adsorb to this resin

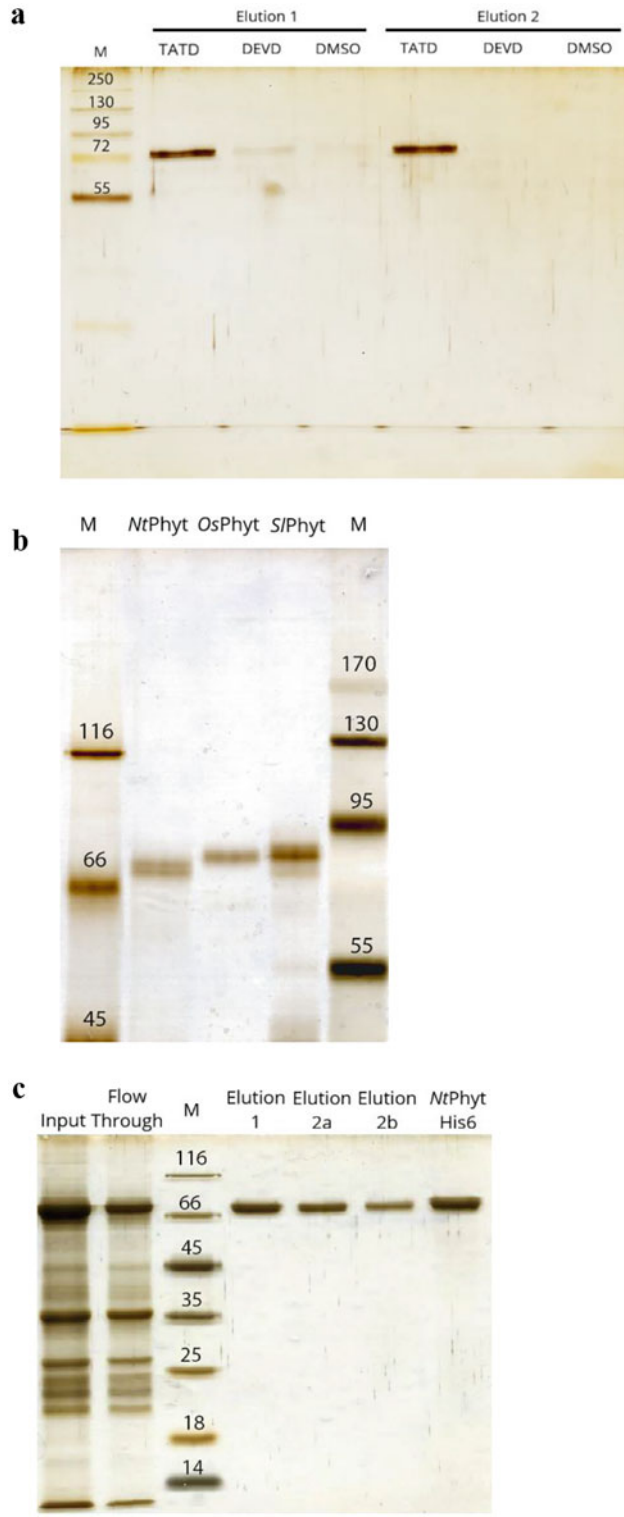


Fig. 3 Purification of phytaspases by affinity chromatography on Avidin resin. (**a**) The bio-TATD-CHO inhibitor determines the specificity of phytaspase isolation by Avidin resin chromatography. Phytaspase-positive fractions eluted from Blue

4 Notes

1. The protocol is not restricted to *N. tabacum* (e.g., Fig. 3b). However to make use of it with new plant species, it is necessary to make sure that the proteolytic activity of phytaspase is clearly detectable in a coarse extract (e.g., using fluorogenic substrates, *see* **Note 6**).
2. Protease inhibitors can be added in order to prevent degradation of phytaspases. However, phytaspase itself needs to remain active and able to bind the aldehyde inhibitor. Phytaspases from many plant tissues are insensitive to the majority of synthetic protease inhibitors. Yet, *Arabidopsis thaliana* phytaspase (AtSBT3.8) is inhibited by PMSF [11]. It is thus recommended to omit protease inhibitors, unless the inhibitor sensitivity of the enzyme under study is known.
3. The molecular mass of phytaspases is approx. 80 kDa, which permits to use concentrators with 50,000 cut-off for fast and convenient concentration and buffer exchange.
4. After thawing the extracts, it is advisable to eliminate the precipitated material by centrifugation.
5. Pellets from fractionated ammonium sulfate precipitation can be stored for extended periods of time at −20 °C.
6. Proteolytic activity of phytaspases can be determined semi-quantitatively by assessing fragmentation of a protein substrate (e.g., EGFP-VirD2Ct protein [4, 8]) by SDS-PAGE. A

Fig. 3 (continued) Sepharose (fractions 18–19, Fig. 2c) were pooled, then split in 3 aliquots and supplemented with either bio-TATD-CHO, or bio-DEVD-CHO, or solvent (DMSO) only. After reaction and binding to Avidin resin, proteins were eluted in two steps: Elution 1 was performed with sodium phosphate buffer at pH 8.0, and the additional Elution 2 was performed with the same buffer containing 5 mM biotin. Eluates were analyzed by 15% SDS-PAGE and silver staining. M, molecular weight markers. (**b**) Phytaspases from leaves of different plant species can be successfully isolated with the current protocol. For tobacco (*Nt*Phyt), rice (*Os*Phyt), and tomato (*Sl*Phyt) phytaspase preparations, eluates from the Avidin resin were fractionated using 10% SDS-PAGE. The gel was stained with silver nitrate. M, molecular weight markers. (**c**) A single affinity chromatography step is sufficient for purification of phytaspase from apoplastic fluid. *Nt*Phyt was overproduced in *N. benthamiana* leaves and extracted by apoplastic wash. After concentration of the extract and buffer exchange, the protein sample was incubated with bio-TATD-CHO and subjected to affinity chromatography on the Avidin resin. Step-wise elution of the bound protein was performed: elution 1 with a pH 8.0 buffer; elution 2a and b with the same buffer containing 5 mM biotin. Aliquots from the input, flow-through, and eluates were analyzed and compared to His-tagged *Nt*Phyt (*Nt*Phyt-His) obtained from the apoplast of overproducing *N. benthamiana* leaves by Ni-NTA agarose affinity chromatography

convenient alternative is to use synthetic fluorogenic peptide substrates of phytaspases and caspases, such as Ac-VEID-AFC and Ac-YVAD-AFC, for quantitative fluorometric determination of phytaspase activity (see the accompanying chapter by Galiullina, Chichkova, Safronov and Vartapetian for a protocol).

7. For a protein to adsorb to an anion exchanger efficiently, the pH of the loading buffer should be ideally 1 pH unit above its isoelectric point (pI) [12]. For phytaspases from tobacco (*Nt*Phyt), tomato (*Sl*Phyt), Arabidopsis (*At*Phyt), and rice (*Os*Phyt) leaves that we have studied, pI values determined by chromatofocusing are 5.0, 4.8, 6.0, and 6.5, respectively. Therefore, *Nt*Phyt and *Sl*Phyt could be readily adsorbed on DE53 resin at pH 5.7, while *At*Phyt and *Os*Phyt required pH 7.0–7.5. We therefore recommend to perform pretests as batch separations at different pH, to find the lowest pH value, at which a particular phytaspase efficiently binds to the anion exchanger.
8. Purification of phytaspases from the apoplastic fluid has the distinctive advantage that the starting material is not contaminated with a large excess of intracellular proteins. This approach is also well-suited for small-scale purification of recombinant untagged phytaspases transiently overproduced in *Nicotiana benthamiana* leaves by means of agroinfiltration. The following protocol works well in our hands:
   - Cut 3 g of phytaspase-overproducing leaves in 0.5 × 2.0 cm pieces and vacuum infiltrate them with Apoplast Wash buffer.
   - Put infiltrated leaf pieces, side by side, onto a Parafilm sheet, then roll up the sheet and place it vertically into a 15 mL Falcon tube. Centrifuge the tube at 2000 × g for 10 min at 4 °C using a fixed-angle rotor.
   - Collect the apoplastic wash (normally about 1.5 mL) from the bottom of the tube.
   - Concentrate the apoplastic wash and transfer it into B1 buffer containing 50 mM NaCl by using a centrifugal concentrator with 50,000 molecular weight cut-off.
   - Proceed as in 3.5. The results of this approach are presented in Fig. 3c.
9. Incubation of the immobilized bio-TATD-phytaspase complex at basic pH leads to hydrolysis of the bond between the enzyme and the inhibitor. Thus, active phytaspase is released and eluted from the resin. However, judging by Fig. 3a, the hydrolysis is incomplete. The non-hydrolyzed portion of the complex can be eluted by addition of biotin to the elution buffer. Re-activation of the enzyme then occurs in the course of elution/storage of the sample.

10. In high-resolution polyacrylamide gels, phytaspase frequently appears as a set of closely spaced bands. This is likely due to the glycosylation pattern of the enzyme.
11. For *Nt*Phyt, which yields approx. 5 μg of pure enzyme from 100 g of leaf material, this step can be omitted. For phytaspases from other species, which may be less abundant or less active with fluorogenic peptide substrates, this step may be required to reach a concentration at which the enzyme can be characterized further.
12. Phytaspases are stable enzymes and can be stored under the indicated conditions for over 1 year without significant loss of activity.

Acknowledgments

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Chapter 4

Characterization of Phytaspase Proteolytic Activity Using Fluorogenic Peptide Substrates

Raisa A. Galiullina, Nina V. Chichkova, Grigoriy G. Safronov, and Andrey B. Vartapetian

Abstract

Within the subtilase family of plant proteolytic enzymes, phytaspases are distinguished by their strict substrate cleavage specificity after an aspartate residue preceded by a characteristic tripeptide amino acid motif. This type of recognition resembles that of animal apoptotic proteases, caspases. Phytaspases attract attention not only because they are critically important for the accomplishment of stress-induced death of plant cells, but also due to their ability to specifically process precursor proteins, thus generating bioactive plant peptide hormones, systemin and phytosulfokine. As the activity of phytaspases appears to be essential for life and death decisions made by the plant cell, elaboration of an approach to characterize and quantitate phytaspase proteolytic activity is of importance. Here we provide a protocol for phytaspase activity determination and characterization using fluorogenic peptide substrates. This approach works well, both with purified phytaspase samples, and with crude extracts from plant tissues. We also discuss advantages of the assay, factors that may influence its sensitivity and specificity, as well as possible pitfalls.

Key words Aspartate cleavage specificity, Cell death, Fluorogenic peptide substrate, Phytaspase, Proprotein processing, Proteolytic activity, Subtilisin-like protease, Subtilase

1 Introduction

Formation of many bioactive plant peptide hormones requires maturation of the respective inactive precursor proteins through precise proteolytic processing [1, 2]. To fulfill such a task, plant proteolytic enzymes possessing high selectivity and specificity of hydrolysis are evidently required. Phytaspases are proteases just of that kind. Although structurally belonging to the family of subtilisin-like proteases (subtilases), many members of which do not possess high cleavage specificity, phytaspases demonstrate high specificity of hydrolysis, introducing a break after an Asp residue in a narrow range of protein substrates. This is due to their substrate interaction mode which involves recognition not only of the Asp

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residue, but of a stretch of 2–3 upstream amino acid residues as well [3, 4]. Depending on the sequence of this upstream motif, phytaspases hydrolyze their substrates with variable efficiencies, or not at all in case of unfavorable context. This type of recognition is very reminiscent of that of animal apoptotic proteases, caspases [5], although structurally phytaspases (subtilases) and caspases bear no resemblance [6, 7]. The preferred recognition motif, as determined by the positional scanning combinatorial substrate library approach is IWLD (Ile-Trp-Leu-Asp) for *Oryza sativa* phytaspase (*Os*Phyt) [4]. This motif is remarkably hydrophobic, in sharp contrast with the highly-charged DEVD motif preferred by animal executioner caspases-3 and -7 [5]. Of note, the latter motif is not recognized by any of the known phytaspases. Initially identified as plant cell death-related proteases [3, 8], phytaspases were later shown to be instrumental also for the processing of precursor proteins of plant peptide hormones, systemin and phytosulfokine [9, 10].

Phytaspases have initially been isolated and characterized by use of their target protein, VirD2 from *Agrobacterium tumefaciens*, as a substrate [3, 8, 9]. However, synthetic fluorogenic peptide substrates with the general formula Ac-X-X-X-Asp-Fluorogenic group (X, any amino acid residues) initially developed for caspases [11] and therefore commercially available, provide a convenient alternative for assessing phytaspase proteolytic activity. The basic principle behind this approach is that phytaspase-mediated cleavage of the bond between the Asp residue and the fluorogenic group renders the latter fluorescent, which can be detected and continuously and quantitatively measured using a fluorescence reader. Coumarin derivatives, such as AFC (7-amino-4-trifluoromethylcoumarin), AMC (7-amino-4-methylcoumarin), and ACC (7-amino-4-carbamoylmethylcoumarin), are frequently employed as fluorogenic groups. Among other advantages of this method, as compared to the use of a protein substrate, is lower interference of other proteases present in the preparation, and the ability to analyze dozens of samples simultaneously. A comparison of the results obtained with protein and fluorogenic peptide-based substrates is shown in Fig. 1.

Within the subtilase family, the number of phytaspases is highly variable between plant species, ranging from one [12], to several [13], or up to a dozen members [14]. As the preferred amino acid motif for cleavage may differ for distinct members of the phytaspase group even within the same plant species [14] (Table 1), the amino acid sequence of the peptide moiety of the fluorogenic substrate may need further optimization. For example, VEID (Val-Glu-Ile-Asp) is recognized fairly well by many different phytaspases, and Ac-VEID-AFC is thus a suitable substrate in these cases. On the other hand, due to strong preference of *Arabidopsis thaliana*

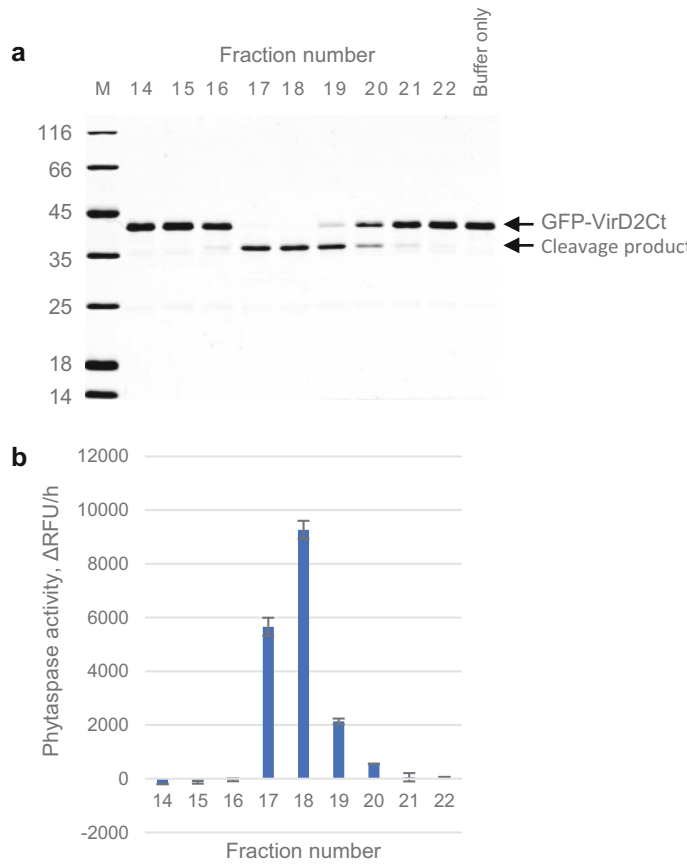


Fig. 1 Determination of phytaspase activity using protein and fluorogenic peptide substrates. *Nicotiana tabacum* phytaspase partially purified from leaf extract was separated by Blue Sepharose column chromatography. Fractions were analyzed for phytaspase activity using the GFP-VirD2Ct protein (**a**, approx. 1–2 μg per sample [8]), or 20 μM Ac-VEID-AFC (**b**) as substrates. The formation of cleavage products was monitored by SDS-PAGE followed by Coomassie blue staining in (**a**) and fluorometrically in (**b**). M, molecular weight markers. ΔRFU/h, increase of relative fluorescence units per hour. Error bars represent standard deviation (s.d.); $n = 3$

phytaspase (*At*Phyt, SBT3.8) for YVAD (Tyr-Val-Ala-Asp) as the recognition motif [12], Ac-YVAD-AFC should be employed as substrate in this particular case.

Here we provide a protocol for phytaspase activity determination and characterization with fluorogenic peptide substrates. This approach works well both with purified phytaspase samples and with crude extracts from plant tissues. We also discuss factors that may influence sensitivity and specificity of the assay, as well as possible pitfalls.

Table 1
Properties of the well-characterized phytaspases

Plant species	Enzyme	Preferred recognition motif	pH optimum	References
Nicotiana tabacum	*Nt*Phyt (SBT 1.9)	VEID>YVAD>IETD~LEHD	5.5–6.0	[3]
Oryza sativa	*Os*Phyt	IWLD[a]> > VEID>IETD~LEHD>YVAD	6.0	[3, 4]
Solanum lycopersicum	*Sl*Phyt 1	VEID~IETD>LEHD> > YVAD	6.0	[9, 14]
	*Sl*Phyt 2	YVAD>VEID~IETD	6.5	[9, 14]
	*Sl*Phyt 4	IETD>WEHD>YVAD~VEID	6.0	[14]
	*Sl*Phyt 5	VEID>WEHD>IETD>YVAD	6.5–7.0	[14]
	P69A	IETD> > VEID	7.5	[14]
Arabidopsis thaliana	*At*Phyt (SBT 3.8)	YVAD>IETD> > VEID	7.0–7.5[b]	[12]

[a]Derived from the analysis of synthetic combinatorial peptide libraries as substrates
[b]At pH below 6.0 *At*Phyt is strictly Asp-specific. However, at neutral and slightly basic pH, it can cleave some synthetic peptide substrates after amino acid residues other than Asp

2 Materials

2.1 Equipment

1. Microcentrifuge tubes (1.5 mL).
2. Bead beater homogenizer (e.g., Minilys, Bertin Instruments) and 1.4 mm ceramic beads.
3. Pestle for 1.5 mL microcentrifuge tubes.
4. Magnetic stirrer and stir bars.
5. Desiccator and vacuum pump.
6. Low-speed centrifuge.
7. Fluorescence reader (e.g., FLUOstar OPTIMA, BMG Labtech).
8. Microplates, 96-well, flat-bottom, with lid.

2.2 Plant Material

Nicotiana benthamiana plants, 6–8 weeks old (*see* **Note 1**).

2.3 Buffers and Solutions

1. Extraction buffer: 20 mM MES, pH 5.5, 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% (v/v) Tween 20.
2. Reaction buffer: 20 mM MES, pH 5.5, 1 M NaCl, 1 mM DTT, 0.1% (v/v) Tween 20 (*see* **Note 2**).
3. 0.2 M PMSF (phenylmethylsulfonylfluoride): prepare 0.2 M stock solution in isopropanol, store at −20 °C (*see* **Note 3**).
4. 5 mg/mL Aprotinin: prepare the stock solution in water, store at −20 °C.
5. 5 mg/mL Leupeptin: prepare the stock solution in water, store at −20 °C.

6. 6 mg/mL Chymostatin: prepare the stock solution in DMSO, store at −20 °C.
7. 3.3 mg/mL E64: prepare the stock solution in water, store at −20 °C.
8. 0.5 M EDTA (ethylenediaminetetraacetic acid): weigh EDTA into a Pyrex beaker, add water and adjust pH to 8.0 with NaOH while stirring. Transfer to a graduated cylinder and adjust to final volume with water.
9. Apoplast wash buffer: 20 mM MES, pH 5.5, 0.1 M NaCl.

2.4 Synthetic Peptides

1. Ac-VEID-AFC, Ac-YVAD-AFC, Ac-IETD-AFC (where AFC is 7-amino-4-trifluoromethylcoumarin) (e.g., California Peptide): fluorogenic peptide substrates; prepare 1 mM stock solutions in DMSO (*see* **Note 4**).
2. Ac-VEID-CHO, Ac-YVAD-CHO, Ac-IETD-CHO, Ac-DEVD-CHO (e.g., Bachem): Phytaspase peptide aldehyde inhibitors; prepare 2 mM stock solutions in DMSO.

3 Methods

3.1 Preparation of Tissue Extracts

1. Put a piece (approx. 100 mg) of plant material (leaf, fruit, etc.) in a 1.5 mL microcentrifuge tube, freeze in liquid nitrogen and grind with a pestle. Suspend the powder in 3 × Vol. of Extraction buffer (300 µL per 100 mg of plant tissue). Alternatively (*see* **Note 5**), freeze plant tissue in liquid nitrogen and disrupt in a bead beater homogenizer using 1.4 mm ceramic beads with two 20 s bursts. Add 3 × Vol of ice-cold Extraction buffer and perform an additional 20-s burst. Optional: Extraction buffer can be supplemented with protease inhibitors, such as PMSF (1 mM) or AEBSF (25 µg/mL), aprotinin (2 µg/mL), leupeptin (6 µg/mL), chymostatin (6 µg/mL), E64 (3 µg/mL), and EDTA (1 mM) to prevent unwanted non-specific proteolysis; but *see* **Note 6**.
2. Incubate the sample on ice for 15–30 min.
3. Centrifuge the sample to remove water-insoluble material at 10,000 × *g* for 10 min at 4 °C.
4. Recover the supernatant into a new microfuge tube.
5. Continue with **step 1** in Subheading 3.3.

3.2 Preparation of Apoplastic Wash

Since the known phytaspases are predominantly apoplastic (extracellular) proteases in non-stressed plant tissues [3, 15, 16], phytaspase activity can also be assayed in an apoplastic wash (extract of the extracellular leaf space).

1. Cut a 100 mg leaf sample in 0.5 × 2.0 cm pieces and collect the pieces into a beaker containing Apoplast wash buffer (*see* **Note 7**).
2. Place the beaker into a desiccator and reduce the pressure to 30 hPa (mbar), for 1 min.
3. Slowly release the vacuum.
4. Blot infiltrated leaf pieces dry, put them, one next to the other, onto a Parafilm sheet, then roll up the sheet and place it vertically into a 1.5 mL centrifuge tube. Centrifuge the tube at 4 °C for 10 min at 2000 × *g* using a fixed-angle rotor.
5. Collect the apoplastic wash (typically 50 μL) from the bottom of the tube and transfer into a new microfuge tube. Samples can be stored at −20 °C before use.
6. Continue with **step 1** in Subheading 3.3.

3.3 Measurement of Phytaspase Proteolytic Activity

1. Dilute the fluorogenic peptide substrate (e.g., Ac-VEID-AFC) to 40 μM in Reaction buffer. Pipette the mixture into the wells of a 96-well microplate, 25 μL per well, one well per reaction. Add 25 μL of the tissue extracts (Subheading 3.1) or apoplastic washes (Subheading 3.2); for controls add 25 μL extraction buffer (final concentrations: 0.5 M NaCl, 20 μM substrate) (*see* **Note 8**) and mix by pipetting up and down.
2. Cover the microplate with the lid to prevent sample evaporation and put it into a fluorescence reader set at 30 °C (*see* **Note 9**).
3. Wavelength settings of the reader depend on the type of fluorophore that you use. Choose 400 nm excitation and 500 nm emission filters for substrates with an AFC fluorescent tag, 350 nm excitation and 450 nm emission filters for AMC and ACC derivatives (*see* **Note 10**).
4. Measure fluorescence intensity of the samples once every 1 h for a 12 h time period. Typical reaction curves look like those shown in Fig. 2a. The higher the phytaspase activity in the sample, the steeper the slope of the curve (*see* **Note 11**).
5. For quantification of enzymatic activity, select a linear section of each curve (usually in the range from 2 to 10 h). Express the rate of hydrolysis of the fluorogenic peptide substrate as an increase of relative fluorescence units per hour (ΔRFU/h, Fig. 2b) (*see* **Note 12**).

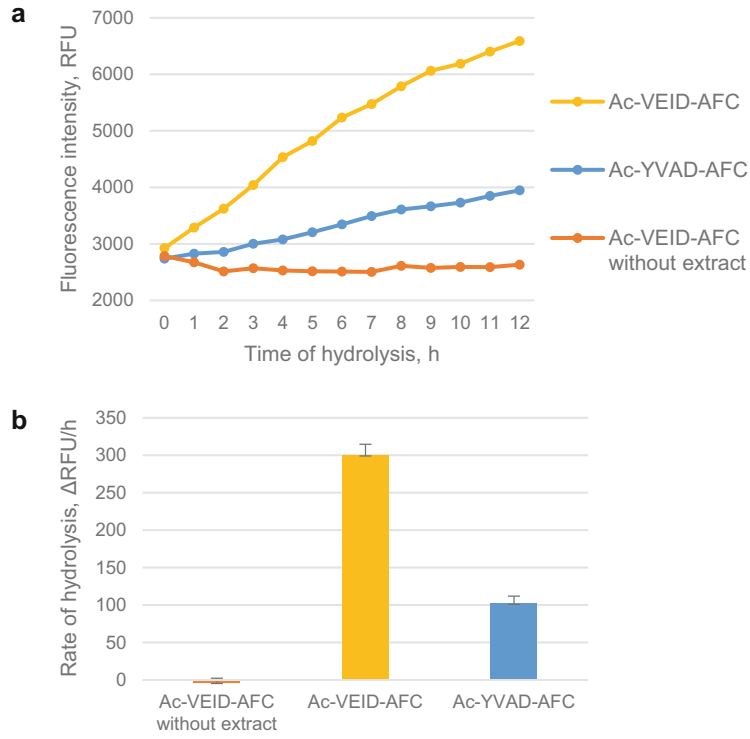


Fig. 2 Phytaspase activity measurement in a crude extract from *Nicotiana benthamiana* leaves. 20 μM Ac-VEID-AFC and Ac-YVAD-AFC were used as substrates. (**a**) Progress curves show the increase in fluorescence intensity as relative fluorescence units (RFU) over time (h). Fluorescence intensities for an Ac-VEID-AFC probe without added extract served as a control. (**b**) Proteolytic activity (the reaction rate) derived from (**a**) is shown as ΔRFU/h, i.e., the increase in relative fluorescence units per hour. Error bars represent s.d. of triplicates

4 Notes

1. *Nicotiana benthamiana* plants were used in the experiment described here. The protocol can be used to determine phytaspase activity in different tissues, in dozens of plant species.
2. The reaction buffer may have to be optimized with respect to pH and ionic strength, depending on the phytaspase under study. While slightly acidic pH is optimal for many phytaspases, for some of them the pH optimum may differ significantly (Table 1). This is also true for the dependence of phytaspase activity on salt concentration. For many (but not all) phytaspases, hydrolytic activity toward peptide substrates is markedly enhanced in the presence of 0.5 M NaCl, even though high salt is detrimental when assessing phytaspase-mediated hydrolysis of a protein substrate [17] (*see* Fig. 3 for pH and salt dependence of NtPhyt proteolytic activity). It may therefore be

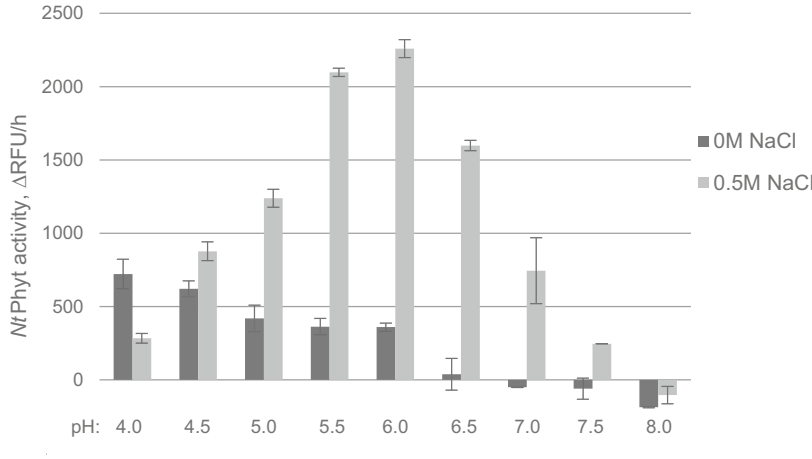


Fig. 3 pH- and salt-dependence of the *N. tabacum* phytaspase proteolytic activity. 20 μM Ac-VEID-AFC was used as a substrate. Activity is shown as increase of relative fluorescence units per hour (ΔRFU/h). Error bars represent s.d. of the mean; $n = 3$ replicates

advisable to perform trial experiments with pH 5.5, 6.5, and 7.5 reaction buffers with high and low ionic strength (500 mM and 50 mM NaCl), respectively.

3. PMSF is toxic; handle with care. PMSF is unstable in aqueous solution. Add to extraction buffer immediately before use. AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride) can be used as a less toxic alternative.
4. When analyzing the phytaspase activity of a new plant species, we recommend to test several phytaspase substrates with different peptide moieties, in order to find the one that works best for the enzyme under study. Based on the data presented in Table 1, we suggest the following substrates: Ac-VEID-AFC, Ac-IETD-AFC, Ac-YVAD-AFC. This may allow to increase the sensitivity of the assay, to confirm that the observed proteolytic activity belongs to a phytaspase, and to provide information on the amino acid motif preferred by the novel enzyme (*see* **Note 13**).
5. Use the bead beater for tough tissues, that are hard to extract (e.g., seeds, stems).
6. If the aim is to isolate the phytaspase from the extract, the use of non-specific protease inhibitors to prevent degradation of the enzyme may be beneficial. However, these inhibitors should leave the activity of the phytaspase under investigation unaffected. Phytaspases from many plant species are insensitive to the majority of synthetic protease inhibitors [8]. Yet some rare exceptions are known [12]. We therefore recommend to omit protease inhibitors at this step until the sensitivity of the enzyme under study is tested.

This can be done by pre-incubation of the extract with a particular inhibitor (or with a mixture thereof (*see* Subheading 2.3 for a list of potential non-specific protease inhibitors, and **3.1.1** for recommended concentrations) at 30 °C for 30 min prior to the addition of the extract to the microplate well. To assess inhibitor sensitivity, compare phytaspase activity in presence and in absence of the inhibitor(s).

7. To obtain a preparative (large-scale) apoplastic wash, see the accompanying chapter by Galiullina, Dyugay, Vartapetian, and Chichkova for a protocol.
8. At 20 μM, substrate concentration is unlikely to be saturating. Therefore, sensitivity of the assay can be increased by increasing the concentration of the fluorogenic peptide substrate to 40 μM.
9. Try to avoid bright illumination of the microplate with samples prior to inserting it into the reader to decrease background fluorescence.
10. Deviation of the filter parameters in your reader by 10–20 nm from the optimal values will not significantly affect the sensitivity of the analysis.
11. If hydrolytic activity of phytaspase in your extract (or apoplastic wash) is very high (off the scale of the reader) the sample needs to be diluted in extraction buffer. As a general rule for enzyme assays, any dilution of the extract should result in a proportional (linear) decrease in activity (ΔRFU/h, in the phytaspase assay described here). For the proteolytic activity of some phytaspases, such concentration dependence does not appear until the extract is diluted 50-fold, which may indicate the presence of an inhibitor in the extract. Therefore, perform serial dilutions of the extract and determine the activity. To setup the assay, choose a dilution factor from the linear range of concentration dependence that allows robust detection of activity.
12. Perform measurements of fluorescence intensities in triplicate and with independently obtained extracts when evaluation of statistical significance of the data is important.
13. To confirm specificity of hydrolysis of a fluorogenic peptide substrate (e.g., Ac-VEID-AFC) in the current assay, a cognate peptide aldehyde inhibitor of phytaspases (e.g., Ac-VEID-CHO) can be used. Add Ac-VEID-CHO (from stock solution in DMSO) to the extract up to the concentration of 80 μM. Supply control samples with an equivalent amount of DMSO, or with Ac-DEVD-CHO (up to 80 μM), the latter amino acid motif is not recognized by any known phytaspase. Incubate the mixtures at 30 °C for 30 min, then add them to the wells of the 96-well plate containing reaction buffer and the fluorogenic substrate to start the reaction. Make sure that Ac-VEID-CHO significantly reduced the detectable level of phytaspase activity compared to the controls.

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Chapter 5

Characterization of Prolyl-4-Hydroxylase Substrate Specificity Using *Pichia pastoris* as an Efficient Eukaryotic Expression System

Gerith Elsäßer, Tim Seidl, Jens Pfannstiel, Andreas Schaller, and Nils Stührwohldt

Abstract

The use of eukaryotic expression systems facilitates the heterologous expression of complex eukaryotic proteins in their post-translationally modified and biologically active state, as a prerequisite for subsequent biochemical characterization and functional analysis. Here we describe the complete workflow for the expression of *Arabidopsis thaliana* prolyl-4-hydroxylases (P4Hs) in the methylotrophic yeast *Pichia pastoris* (renamed as *Komagataella phaffii*), for the extraction of the recombinant enzymes, purification by affinity chromatography, and characterization of P4H activity and specificity toward oligopeptide substrates by mass spectrometry. We expressed eight of the 13 Arabidopsis P4Hs and show that they are all active against proline-rich extensin-derived peptides. However, three of them differed in substrate specificity and were also able to hydroxylate the CLEL9 signaling peptide, featuring a single proline within its mature peptide sequence.

Key words CLEL9, Extensin, Prolyl-4-hydroxylase, Proline hydroxylation, Post-translational modification, Heterologous expression, *Pichia pastoris*, NiNTA chromatography, Enzyme assay, Quantitative mass spectrometry

1 Introduction

The formation of small post-translationally modified peptides (SPMPs) requires proteolytic processing of the respective precursor proteins to release the peptide moiety. Proteolytic processing may occur within the secretory pathway or extracellularly, when the peptides reach the apoplast as their final destination [1, 2]. SPMPs are subject to further post-translational modification during passage through the secretory pathway including tyrosine sulfation and/or proline hydroxylation which may be followed by

Gerith Elsäßer and Tim Seidl have contributed equally to this work.

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O-glycosylation [3]. Recent progress has deepened our understanding of the how, why, and where of proteolytic processing [4]. Likewise, many of the enzymes responsible for the other post-translational modifications have already been identified and characterized. This includes tyrosyl protein sulfotransferase (TPST) for sulfation of tyrosine residues in the Golgi [5], and several Golgi-localized hydroxyproline *O*-arabinosyltransferases (HPATs) for glycosylation. HPATs initiate a step-wise arabinosylation cascade that typically results in a tri-arabinoside chain linked to hydroxyproline (Hyp) [6, 7]. However, how prolines are hydroxylated to generate Hyp as the substrate for subsequent glycosylation by HPATs is still unknown. Proline residues are abundant in SPMPs, but only some of them are hydroxylated, while others are not. How prolines are selected for hydroxylation is unclear. To answer these questions, the enzymes responsible for proline hydroxylation need to be identified and characterized with respect to substrate selectivity.

There are 13 genes coding for putative prolyl 4-hydroxylases (P4H1 to P4H13) in Arabidopsis. They all carry predicted signal peptides targeting them to the secretory pathway, where they are located predominantly in the Golgi apparatus [8, 9]. P4Hs have been implicated in the post-translational modification of hydroxyproline-rich glycoproteins (HRGPs), extensins (EXTs), and arabinogalactan proteins [9]. Biochemical studies confirmed that P4H2 requires a stretch of at least three consecutive proline residues for efficient hydroxylation in vitro [10]. The only other biochemically characterized family member is P4H1, which was shown to hydroxylate poly-L-proline, collagen-like peptide ((Pro--Pro-Gly)$_{10}$), as well as proline-rich peptides derived from arabinogalactan proteins and EXTs [10, 11]. SPMPs have implicitly been assumed to be P4H substrates as well. However, P4H-mediated hydroxylation has not been shown for any of the SPMPs. Further, poly-proline motives with three or more consecutive proline residues are rare in SPMPs, and not required for proline hydroxylation.

To gain further insight into the biogenesis of plant signaling peptides, we addressed the question whether the putative Arabidopsis P4Hs are able to hydroxylate the prolines in SPMPs, and if so, whether there is a preference for prolines in a certain sequence context. To this end, we analyzed the activity and specificity of recombinant P4Hs toward synthetic oligopeptide substrates. In order to obtain Golgi-resident P4Hs in the native form, we decided to use an eukaryotic expression system. *E. coli* has been widely and very successfully used as prokaryotic host for protein expression because of its ease of use and high protein production rate. Yet, prokaryotes are limited with respect to the ability to carry out post-translational modifications, and they lack the secretory pathway of eukaryotic cells. This sets limits to the expression of complex eukaryotic proteins in prokaryotes and calls for alternatives. A

well-established eukaryotic host, comparably easy to grow and maintain as *E. coli*, is the unicellular yeast *Komagataella phaffii*. Also known as *Pichia pastoris*, this yeast has been widely adopted as an efficient eukaryotic expression system, particularly for the production of post-translationally modified secretory proteins, including plant proteins [12, 13].

Since *P. pastoris* does not possess its own stable plasmids, expression constructs need to be integrated into the genome to express the genes of interest. Therefore, gene constructs as well as vectors were linearized before transformation into competent yeast cells. Our expression constructs are under control of the strong and tightly regulated AOX1 promotor for induced expression of the protein of interest when the methylotrophic yeast cells are grown on methanol as the sole carbon source. Thereby cultures can be grown to high cell densities prior to methanol-induced protein production. We used this system for the expression of eight Arabidopsis P4Hs (P4H1, 3, 4, 5, 7, 9, 10, and 12). Recombinant P4Hs were purified by metal chelate affinity chromatography on NiNTA-agarose. Expression and purification were monitored by SDS-PAGE analysis (Fig. 1).

The activity of recombinant P4Hs was tested in an enzyme assay based on Velasquez et al. (2014). Since proline hydroxylation adds the mass of an oxygen atom to that of the substrate, product formation can be monitored and quantified by mass spectrometry using a high-resolution tandem mass spectrometer (MS) coupled to a reversed-phase nano-HPLC system. Analysis of the obtained fragment spectra revealed proline hydroxylation for some of the peptide/P4H combinations, and the efficiency of hydroxylation was assessed by quantifying the abundance of modified peptides using full scan spectra and extracted ion chromatograms (XICs) (Fig. 2).

Using a synthetic oligopeptide derived from the EXT sequence as the substrate, we demonstrated P4H activity for all the enzymes tested (Fig. 3) suggesting that EXT can be considered as a general substrate of all Arabidopsis P4Hs. Using a peptide derived from the CLEL9 sequence as the substrate we observed a more refined substrate specificity. Only three out of the eight tested P4Hs (P4H1, 9, and 10) were found to hydroxylate CLEL9 at its single proline residue (Fig. 4). The results indicate that Arabidopsis P4Hs differ substantially in substrate specificity, and they suggest that specific P4Hs may be responsible for the hydroxylation of different plant signaling peptides.

Here, we provide detailed protocols for the expression in *P. pastoris*, the purification, and the characterization of Arabidopsis P4Hs. Not included are protocols for the cloning of the expression constructs, as this depends largely on the specific research question. We start out with a protocol for linearization of the expression construct, transformation and integration into the genome of

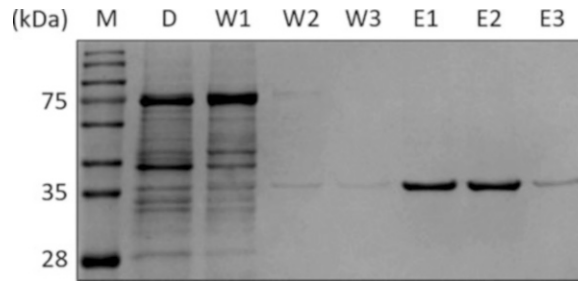


Fig. 1 SDS-PAGE analysis of P4H purification. Arabidopsis P4H10 was expressed in *P. pastoris* and purified from the culture supernatant by affinity chromatography on NiNTA agarose. Proteins in the flow-through fraction (D), three wash fractions (W1-W3), and three eluate fractions (E1-E3) were separated by SDS-PAGE on a 12% gel stained with Coomassie Brilliant Blue. The mass of selected marker proteins (M) is indicated in kDa

P. pastoris (Subheadings 3.1–3.3), followed by the expression and purification of the recombinant protein (Subheadings 3.4–3.9). Setup of the P4H enzyme assay is described in Subheading 3.10 and the characterization of the reaction products by mass spectrometry including analysis of the data in Subheadings 3.11–3.13.

2 Materials

2.1 Cloning and Expression of P4Hs

1. Expression construct for your protein of interest in a vector from the pPiCZα series (*see* **Note 1**). In the experiment described here, we used pPiCZαB to express the open reading frames (ORFs) of Arabidopsis P4Hs (P4H1, 3, 4, 5, 7, 9, 10, and 12) (*see* **Note 2**).
2. Gel extraction kit (e.g., QIAquick gel Extraction Kit, Qiagen).
3. Restriction enzymes with corresponding 10× buffers: restriction enzymes are chosen from the multiple cloning site of the pPiCZα vector that are not present in the ORF of the gene of interest.
4. Horizontal agarose gel electrophoresis system, with power supply, ethidium bromide (or alternative) nucleic acid stain, DNA size marker.
5. *E. coli* DH10B cells, chemically competent.
6. Plasmid miniprep kit.
7. 1:1 mixture of water-saturated phenol and chloroform.
8. 2.8 L Fernbach flasks.
9. Shaking incubator for microbial cultures.
10. 1 M DTT.
11. 1 M sorbitol, autoclaved or filter-sterilized.

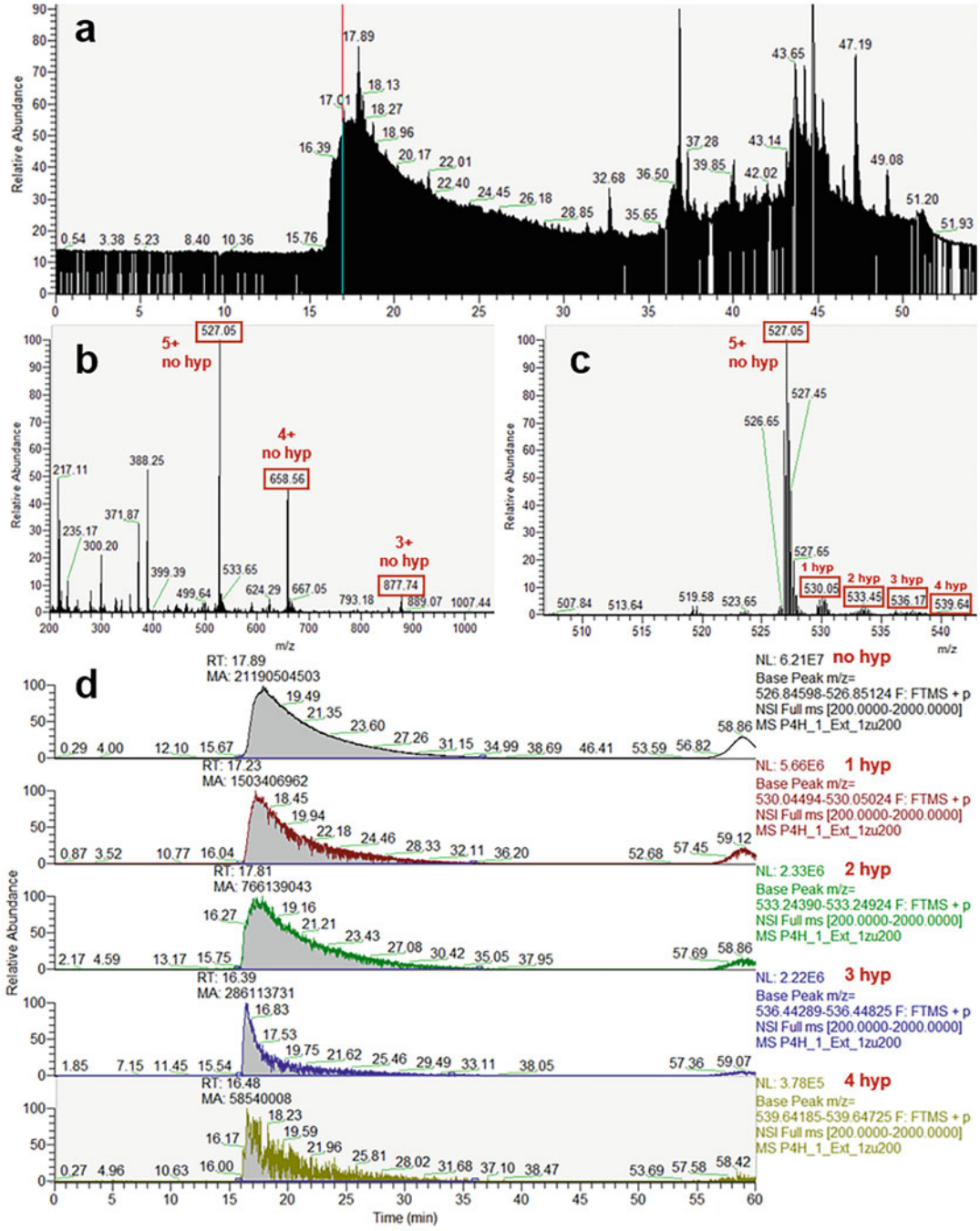


Fig. 2 Quantification of P4H1-mediated proline hydroxylation of the EXT peptide. (**a**) Total ion chromatogram (TIC) of reaction products from the hydroxylation assay (procedure 3.10) using Arabidopsis P4H1 with the EXT peptide as substrate. Relative abundance of 100% was assigned to the highest peak. (**b**) Full scan MS spectrum of the time point marked with the red line in (a) showing three charge states (+3, +4, +5) of the non-hydroxylated (no hyp) EXT substrate, with +5 as the most abundant one (100%). Hence, the +5 charge state was used for quantification (**c**) Detail of (b), zooming in on the +5 charge state. Peaks are detected for the native state (no hyp) as well as for onefold (1 hyp) to fourfold (4 hyp) hydroxylated peptides. (**d**) Extracted ion chromatograms (XICs) for the ions shown in (b) The area under these curves (NL) was used for

12. 250 mL centrifuge bottles.
13. 30 mL Oakridge tubes.
14. Electroporator.
15. LB medium: dissolve 10 g tryptone, 5 g sodium chloride, and 5 g yeast extract in 950 mL of water. Adjust pH to 7.5 with 1 M NaOH. Bring the volume up to 1 L with water. Autoclave for 20 min at 121 °C.
16. Low salt LB Medium (1 L) and plates: dissolve 10 g tryptone, 5 g sodium chloride, and 5 g yeast extract in 950 mL ddH_20. Adjust pH to 7.5 with 1 M NaOH. Bring the volume up to 1 L. For plates, add 15 g agar per liter of medium. Autoclave at 121 °C for 20 min. Cool down to about 60 °C before adding zeocin, then pour plates in 94 mm Petri dishes.
17. Glycerol stock of *P. pastoris* GlycoSwitch® (BioGrammatics Inc.) [14, 15].
18. YPD medium and plates: dissolve 10 g of yeast extract and 20 g peptone in 900 mL of water. For plates add 20 g agar. Sterilize by autoclaving for 20 min at 121 °C. Add 100 mL filter-sterilized 20% (w/v) glucose. Pour plates in 94 mm Petri dishes when the medium has cooled down to about 60 °C.
19. YPDS plates with 100 μg/mL zeocin: dissolve 10 g of yeast extract, 20 g peptone, and 182.2 g sorbitol in 900 mL of water, add 20 g agar and autoclave for 20 min at 121 °C. Add 100 mL filter-sterilized 20% (w/v) glucose. Cool down to about 60 °C, add 1 mL of 100 mg/mL zeocin, pour plates in 94 mm Petri dishes.
20. BMGY-Medium: dissolve 10 g of yeast extract and 20 g peptone in 798 mL ddH_2O. Add 100 mL 1 M potassium-phosphate-buffer, pH 6.0, and 10 mL glycerol. Autoclave for 20 min at 121 °C. Add 100 mL filter-sterilized 10× YNB (1.34% (w/v) yeast nitrogen base with ammonium sulfate in 1 L of water) and 2 mL 0.02% (w/v) filter-sterilized biotin.
21. BMMY-Medium: dissolve 10 g yeast extract and 20 g peptone in 793 mL ddH_2O, add 100 mL 1 M potassium-phosphate-buffer, pH 6.0. Autoclave for 20 min at 121 °C. Add 100 mL filter-sterilized 10× YNB, 2 mL 0.02% (w/v) filter-sterilized biotin, and 5 mL methanol.

Fig. 2 (continued) quantification of the substrate (no hyp) and product (1 hyp – 4 hyp) peptides. The fraction $NL_{hyp} \times 100/NL_{no\ hyp}$ was calculated as a measure of hydroxylation efficiency in percent, as shown in Figs. 3 and 4

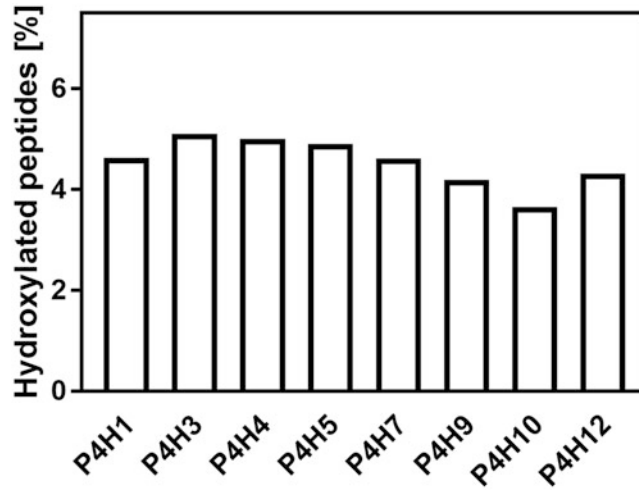


Fig. 3 Hydroxylation of the EXT peptide by Arabidopsis P4Hs. EXT was used as a substrate for indicated Arabidopsis P4Hs in the hydroxylation assay (procedure 3.10). The percentage of the hydroxylated relative to the non-hydroxylated peptide was calculated for the no-enzyme control and subtracted from the value obtained for the P4H-treated sample

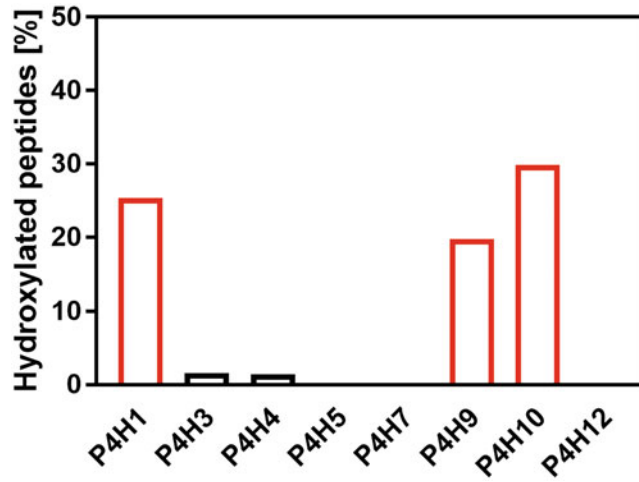


Fig. 4 Hydroxylation of the CLEL9 peptide by Arabidopsis P4Hs. CLEL9 was used as a substrate for indicated Arabidopsis P4Hs in the hydroxylation assay (procedure 3.10). The percentage of the hydroxylated relative to the non-hydroxylated peptide was calculated for the no-enzyme control and subtracted from the value obtained for the P4H-treated sample

2.2 Protein Purification and Dialysis

1. NiNTA Agarose (Qiagen).
2. Polypropylene chromatography column (e.g., Econo-Pac, BioRad).

3. Lysis buffer: 50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl. Immediately before the experiment, add zymolyase and DNase (a spatula tip for 10 mL), PMSF (100 μL of a 100 mM ethanolic stock solution for 10 mL), and a cocktail of protease inhibitors (containing 23 mM AEBSF, 100 mM EDTA, 2 mM bestatin, 0.3 mM pepstatin and 0.3 mM E-64; e.g., Sigma-Aldrich P8465 used according to the manufacturer's instructions).
4. Equilibration buffer: 50 mM sodium phosphate buffer, pH 7.0.
5. Wash buffer: 50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl, 20 mM imidazole.
6. Elution buffer: 50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl, 400 mM imidazole.
7. Overhead-shaker.
8. 0.45 μm nitrocellulose membrane filters.
9. Sonicator with tip probe.
10. 30% (v/v) ethanol.
11. Dialysis tubing (6 kDa MWCO; can be adjusted according to the mass of the protein of interest), and clamps.
12. 10% (w/v) Na_2CO_3.
13. TE buffer: add 5 mL 0.5 M EDTA and 2.5 mL 1 M Tris/HCl, pH 8.0, to 242.5 mL of water.
14. Dialysis buffer: 25 mM Tris/HCl, pH 8.0.

2.3 SDS-PAGE and Hydroxylation Assay

1. Standard equipment for SDS-PAGE analysis, including glass plates, spacers, and combs (e.g., Mini-PROTEAN® Tetra Handcast System; Bio-Rad), electrophoresis tank and power supply.
2. 40% (w/v) Acrylamide:bisacrylamaide (37.5:1) stock solution (e.g., Rotiphorese Gel 40; Carl-Roth GmbH).
3. Stacking gel buffer: 0.5 M Tris/HCl, pH 6.8, 0.4% (w/v) sodium dodecyl sulfate (SDS).
4. Separating gel buffer: 1.5 M Tris/HCl, pH 8.8, 0.4% (w/v) SDS.
5. 10% (w/v) ammonium persulfate (APS).
6. N,N,N′,N′-tetramethylethylenediamine (TEMED).
7. Loading buffer (4×): 200 mM Tris/HCl, pH 6.8, 400 mM 1,4-dithiothreitol, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 40% (v/v) glycerol.

8. Running buffer: 0.25 M Tris base, 1.92 M glycine, 1% SDS. pH should be close to 8.3, no adjustment required. Dilute 1:10 before use.
9. Pre-stained protein marker; use according to the manufacturer's instructions.
10. Commercial Coomassie Brilliant Blue protein stain; use according to the manufacturer's instructions.
11. CLEL9 peptide (sequence: DVDGLMDMDY(SO_3H)NSANKKRPIHNR). Take up the lyophilized peptide in sterile ddH_2O to result in a 1 mM stock solution and store it at −20 °C. Avoid repeated freeze-thaw cycles.
12. EXT peptide (sequence: SPPPPYVYSSPPPPYYHHHHHH). Take up the lyophilized peptide in sterile ddH_2O to result in a 1 mM stock solution and store it at −20 °C. Avoid repeated freeze-thaw cycles.
13. P4H reaction buffer: 25 mM Tris/HCl, pH 8.0, 100 mM DTT, 300 mM 2-oxoglutarate, 50 mM $FeSO_4$, 200 mM ascorbic acid.
14. 0.5 M EDTA: Weigh in 18.612 g disodium EDTA dihydrate and dissolve in 80 mL of water. Adjust pH to 8.0 with NaOH while stirring until completely dissolved. Add water up to 100 mL. Store at room temperature.
15. Spectrophotometer.

2.4 Mass Spectrometry and Data Analysis

1. Incubation shaker for microfuge tubes.
2. Vacuum concentrator.
3. PTFE (polytetrafluorethylene; Teflon™) membranes with embedded C18 beads (e.g., Supelco Empore™ solid phase extraction discs; Sigma-Aldrich/Merck).
4. Hypodermic needle: 17- or 18-gauge blunt-tipped syringe needle (e.g., B.Braun Sterican®).
5. Solvent A: 80% (v/v) acetonitrile, 0.5% (v/v) acetic acid in UHPLC-grade water.
6. Solvent B: 0.5% (v/v) acetic acid in UHPLC-grade water.
7. 50% acetonitrile (ACN), UHPLC-grade, in UHPLC-grade water.
8. Water, UHPLC grade.
9. Formic acid (FA), LC-MS grade.
10. 0.1% (v/v) trifluoroacetic acid (TFA), LC-MS grade.
11. High-resolution tandem mass spectrometer (e.g., Orbitrap Exploris 480; Thermo Fisher Scientific) coupled to a nano-high performance liquid chromatography (nano-HPLC)

system (e.g., Ultimate 3000 RSLCnano; Dionex, Thermo Fisher Scientific).

12. Reversed-phase chromatography trap column (μ-precolumn C18 PepMap100, 300 μm, 100 Å, 5 μm × 5 mm; Thermo Fisher Scientific) and analytical column (NanoEase M/Z HSS C18 T3, 1.8 μm 100 Å 75 μm × 250 mm, Waters, Germany) suitable for the corresponding nano-HPLC system.
13. Solvent C: 0.1% (v/v) formic acid, in UHPLC-grade water.
14. Solvent D: 0.1% (v/v) formic acid in 80% (v/v) acetonitrile, in UHPLC-grade water.
15. Personal computer with software package for peptide identification by MS/MS (Mascot 2.6; Matrix Science Ltd., London, UK or other search engines), peptide quantification (Xcalibur; Thermo Fisher Scientific), data visualization (Scaffold; Proteome Software, Portland, OR), and data analysis (Microsoft Excel).

3 Methods

The protein of interest (here the open reading frame of Arabidopsis P4Hs lacking start codon and signal peptide, equipped with a C-terminal His tag) was cloned into the yeast expression vector pPiCZαB, in frame with the α-factor signal peptide provided by the vector (*see* **Note 2**). Alternative constructs can be used depending on the specific research question. The expression constructs are linearized and transformed into *P. pastoris*.

3.1 Linearization of the pPiCZαB Vector

1. Inoculate 3 mL low-salt LB containing 25 μg/mL zeocin in five (*see* **Note 3**) 15 mL culture tubes each with a single colony of *E. coli* DH10B carrying the pPICZaB expression vector. Incubate overnight at 37 °C, 200 rpm.
2. Isolate the plasmid DNA from the overnight cultures using a commercial miniprep kit (*see* **Note 3**).
3. Determine the DNA concentration by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (1 A_{260} unit corresponds to 50 μg/mL plasmid DNA).
4. Combine 5–10 μg plasmid DNA, approximately 10 Units of the restriction enzyme (*see* **Note 4;** here *Sac*I, *Pst*I, *Bst*XI), 5 μL enzyme buffer and ddH_2O for a total volume of 50 μL in a reaction tube, incubate for 2–3 h at 37 °C, then stop the reaction at 70 °C for 5 min.
5. Add 50 μL phenol/chloroform, shake vigorously, centrifuge for 2 min at full speed in a microcentrifuge.
6. Transfer the upper aqueous phase into a new tube.

7. Add 2.5 × volumes of ethanol (p.a.) and 1/10 volume of 3 M sodium acetate.
8. Incubate at 4 °C for 15 min, then centrifuge at 4 °C, 15 min at full speed in a microcentrifuge.
9. Discard the supernatant.
10. Wash the pellet by adding 500 µL 70% ethanol, vortex and centrifuge at 4 °C for 15 min at full speed in a microcentrifuge.
11. Discard the supernatant, let the pellet dry and resuspend the DNA in 10 µL ddH_20. Store at −20 °C.

3.2 Production of Electro-Competent P. pastoris Cells

1. Prepare an overnight culture of *P. pastoris* GlycoSwitch. Inoculate 5 mL of YPD medium in a 15 mL culture tube and grow overnight at 30 °C, 200 rpm.
2. Inoculate 500 mL of YPD medium in a 2.8 L Fernbach flask with 0.1–0.5 mL of the overnight culture.
3. Incubate the culture at 30 °C, 300 rpm, to reach a density of 5–7 × 10^7 cells/mL (OD_{600} = 1.6–1.7; doubling time is approximately 2 h at 30 °C).
4. Decant the cell suspension into two sterile 250 mL centrifuge bottles and sediment the cells by centrifugation at 3000 × *g* for 5 min at 4 °C.
5. Carefully pour off and discard the supernatant.
6. Add 50 mL of sterile YPD to each of the bottles and vortex to resuspend the cells; add 1.25 mL of 1 M DTT to each bottle; mix gently.
7. Incubate the cells for 15 min at 30 °C.
8. Add 200 mL of sterile, ice-cold 1 M sorbitol to each centrifuge bottle. Collect the cells by centrifugation at 3000 × *g* for 5 min at 4 °C; pour off and discard the supernatant.
9. Add ~50 mL of sterile, ice-cold 1 M sorbitol to each of the bottles and vortex to resuspend the cells. Bring the volume in each of the centrifuge bottles to 250 mL with sterile, ice-cold 1 M sorbitol.
10. Collect the cells by centrifugation at 3000 × *g* for 5 min at 4 °C; pour off and discard the supernatant.
11. Resuspend each cell pellet in 10 mL of sterile, ice-cold 1 M sorbitol and pool in a chilled 30 mL Oakridge tube. Collect the cells by centrifugation at 3000 × *g* for 5 min at 4 °C; pour off and discard the supernatant.
12. Resuspend the cell pellet in 0.5 mL of sterile, ice-cold 1 M sorbitol; the final volume should be ~1.3 mL, and the cell concentration ~ 1 × 10^9 cells/mL. Keep the cells on ice and use immediately for electroporation.

3.3 Transformation by Electroporation

1. Mix 80 µL of competent *P. pastoris* cells with 5–10 µg of linearized vector (Subheading 3.1, **step 11**) in a 1.5 mL microfuge tube.
2. Transfer the suspension into an electroporation cuvette on ice.
3. Incubate the cuvette with the cells for 5 min on ice.
4. Pulse the cells using the electroporator. Use the settings for yeast transformation as recommended by the manufacturer.
5. Immediately add 1 mL of ice-cold 1 M sorbitol into the cuvette (*see* **Note 5**).
6. Transfer the cuvette content into a 15 mL culture tube.
7. Incubate at 30 °C without shaking for 1–2 h.
8. Spread 200 µL of the cell suspension on a YPDS plate containing 100 µg/mL zeocin.
9. Incubate the plate at 30 °C for 2–3 days until colonies form.
10. Pick at least 4 colonies and streak them on fresh YPDS plates containing 100 µg/mL zeocin.

3.4 Expression in P. pastoris

There usually is substantial variation in expression levels between different *Pichia* transformants. Therefore, in order to identify the clone with highest expression level, the protocols provided under Subheadings 3.4 to 3.9 should be completed for all four colonies obtained in the previous step.

1. Pre-culture: Inoculate 10 mL BMGY medium with each of the *P. pastoris* strains containing your P4H construct of interest and one untransformed control and incubate at 30 °C, 220 rpm, overnight until OD_{600} reaches 2–6 (*see* **Note 6**).
2. Harvest the cells by centrifugation at 1500 × *g* for 5 min at room temperature.
3. Main culture: Discard the supernatant and resuspend the pellet to an OD_{600} of 1.0 in BMMY medium (approximately 20–60 mL).
4. Transfer the cell suspension into a 500 mL flask and incubate at 30 °C, 220 rpm.
5. After 24 h, add methanol to a final concentration of 0.5% (v/v), sufficient for induced expression.
6. Harvest the cells after 48 h by centrifugation at 1500 × *g* for 5 min (*see* **Note 7**).
7. Transfer the supernatant to a fresh tube, filter through a 0.45 µm nitrocellulose membrane filter, and store at 4 °C before continuing with protein purification (Subheading 3.6).
8. The pellet can be stored at −20 °C or used directly for protein purification (Subheading 3.5).

3.5 Preparation of Proteins for Purification from the Cell Pellet

The secretion of the expressed protein into the culture supernatant may not be very efficient. We therefore recommend to test whether the target protein is present in the supernatant or still contained in the cell pellet. We thus present two protocols, one for protein purification from the cell pellet under this subheading, and one for protein purification from the culture supernatant under Subheading 3.6. We also recommend to perform a mock purification with an untransformed control culture.

1. Resuspend the pellet from 20 to 60 mL cell culture in 5 mL lysis buffer by vortexing.
2. Transfer the cell suspension into an Oak-Ridge tube.
3. Place the tube into an ice box and put the probe tip of the sonicator into the cell suspension, without touching the bottom of the tube.
4. Sonicate with at least three 30-s pulses with 30-s intervals, while keeping the tube on ice.
5. Centrifuge for 10 min at 21,000 × *g* and 4 °C.
6. Transfer the supernatant to a fresh tube.
7. If the supernatant still appears cloudy, centrifuge again as in **step 5** until the solution is clear.
8. Continue with Subheading 3.7.

3.6 Preparation of Proteins for Purification from the Culture Supernatant

1. Adjust the pH of the supernatant with 1 M KOH to pH 7.0 (the pH of the equilibration buffer).
2. To equilibrate the centrifugal concentrators, wash them twice by adding 20 mL of ddH_2O and centrifugation at 500 × *g* for 5 min at 4 °C.
3. Add 20 mL of equilibration buffer and centrifuge as before.
4. Add 20 mL of the filtered supernatant containing your protein of interest (Subheading 3.4, **step 7**), centrifuge at 500 × *g* at 4 °C (*see* **Note 8**).
5. Gradually add the rest of the supernatant, until the volume is reduced from 20–50 mL to 10 mL (it takes about 40 min per 10 mL to run through, depending on the protein concentration) (*see* **Note 8**).
6. Add equilibration buffer and centrifuge as before to reduce the volume to 10 mL. Repeat this step at least three times in order the exchange the BMMY medium of the supernatant with equilibration buffer.

3.7 Protein Purification by NiNTA-Agarose Chromatography

1. Add 1 mL of the resuspended NiNTA matrix (50% slurry) into each of two columns, let the buffer run out, resulting in 0.5 mL settled bed volume.
2. Wash the columns with 4 mL equilibration buffer.
3. Wash the columns 3 times with 6 mL wash buffer, then close the bottom end of the columns.
4. Load the protein solution prepared from cell pellet (Subheading 3.5, **step 7**) onto the first, and that from the culture supernatant (Subheading 3.6, **step 6**) onto the second column.
5. Close the columns, place them in an overhead shaker for at least 2 h (up to overnight) at 4 °C.
6. Stop the shaker and let the NiNTA agarose settle before opening the lids (around 10 min).
7. Collect the flow-through into a 15 mL culture tube (*see* **Note 9**); be careful to not let your columns run dry!
8. Wash the columns 3× with 4 mL wash buffer. Collect the wash fractions.
9. Add 400 µL elution buffer and collect the efflux in microfuge tubes (since the bed volume is 500 µL, this first elution step will not release much protein).
10. Add 500 µL elution buffer, collect the efflux (this fraction will include most of the His-tagged P4H).
11. Repeat **step 10**.
12. Apply 8 mL elution buffer to clean the columns.
13. Apply 8 mL column wash buffer.
14. Apply 8 mL 30% (v/v) ethanol to prevent microbial growth.
15. Store the columns at 4 °C.
16. To check for expression of the protein of interest, mix aliquots of the flow-through, the wash steps and the eluted fractions with 4× loading buffer and analyze them by SDS-PAGE (Subheading 3.8, starting at **step 2**).
17. Of the two eluate fractions, keep the one that contains most of the protein of interest as judged by SDS-PAGE analysis (Fig. 1). If both fractions contain substantial amounts of protein, pool them and store at 4 °C for further experiments.

3.8 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Mix 15 µL of the purified enzymes (i.e., the eluates from Subheading 3.7, **step 17**) with 5 µL of 4× loading buffer.
2. Incubate the samples at 95 °C for 10 min, then chill on ice.
3. Spin briefly to collect the condensate and sample at the bottom of the tube.

4. Mix 5.4 mL acrylamide solution with 4.5 mL separating gel buffer and 8.1 mL ddH_2O. Add 180 μL 10% APS and 18 μL TEMED, mix well and cast the separating gel (12%; volumes are sufficient for two standard-size mini gels, using, e.g., the Mini-PROTEAN® Tetra Handcast System). Overlay with isopropanol. Allow the gel to polymerize for at least 1 h. Remove the isopropanol. Overlay with stacking gel consisting of 1.7 mL stacking gel buffer, 0.75 mL acrylamide solution, 4.25 mL ddH_2O mixed with 70 μL 10% APS and 12 μL TEMED. Insert 1.5 mm, 10-well sample comb; polymerize for at least 1 h.
5. Remove the sample comb, place the gel cassette into the electrophoresis tank, add running buffer, and rinse the wells with running buffer using a blue-tip pipette.
6. Carefully load the samples into the wells next to the pre-stained protein marker, using a yellow-tip pipette.
7. Run the samples into the stacking gel for about 10 min at 80 V.
8. Continue electrophoresis at 120 V until the blue dye front runs out of the gel.
9. Dismantle the gel cassette.
10. Stain the gels with Coomassie Brilliant Blue, then destain following the manufacturer's instructions.

3.9 Dialysis

1. To prepare the dialysis tubing cut pieces sufficient to hold the eluate fractions (Subheading 3.7, **step 17**) and boil the tube in 10% Na_2CO_3 for 10 min.
2. Rinse the tubing in cold ddH_2O, then store in TE buffer.
3. Close one end of each piece of tubing with a knot or using a clamp, then fill with TE to make sure there are no leaks.
4. Remove the TE, then use a pipet to fill in the eluate fractions containing the purified proteins, close the tubing at the top end and place it into a beaker containing 1 L of dialysis buffer (*see* **Note 10**).
5. Incubate at 4 °C with gentle stirring using a magnetic stir bar. Change the dialysis buffer twice during dialysis (e.g., first change after 2 h, second change after overnight dialysis).
6. Transfer the dialyzed samples into 1.5 mL reaction tubes and centrifuge at maximum speed in a microfuge to sediment any insoluble material or precipitates, then transfer the supernatant into a fresh tube.

3.10 Hydroxylation Assay

1. Setup the P4H activity assay by mixing 85 μL of P4H reaction buffer and 5 μL of the peptide substrate (either the EXT or the CLEL9 peptide) in a microfuge tube.

2. Start the reaction by adding 10 µL of the purified P4H enzyme (from Subheading 3.9, **step 6**), or 10 µL of reaction buffer for the no-enzyme control.
3. Incubate at 25 °C for 2 h.
4. Stop the reaction by adding 10 µL 0.5 M EDTA.

3.11 Sample Preparation for Mass Spectrometry (MS)

Prior to MS analysis, peptides need to be purified from the reaction mixture, desalted, and concentrated by solid phase extraction on C18 ZIP tips or StageTips [16].

1. To prepare StageTips, use a hypodermic needle to punch out small disks from PTFE membranes with embedded C18 beads, and place them into one 200 µL "yellow" pipet tips. Use two discs per tip. Prepare one tip per sample to be purified (*see* **Note 11**).
2. Add 50 µL solvent A to each of the StageTips and spin in a microfuge tube (*see* **Note 11**) for 1 min at 2300 × *g*, 4 °C, then discard the flow-through.
3. Wash StageTips twice by adding 100 µL solvent B and spin as above, discard the flow-through.
4. Acidify the samples (from Subheading 3.10, **step 4**) to about pH 2 by adding 5 µL of TFA (*see* **Note 12**).
5. Load the entire sample onto the stage tips, spin for 1 min at 800 × *g* (*see* **Note 13**).
6. Wash twice by adding 150 µL solvent B, and spinning for 1 min at 2300 × *g*.
7. Transfer the StageTips to new reaction tubes.
8. Elute the samples from the filter by adding 20 µL of 50% acetonitrile and spinning at 800 × g for 1 min (*see* **Notes 14** and **15**).
9. Repeat **step 8**, resulting in a final volume of 40 µL of eluate.
10. Vacuum dry the samples and store at −20 °C until further analysis by mass spectrometry.

3.12 Nano-LC-ESI-MS/MS Analysis

The exact procedure will have to be adjusted to the equipment available in your laboratory or at the local mass spectrometry facility. Here, we used an Ultimate 3000 RSLCnano system (Dionex, Thermo Fisher Scientific) with a precolumn (µ-precolumn C18 PepMap100, 300 µm, 100 Å, 5 µm × 5 mm, Thermo Fisher Scientific) and a NanoEase analytical column (NanoEase M/Z HSS C18 T3, 1.8 µm, 100 Å, 75 µm × 250 mm column, Waters) operated at constant temperature of 35 °C. The RSLCnano system was coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific) using a NanosprayFlex source (Thermo Fisher Scientific). The Orbitrap Exploris 480 was operated under

the control of Xcalibur software (version 4.4., Thermo Fisher Scientific). Internal calibration was performed using lock-mass ions from ambient air [13].

1. Prepare an inclusion list with the mass-to-charge ratios of all peptides that should be selected for fragmentation by MS/MS (*see* **Notes 16–20**).
2. Adjust the flow rate of the HPLC system to 300 nL/min of solvent C.
3. Resuspend the samples (Subheading 3.11, **step 10**) in 50 μL 0.1% TFA (*see* **Note 12**) and prepare 100-fold to 200-fold dilutions in 0.1% TFA.
4. Inject 1 μL into the precolumn for nano-LC-ESI-MS/MS analysis.
5. Perform gradient elution with the following profile: 2–55% solvent D in 30 min, 55–95% solvent D in 10 min, 5 min isocratic at 95% solvent D, 10 min from 95% to 2% D and then re-equilibration for 10 min with 2% D.
6. Collect survey spectra ($m/z = 200–2000$) at a resolution of 60.000 at $m/z = 200$ using a fill time of 50 ms and a normalized AGC target of 300% (*see* **Note 21**).
7. Generate data-dependent MS/MS mass spectra for the 30 most abundant peptide precursors using high energy collision dissociation (HCD) fragmentation at a resolution of 15,000 with normalized collision energy of 30, 50 ms fill time, and a normalized AGC target of 50% (*see* **Note 21**).
8. Use the Mascot 2.6 (Matrix Science) (*see* **Note 22**) search engine for peptide identification, by searching spectra against a customized protein database (here the CLEL9 and EXT peptide sequences in FASTA format) including a suitable Uniprot database as background (here the *Arabidopsis thaliana* protein database).
9. As search parameters, do not specify a specific enzyme or the number of missed cleavages ("no enzyme"). Set mass tolerance at 5 ppm for peptide precursors and 0.02 Da for fragment ions. Do not specify fixed modifications except carbamidomethylation of cysteine, if alkylation of cysteine has been performed during sample preparation. Allow proline hydroxylation (oxidation (P)) and sulfation of serin, threonine, and tyrosin as variable modifications.
10. Transfer Mascot search results to Scaffold™ 4.10.0 (Proteome Software) for visualization (*see* **Note 23**).
11. Compare results from the database search with expected reaction products (here the CLEL9 or EXT peptides and their hydroxylated derivatives). m/z values of product peptides

should match the expectation within the specified mass accuracy. Validate peptide identification results by evaluation of MS/MS spectra as described under Subheading 3.13.

3.13 Analysis of the Data Obtained from MS

1. To confirm the presence of hydroxylated proline residues in the peptide samples, evaluate the MS data using the software Scaffold 4, with a protein and peptide threshold of 95.0% and the minimum number of peptides set to 1.
2. Select "Proteins" on the left side of the Scaffold window and check the peptides that are shown for possible proline-hydroxylation (*see* **Notes 24–27**).
3. Still on "Proteins", verify the position of the hydroxyproline within the peptide sequence and check the reliability of peptide identification using the "mascot ion score".
4. Select the tabs "spectrum" and "fragmentation table" for more detailed information on peptide identification (*see* **Note 25**).
5. For quantitative analysis of the MS data (Fig. 4), open the Qual Browser in the software Xcalibur and load the raw data file.
6. For the following steps, choose the mass corresponding to the charge state with the highest relative intensity for each target peptide (*see* **Notes 26–28**).
7. To load the extracted ion chromatograms (XICs) of the peptides of interest enter their mass-to-charge ratio (in the native and modified forms) as mass range with the following settings: Scan filter: FTMS + p NSI Full ms [200.000–2000.000]; Plot-type: Base peak; Gaussian Smoothing: 9 Points; Mass tolerance: 5 ppm (*see* **Note 29**).
8. Integrate the peaks of the chromatogram (*see* **Note 30**) and export the peak list to Microsoft Excel.
9. Continue the data analysis in Excel: divide the peak area of the modified peptide by the area of the corresponding native peptide to obtain the fraction of the modified peptide in the sample. The fraction of modified peptide in the no-enzyme control is considered as background noise and subtracted from all other samples (*see* **Note 31**).
10. Plot the results as shown in Figs. 3 and 4.

4 Notes

1. The pPICZα vector series (ThermoFisher) is used for the expression in *Pichia pastoris* of recombinant proteins fused at the N-terminus to the secretion signal of yeast α-factor, and at the C-terminus to a c-myc epitope and hexa-His tag. In-frame cloning with the C-terminal tag is facilitated by the three

reading frames provided in pPICZαA, B, C. In order to avoid any additional amino acids between the protein of interest and the C-terminal tags, we did not use the tags provided by the vector but incorporated the His tag in the primers used for PCR-amplification of the P4H ORF). The vector includes the AOX1 promoter for tightly regulated, methanol-inducible expression of the gene of interest and a Zeocin™ resistance gene for selection in both *E. coli* and *Pichia*.

2. To generate our P4H expression constructs, we used *Arabidopsis thaliana* flowers as starting material, i.e., the tissue in which these enzymes are most highly expressed. Total RNA was extracted from around 2–5 g of Arabidopsis (Col-0) flowers and used for first-strand cDNA synthesis with oligo-dT primers. The open reading frame of Arabidopsis P4Hs (P4H1, 3, 4, 5, 7, 9, 10, 12) lacking the N-terminal signal peptide and transmembrane helix, but including a C-terminal hexa-His tag, was amplified by PCR and cloned into pCR2.1-TOPO Invitrogen). The construct was amplified in *E. coli* DH10B and verified by sequencing. Positive clones served as starting material for cloning into the pPICZα expression vector.
3. Performing the plasmid miniprep requires a high amount (> 5–10 μg) of plasmid DNA for yeast transformation. To achieve this, combine multiple minipreps or perform one large-scale DNA preparation.
4. To promote integration, we recommend that you linearize your pPICZ construct within the 5′ AOX1 region. The vector map lists unique sites that may be used to linearize pPICZ prior to transformation. Other restriction sites are possible. Note that for the enzymes listed in the vector map, the cleavage site is the same for versions A, B, and C of pPICZ. Be sure that your insert does not contain the restriction site you wish to use to linearize your vector.
5. After electroporation, sorbitol should be added immediately to minimize cell death and to prevent leaking of yeast intracellular compounds.
6. Depending on the construct, growth of transformed yeast cells can vary substantially. Should the optical density (OD_{600}) be significantly above 6, the viability of the yeast culture is at risk, which can have a negative impact on product quality. In this case, it is recommended to inoculate a new culture at lower cell density.
7. To test which time of expression is most suitable for your protein of interest, take aliquots of 1 mL before and after 24 and 48 h of induction, centrifuge at 500 × *g* and store pellet and supernatant separately at −20 °C.

8. The concentrating and rebuffering steps on centrifugal concentrators can take quite some time (approximately 5–6 h, depending on the viscosity of the sample). Make sure to have sufficient time to complete this step without interruptions. If there is not sufficient time to perform the NiNTA-purification directly afterward, the protein solution can be loaded onto the column and incubated in an overhead shaker overnight at 4 °C.
9. During the entire procedure of NiNTA-purification, ensure that the column never runs dry, as this can prevent the protein from eluting off the resin.
10. The dialysis buffer should match the reaction buffer used in the subsequent hydroxylation assay.
11. Softeners in laboratory plasticware may dissolve in acetonitrile. Therefore, use high-quality plasticware (tips and tubes) with a track record of acetonitrile compatibility (e.g., from Eppendorf) to avoid any plasticizer leaking into the sample, which is detrimental to subsequent MS analysis.
12. TFA acid is corrosive; wear gloves and avoid inhalation of fumes.
13. Ensure to place the entire sample volume on the C18 stage tips, to avoid losing product. Should the final volume exceed the capacity of the tips, the solution can be added gradually between each centrifugation step.
14. In case the stage tips get clogged, the C18 material can be removed, washed with $_{dd}H_2O$ and inserted into the stage tip again.
15. Using only 50% acetonitrile for elution will elute only small peptides, which is beneficial in our case. Proteins of larger molecular weight are not eluted.
16. The inclusion list is used to increase the sensitivity of the method. It contains the m/z-ratios of both modified and native peptides to be preselected as ions of interest for fragmentation. To calculate the masses of the peptides, use one of many available web-based programs, for example, PeptideMass (expasy.org).
17. An inclusion list will introduce some bias into the analysis, as only the listed ions can be found. Therefore, try to cover as many peptide charge and modification states as possible, in order not to miss unexpected variants. Be aware, that depending on the ionization method used, the mass of the peptides can change, for example, if a positive charge is transferred by a proton.
18. For the peptide masses included in the list, the position of the modification does not matter, as it does not change the overall mass of the peptide. Consequently, adding the mass of an

oxygen covers the hydroxylation of any one of the prolines, if more than one are present in the peptide of interest.

19. Ions with lower charge state will usually give fragment spectra with higher quality.
20. The inclusion list only comprises monoisotopic masses. Deviations of values in the full scan spectra from the inclusion list arise due to naturally occurring heavy isotopes in the peptides.
21. AGC target values, fill times, and HCD collision energy are specific settings for Orbitrap mass spectrometers (Thermo Fisher Scientific). However, these experiments can also be performed on modern high-resolution Q-TOF mass spectrometers using appropriate instrument settings.
22. Mascot Server is a commercial product from Matrix Science Ltd. (London, UK). For the analysis of low-complexity samples like peptide mass fingerprints, Mascot Server can be used on the company's server without purchasing a license. However, this is of limited value for the LC-MS experiments described here, because the "no enzyme" search function is only available in the licensed version of Mascot. MS data analysis can also be performed using other commercial search algorithms (Sequest (Thermo Fisher Scientific), Byonic (Protein Metrics)) or freeware programs like Andromeda/MaxQuant, X! Tandem, or MSFragger/FragPipe.
23. This step is not essential, but facilitates data analysis.
24. To get an overview about possible modifications, you may want to reduce the threshold to 90, 85, or 80%. To conveniently screen through the sample, you can group the identified peptides by type of modification.
25. Additional validation parameters that could be used in Scaffold are the peptides spectrum/model error and the TIC intensity. Furthermore, some cross-validation can be conducted by comparing the peptides retention time with the retention time of the peaks used for the quantitative analysis.
26. As different modification variants usually have almost identical physico-chemical properties, it can be difficult to separate them by chromatography. This may result in superimposition of different variants in the same fragment spectrum.
27. To make sure not to miss any of the variants, the fragment spectrum can also be assigned manually.
28. Performing the analysis for multiple charge states of one peptide may result in more reliable outcomes.
29. To reduce workload, you may want to create a "layout" in Xcalibur comprising the settings mentioned and apply it to every sample.

30. As the integration limits are set manually, try to be consistent and set them as similar as possible for each sample.
31. Calculating the quotient of native and modified peptide abundance for each sample renders the samples comparable to each other. It also cancels out any dilutions of the samples that may have been done to increase sensitivity in MS analysis (Subheading 3.12, **step 3**).

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Chapter 6

Extraction of Apoplastic Peptides for the Structural Elucidation of Mature Peptide Hormones in *Arabidopsis*

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Abstract

Various secreted peptides, including peptide hormones, are present in the apoplast, but their biochemical characterization remains a challenge due to their low abundance, difficulty in extraction, and interference from numerous secondary metabolites. Here, we describe a simple and straightforward protocol for the extraction of apoplastic peptides with a high purity. This protocol takes advantage of the fact that apoplastic peptides diffuse and accumulate in the culture medium when *Arabidopsis* seedlings are subjected to whole-plant submerged culture. The peptides in the culture medium are efficiently recovered by *o*-chlorophenol extraction followed by acetone precipitation. The recovered peptides can be subjected to nano-liquid chromatography coupled to tandem mass spectrometry (nano-LC-MS/MS) without any additional clean-up. This procedure enables the structural elucidation of mature peptide hormones in the apoplast with the use of *Arabidopsis* plants that overexpress peptide hormone genes.

Key words Peptide hormone, Post-translational modification, Apoplast, Mass spectrometry, Peptidomics

1 Introduction

The apoplast is the free diffusional space between cells that is integral to intercellular signaling, and is indispensable for plant growth and development. One of the key signaling components in the apoplastic space are secreted peptides, which include peptide hormones that mediate a wide variety of biological processes through binding to their specific receptors [1]. In general, secreted peptides are initially translated as prepropeptides, then the N-terminal signal peptide is removed by signal peptidases to afford propeptides. Propeptides are further structurally modified by several modification enzymes to give biologically active mature peptides.

There is one structurally distinct group of secreted peptides that comprises those with a small size (less than 25 amino acid residues) due to proteolytic processing, and the presence of

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post-translational modifications, such as tyrosine sulfation, proline hydroxylation, and hydroxyproline arabinosylation. More than 10 currently known peptide hormone families are included in this group. Another major group comprises cysteine-rich peptides that are characterized by the presence of an even number of cysteine residues (typically 6 or 8) that participate in the formation of intramolecular disulfide bonds. In both cases, the activity of the peptides is critically affected by the peptide chain length, and the type and site of the modifications.

In general, the mature structures of peptide hormones cannot be predicted from the primary amino acid sequences of the propeptides; therefore, biochemical detection and structural elucidation of naturally occurring peptides in the apoplast are needed to clarify the exact function of peptide hormones. The conventional apoplastic fluid isolation technique often employs mechanical treatments, such as vacuum infiltration or centrifugation, which may lead to contamination by cytosolic components due to the rupturing of a certain fraction of cells [2]. Here, we describe a protocol that combines the whole-plant submerged culture technique and *o*-chlorophenol extraction as a simple and straightforward method for collecting apoplastic peptides with a purity high enough for direct analysis by nano-LC-MS [3]. This protocol enabled the determination of the mature structures of peptide hormones, such as CEP [3], RGF [4], CIF [5], and PSY [6], with the use of *Arabidopsis* plants overexpressing individual peptide hormone genes.

2 Materials

Prepare all solutions using ultrapure water and analytical-grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Whole-Plant Submerged Culture

1. *Arabidopsis* seeds (≈60 seeds) overexpressing the peptide hormone gene of interest.
2. 70% (v/v) ethanol.
3. Sterilization solution: 5% (v/v) sodium hypochlorite, 0.3% (v/v) Tween 20 in water.
4. Sterile water.
5. Half-strength MS medium plate: Half-strength MS medium supplemented with 1.0% (w/v) sucrose and solidified with 0.7% (w/v) agar in a 120 × 120 mm square Petri dish.
6. Erlenmeyer flask, 300 mL.
7. Porous silicone cap.

8. Filter-sterilized half-strength MS liquid medium: Half-strength MS medium supplemented with 1.0% (w/v) sucrose and 25 mM $CaCl_2$ (*see* **Note 1**).
9. Stainless steel forceps.

2.2 o-chlorophenol Extraction

1. Rotary evaporator.
2. Recovery flask, 200 mL.
3. Conical culture tubes, 50 mL.
4. *N*-ethylmorpholine (NEM): 1% (v/v) aqueous solution.
5. *o*-chlorophenol.
6. Parafilm.
7. 1% (w/v) Ficoll: Add 0.5 g of Ficoll 400–50 mL water and mix well.
8. Acetone.
9. Vacuum desiccator with pump.
10. Trifluoroacetic acid (TFA): 0.1% (v/v) in water.
11. Centrifuge for conical culture tubes.
12. Pasteur pipettes.
13. Sonicator bath.

2.3 Nano-LC-MS/MS Analysis

1. Nano-HPLC system and mass spectrometer.
2. C_{18} trap column (0.5 mm i.d. × 1 mm cartridge).
3. C_{18} nano-column (100 μm i.d. × 150 mm).
4. Solvent A: 0.1% (v/v) formic acid in HPLC-grade water.
5. Solvent B: 0.1% (v/v) formic acid in HPLC-grade acetonitrile.
6. Glass syringe with a blunt tip stainless steel needle.

3 Methods

3.1 Whole-Plant Submerged Culture

1. Transfer ≈60 *Arabidopsis* seeds into a 1.5-mL microcentrifuge tube.
2. Add 1 mL 70% (v/v) ethanol to the tube and vortex for 10 s.
3. Remove ethanol with micropipette, add 850 μL sterilization solution and mix for 10 min at room temperature on a rotator.
4. Spin briefly and discard supernatant with micropipette.
5. Wash seeds with 850 μL sterilized water 3 times.
6. Place the tube containing the seeds in sterile water for 2 days at 4 °C in the dark for stratification.
7. Distribute ≈60 seeds onto a half-strength MS medium plate using a 1 mL micropipette.

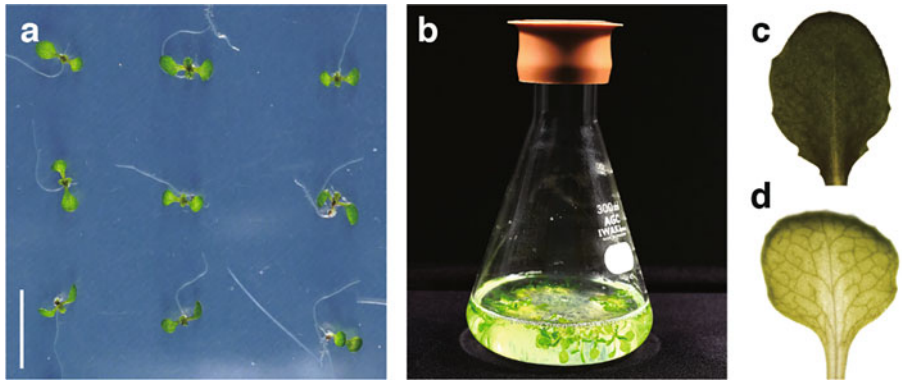


Fig. 1 Whole-plant submerged culture. (**a**) *Arabidopsis* seedlings grown on agar plate at 6 days after germination (scale bar, 10 mm). (**b**) Whole-plant submerged culture at 12 days after transfer. (**c**) Normal leaves grown in aerial condition. (**d**) Hyperhydric leaves grown under submerged culture condition

8. Incubate the plate at 22 °C for 6 days under continuous light (4000 lux, 50 μmol m^{-2} s^{-1}) (Fig. 1a).
9. Transfer 50 six-day-old seedling into 100 mL liquid half-strength MS medium in a 300 mL Erlenmeyer flask. Use forceps and be careful not to damage the seedlings.
10. Incubate the fully submerged seedlings for 12 days without shaking under continuous light (4000 lux, 50 μmol m^{-2} s^{-1}) at 22 °C (Fig. 1b). Leaves will become hyperhydric with large water-filled intercellular spaces and will thus appear translucent (Fig. 1c, d).

3.2 o-chlorophenol Extraction

1. In order to prepare water-saturated *o*-chlorophenol (*see* **Notes 2** and **3**), place 10 mL 1% (v/v) NEM and 20 mL *o*-chlorophenol into a 50 mL culture tube. Close tube and seal around lid with Parafilm (Fig. 2a).
2. Wrap the tube in a paper towel and place it inside a zipper bag (*see* **Note 4**) (Fig. 2b). Shake vigorously for 5 min.
3. Centrifuge the tube at 350 × *g* for 5 min and store at 4 °C until use. Note that water-saturated *o*-chlorophenol separates into the lower layer (Fig. 2a).
4. Transfer the 12-day culture medium (≈100 mL; step **3.1.10**) by decantation into a 200 mL recovery flask.
5. Concentrate to 1/5 volume (≈20 mL) using a rotary evaporator on a water bath at 37 °C.
6. Transfer the concentrate to a 50 mL conical culture tube and centrifuge at 3800 × *g* for 5 min.
7. Transfer the supernatant to a new 50 mL tube (Fig. 3a).

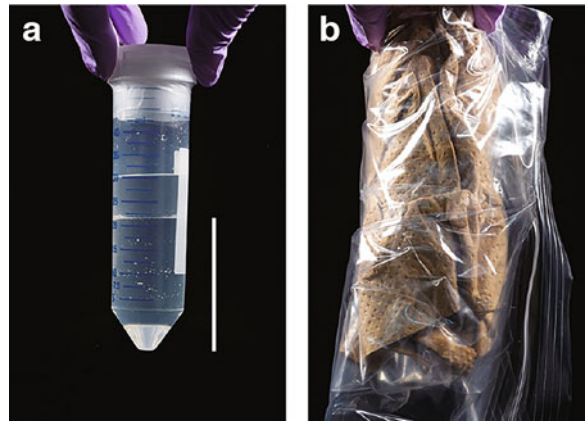


Fig. 2 Preparation of water-saturated *o*-chlorophenol. (**a**) When handling the tubes containing *o*-chlorophenol, seal around lid with Parafilm before shaking to avoid leakage. Bar represents *o*-chlorophenol layer. (**b**) Cover the entire tube with a paper towel and place it inside a zipper bag before shaking

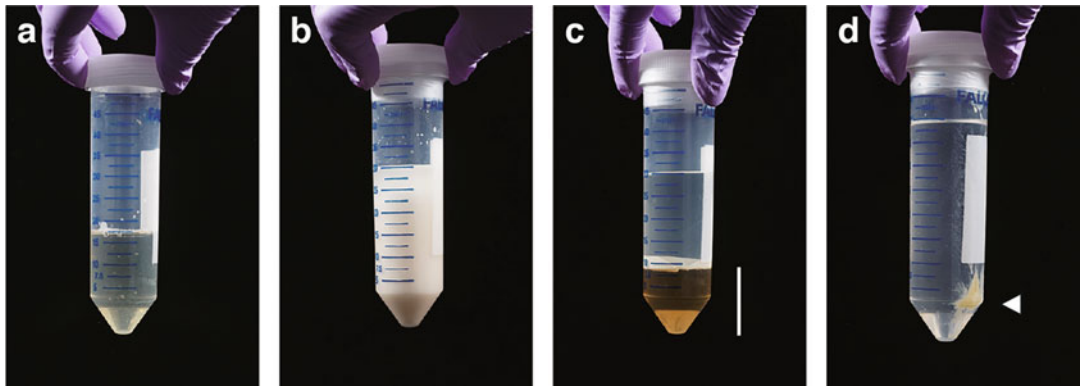


Fig. 3 *o*-Chlorophenol extraction of the peptides. (**a**) Appearance of concentrated culture medium sample. (**b**) Appearance of the sample just after shaking with *o*-chlorophenol. (**c**) Appearance of the sample after centrifugation. Bar represents *o*-chlorophenol layer. (**d**) Appearance of the tube after acetone precipitation. Arrowhead represents precipitated peptides

8. Add NEM to the concentrated culture medium at a final concentration of 1% (v/v). Typically, add 200 µL NEM for 20 mL concentrated media.
9. To the concentrated culture medium in a 50 mL centrifuge tube, add 10 mL of water-saturated *o*-chlorophenol (lower layer from step 3 above) using a Pasteur pipette and shake vigorously by hand for 1 min (*see* **Notes 2** and **5**) (Fig. 3b).
10. Centrifuge at 10,000 × *g* for 20 min at room temperature. *o*-chlorophenol separates into the lower layer (Fig. 3c).
11. Collect the *o*-chlorophenol layer (≈10 mL) using a Pasteur pipette and divide into two new 50 mL culture tubes

(≈4.5 mL each) (*see* **Note 6**). Be careful not to aspirate boundary layer impurities.

12. Add 10 volumes of acetone (≈45 mL).
13. Incubate at −20 °C overnight to precipitate peptides.
14. Centrifuge at 10,000 × *g* for 10 min at 4 °C (Fig. 3d).
15. Carefully remove the supernatant by decantation and combine the pellets (in two 50 mL culture tubes) into one new 50 mL centrifuge tube using a pipette with a small amount of acetone.
16. Wash pellets with 30 mL of acetone and centrifuge at 10,000 × *g* for 10 min at 4 °C. Carefully remove the supernatant by decantation.
17. After air-drying the pellets in ambient air for 15 min, add 500 μL of 0.1% TFA, dissolve the precipitate in a sonicator bath and transfer to a 1.5 mL tube.
18. Centrifuge at 15,000 rpm in a microfuge for 5 min at room temperature and transfer the supernatant to a new 1.5 mL tube.
19. Aliquots of the peptide fractions (5 μL) are analyzed by conventional nano-LC-MS/MS (*see* **Note 7**).

4 Notes

1. Cationic basic peptides are often captured by the negatively charged pectin in the cell wall. Calcium binds strongly to pectin, facilitating the diffusion of basic peptides into the medium.
2. The entire protocol should be performed under a fume hood. *o*-chlorophenol has a very strong odor and is extremely hazardous. Always wear a lab coat, certified safety glasses, and suitable gloves during experiments.
3. Prepare water-saturated *o*-chlorophenol at the time of use.
4. Paper towels change color as they absorb liquid, making it easy to notice leaks (Fig. 2a, b).
5. The mixture often emulsifies and becomes cloudy. Some culture media may be colored during this step. Neither of these affect extraction efficiency.
6. The lower layer (phenolic layer) may vary in color depending on the culture media conditions.
7. The conditions may have to be adapted to the nano-LC ESI-MS/MS system to be used. Our nano-LC conditions are as follows: Aliquots of peptide fractions (5 μL) are loaded onto a C_{18} trap column (0.5 mm i.d. × 1 mm cartridge; KYA Technologies) and washed with 10 μL of 0.1% formic acid.

Peptides are subsequently eluted from the precolumn and separated on a MonoCap C_{18} Fast-flow nano-column (100 μm i.d. × 150 mm; GL Sciences) using a gradient of 2–35% acetonitrile containing 0.1% formic acid for 30 min at a flow rate of 500 nL/min. Tandem mass spectra were obtained by scanning the mass range from m/z 350 to m/z 1500 using data-dependent acquisition methods with HCD fragmentation at a normalized collision energy of 30 or 35.

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Part II

Peptide Activity and Signaling



Chapter 7

Assaying the Effect of Peptide Treatment on H^+-Pumping Activity in Plasma Membranes from Arabidopsis Seedlings

Nanna Weise Havshøi and Anja Thoe Fuglsang

Abstract

Extracellular acidification or alkalization is a common response to many plant-signaling peptides and microbial elicitors. This may be a result of peptide-mediated regulation of plasma membrane-localized ion transporters, such as the plasma membrane H^+-ATPase. Early responses to some signaling peptides can therefore be analyzed by assaying H^+-pumping across the plasma membrane.

We describe a set-up suited for the purification of plasma membranes by aqueous two-phase partitioning from a small sample of Arabidopsis seedlings. Seedlings are grown in a liquid culture, suited for the analysis of in vivo peptide treatment. Additionally, we describe how to measure the H^+-pumping activity of the plasma membrane H^+-ATPase using the fluorescent probe ACMA.

Key words Plasma membrane, Membrane vesicles, Liquid seedling culture, H^+ pumping assay, H^+-ATPase, In vivo peptide treatment, Two-phase partitioning

1 Introduction

The plant plasma membrane is the outermost membrane of the cell, all information and nutrients targeted for the cell must therefore pass the plasma membrane. This is taken care of by membrane proteins such as receptors and ion transporters. Of particular importance is the plasma membrane H^+-ATPase, it contributes to maintenance of the low pH in the apoplastic space. The proton pump creates a H^+-gradient across the plasma membrane and thereby contributes to the plasma membrane potential, required for an efficient nutrient uptake into the cells. A lower apoplastic pH is suggested as required for loosening of the cell wall during cell elongation and growth, and lastly as a defense response during pathogen attack [1–3].

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Some plant signaling peptides modulate cellular ion fluxes by inducing post-translational modifications (e.g., phosphorylation) of ion transporters in the plasma membrane, including the H^+-ATPase. This causes disturbance of the membrane potential and pH in the cytosol and apoplast, which can subsequently translate into other cellular responses. Among these signaling peptides are endogenous peptides like systemin, RALF, PSK, and PSY peptides, as well as microbe- and damage-associated molecular patterns like flg22, that are suggested to modify apoplastic pH by regulation of the plasma membrane H^+-ATPase [4–8], or other H^+-coupled ion transporters. The apoplastic pH effect can be caused by the activation/de-activation of the plasma membrane H^+-ATPase. This can be achieved by direct interaction of the receptor-peptide complex with the ion transporter, as seen for PSY1R and AHA2/1 [6], or indirectly via a receptor/peptide-activated signal transduction pathway leading to the activation of the ion transporter or channel [4, 5, 9]. Rapid alkalinization factor (RALF) peptides, for example, increase the pH of the apoplast (hence their name) by a signaling pathway, containing a calcium-stimulated protein kinase where the intracellular calcium spike is a pre-requisite for the de-activation of the H^+-ATPase [6, 10].

Here we describe a protocol to assay the effect of plant peptide signals and growth factors, and of pathogen-derived elicitors on plasma membrane H^+-ATPase activity. The method presented allows to test the effect of peptides applied in vitro or in vivo and, additionally, we previously used it to assess the effect of nutrient treatments [11]. Biochemical characterization of plasma membrane proteins in *Arabidopsis thaliana* can be a challenge because of the time required to produce sufficient amounts of the plant material and for plasma membrane preparation. In this paper, we present a method suited for the generation of plasma membranes from a small sample of Arabidopsis seedlings in a relatively fast manner: growth of seedlings and purification of plasma membranes, by two-phase partitioning, in 2 weeks. We describe how to grow the seedlings in liquid culture, suited for the analysis of in vivo peptide treatment. We then describe how to isolate plasma membranes from this small amount of plant material by aqueous two-phase partitioning. Aqueous two-phase partitioning is the most efficient way to obtain relatively pure plasma membranes from plant material. The method presented here is based on the most common biphasic system formed by two polymers, polyethylene glycol (PEG) and dextran [12, 13] (*see* **Note 1**).

Finally, we describe how to measure the H^+ pumping activity of the plasma membrane H^+-ATPase. We utilize the fluorescent probe 9-amino-6-chloro-2-methoxyacridine (ACMA) for the detection of H^+ transport into inside-out vesicles (*see* **Note 2**). Fluorescent amines are widely used to detect differences in transmembrane proton concentration (ΔpH) in natural and model membrane

systems, as they are freely permeable through the membrane phase in their neutral form and, therefore, re-distribute between the inner and outer aqueous compartments following the generation of a transmembrane ΔpH (acidic inside). Upon protonation the probe is caught inside the vesicle leading to quenching of the fluorescent signal. The decrease in fluorescent signal is therefore a direct measure for the transport of H^+ into the vesicle [14, 15].

2 Materials

2.1 Arabidopsis thaliana *Growth and In Vivo Peptide Treatment*

1. 1 L Erlenmeyer bottles without baffles.
2. Orbital shaker adequate for Erlenmeyer flasks in plant climate chamber (24 °C).
3. Horizontal Tube roller mixer.
4. 50 mL culture tubes.
5. Sterilization of Arabidopsis seeds: 3 − < 5% (w/w) sodium hypochlorite solution, 37% HCl, 50 mL glass beaker, airtight container, and 50 mg seeds in a microfuge tube (*see* **Note 3**).
6. Growth medium: ½-strength Murashige and Skoog Basal Medium (MS), 1% (w/v) sucrose, pH 5.7; add 2.165 g MS salts including vitamins and 10 g sucrose to 1 L deionized water, adjust to pH 5.7 with NaOH. For solid medium, add 7 g/L plant agar. Autoclave for 12 min, cool down to about 60 °C, then pour into Petri dishes (Optional: add 100 mg/L ampicillin to avoid contaminations).
7. RALF33, RALF36: Peptides were custom synthesized at >95% purity. Prepare stock solutions of custom-synthesized peptides in sterile deionized water.
8. Peptide treatment solution: Dilute RALF peptides to 1 μM in growth medium. You will need 20 mL per treatment.

2.2 Plant Tissue Homogenization and Microsomal Isolation

1. Liquid nitrogen.
2. Porcelain mortar and pestle.
3. Ultracentrifuge, with rotor and tubes (e.g., 26.3 mL ultracentrifuge tubes from Beckman Coulter).
4. 2 mL Dounce glass homogenizer with Teflon pestle.
5. Crushed ice and ice buckets.
6. Homogenization buffer: 50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 0.33 M sucrose.
7. Protease inhibitor: 0.1 M phenylmethylsulphonyl fluoride (PMSF) stock in isopropanol.
8. 330/5 buffer: 330 mM sucrose, 5 mM potassium phosphate buffer, pH 7.8.

9. 100 mM DTT.
10. 500 mM EDTA, pH 8.0.

2.3 Aqueous Polymer Two-Phase Partitioning and Isolation of Plasma Membranes

1. Tube roller mixer.
2. Ultracentrifuge, with rotor and tubes (e.g., 26.3 mL ultracentrifuge tubes from Beckman Coulter).
3. 1 mL Dounce glass homogenizer with Teflon pestle.
4. 20% (w/w) dextran T500.
5. 40% (w/w) PEG4000.
6. 0.2 M potassium phosphate buffer, pH 7.8.
7. 2 M KCl.
8. Standard protein assay, e.g., Pierce™ BCA™ Protein assay kit.
9. Homogenization buffer: 50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 0.33 M sucrose.

2.4 Measurement of H^+-Pumping

1. Spectrofluorometer with cuvettes (200 µL–1 mL), or fluorescence plate reader with black 96-well plates.
2. 0.1 M ATP-KOH.
3. H^+-Pumping buffer: 20 mM MOPS/KOH, pH 7, 40 mM K_2SO_4, 25 mM KNO_3, 1 µM 9-Amino-6-Chloro-2-Methoxyacridine (ACMA), 60 nM valinomycin, 0.05% (w/v) polyethylene glycol hexadecyl ether (Brij-58).
4. $MgSO_4$ stock: 0.5 M $MgSO_4$ (for cuvette assays), or 60 mM $MgSO_4$ (for 96-well plate assay).
5. Nigericin stock: 40 µM nigericin (for cuvette assays), or 6 µM nigericin (for 96-well plate assays).

3 Method

3.1 Arabidopsis thaliana Growth and In Vivo Peptide Treatment

1. Day 1: Surface sterilize seeds by gas; add 50 mL hypochlorite solution into a glass beaker in an airtight container, add 50 mg of seeds into a microfuge tube and place it with open lid in the container. Quickly add 1 mL of 37% HCl and close the airtight container to allow chlorine gasses to form. Sterilize seeds for 2 h.
2. Transfer sterilized seeds (*see* **Note 3**) to growth plates (*see* **Note 4**) and incubate 1 day at 4 °C in darkness (see Fig. 1 for experimental setup).
3. Day 2: Shift plate(s) to long day climate chamber (16 h light/8 h dark) and incubate vertically for 7 days at 24 °C for initial growth (*see* **Note 5**).

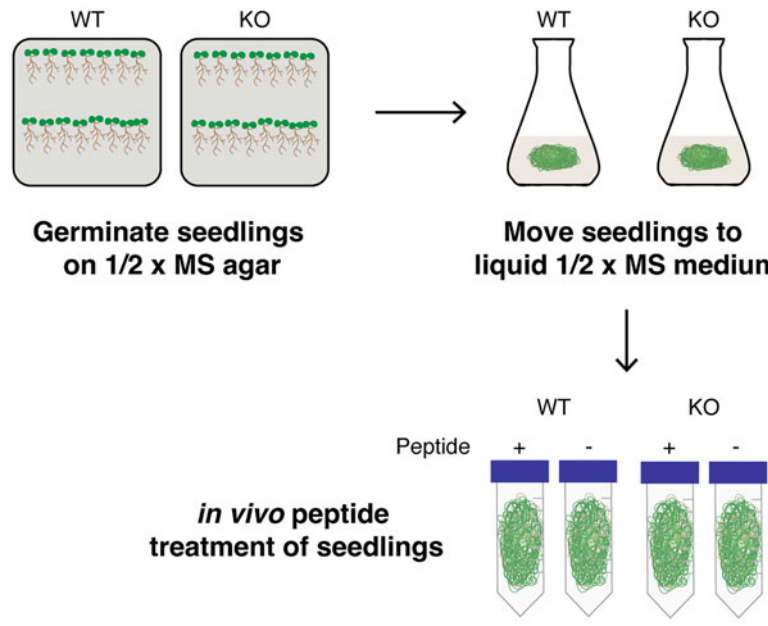


Fig. 1 Plant growth and in vivo treatment. Germinate seedlings on ½ × MS agar plates, this will increase the germination rate compared to germination in liquid culture. Transfer the seedlings to liquid cultures after germination and grow for additionally 3 days to increase material needed for plasma membrane purification. In vivo peptide treatment of *A. thaliana* seedlings before tissue homogenization, to see effect on the plasma membrane H^+-ATPase activity. The treatment can also be done with other compounds regulating the activity of the plasma membrane H^+-ATPase

4. Day 9: Transfer seedlings to 500 mL growth medium in a 1 L Erlenmeyer flask (Fig. 1) and grow for an additional five days (*see* **Note 6**) at 150 rpm in a growth chamber (24 °C, 24 h light).
5. Day 14: Harvest seedlings from liquid medium and weigh plant material (>10 g seedlings are needed for plasma membrane isolation).
6. Transfer harvested seedlings to 20 mL peptide treatment solution in a 50 mL culture tube and incubate on a roller mixer for 30 min.
7. Remove peptide treatment solution and continue to tissue homogenization (Fig. 1).

3.2 Preparation of Polymer Solution for Aqueous Two-Phase Partitioning

1. Day 13 (1 day before plasma membrane purification): To prepare polymer solution, weigh in 3.72 g of 20% (w/w) Dextran T500, 1.86 g of 40% (w/w) PEG4000, 1.017 g of sucrose. Add 225 µL of 0.2 M potassium phosphate pH 7.8, 18 µL of 2 M KCl, fill up with water to 9 g. Final concentration in the polymer solution: 6.2% Dextran T500, 6.2% PEG4000,

0.33 M sucrose, 5 mM potassium phosphate pH 7.8, 3 mM KCl (*see* **Notes 7** and **8**).

2. Mix gently (*see* **Note 9**) overnight in the cold (4–10 °C).

3.3 Plant Tissue Homogenization and Microsomal Isolation

1. Day 14: Flash freeze seedlings in mortar with liquid N_2 and grind seedlings using pestle (*see* **Note 10**), add 1 mL homogenization buffer per g seedlings, when the ground seedlings begin to thaw.
2. Continue homogenization until sample is thawed and completely homogenized.
3. Add PMSF to a final concentration of 0.1 mM.
4. Centrifuge sample at 10,000 × *g* for 15 min at 4 °C to remove cell wall debris.
5. Transfer supernatant to ultracentrifuge tube (*see* **Note 11**) and centrifuge at 50,000 × *g* for 30 min at 4 °C (*see* **Note 12**).
6. Remove the supernatant (soluble proteins) and collect the pellet (microsomal fraction) by suspending in 2 ml 330/5 buffer, supplemented with 1 mM DTT and 0.1 mM EDTA pH 8 (*see* **Note 13**).
7. Transfer microsomal fraction to Dounce glass homogenizer and homogenize the sample on ice (*see* **Note 14**).
8. Continue with two-phase partitioning (Subheading 3.4) on the same day.

3.4 Aqueous Two-Phase Partitioning and Isolation of Plasma Membranes

The separation of plasma membranes should be done on ice or in a cold room, to ensure the activity of the plasma membrane H^+-ATPase.

1. Dilute the microsomal fraction to just above 3 mL (*see* **Note 15**).
2. Transfer the microsomal fraction to the top layer of a 9 g partitioning system (Fig. 2a) to a final weight of 12 g (9 g polymers/3 g microsomal fraction; *see* **Note 16**).
3. Mix the sample 10 times as shown in Movie 1 (*see* **Note 17**).
4. Centrifuge samples at 1000 × *g* for 5 min at 4 °C and collect the top phase (plasma membrane fraction, T1) (*see* **Note 18**; Fig. 2a). If T1 is still green or greenish, it needs to be further purified (*see* **Note 19**). Alternatively, if T1 is milky-colored and clear, proceed with **step 5**.
5. Use a pipet to carefully transfer the top phase with plasma membranes to an ultracentrifuge tube, dilute sample with 330/5 buffer until the ultracentrifuge tube is full, and centrifuge at 100,000 × *g* for 1 h at 4 °C to pellet the plasma membranes.

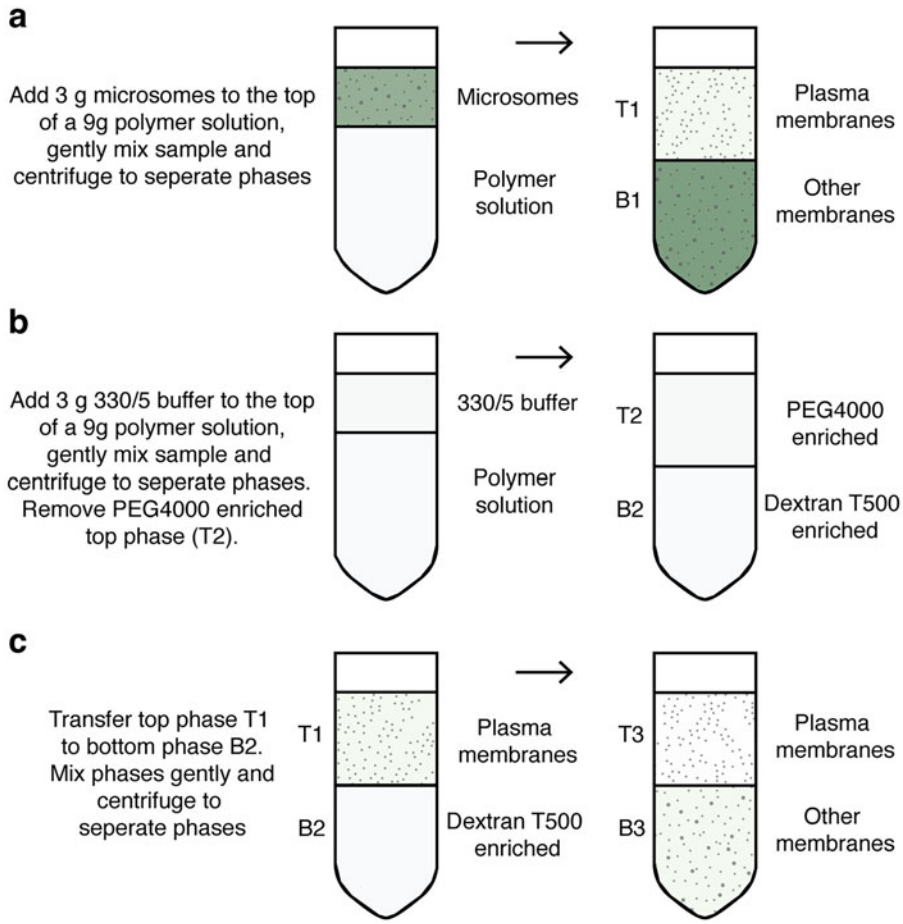


Fig. 2 Aqueous two-phase separation steps. (**a**) Layer microsomal fraction on top of a polymer solution, mix and centrifuge to separate plasma membranes from other internal membranes. (**b**) Preparation of Dextran-enriched bottom phase (B2), by layering 330/5 buffer onto a polymer solution. Removal of the PEG4000-enriched top phase (T2) after mixing and centrifugation. (**c**) Further purification of plasma membranes top phase (T1) by layering on top of a dextran-enriched bottom phase (B2)

6. Resuspend the plasma membranes in 250–500 μL 330/5 buffer supplemented with 50 mM KCl and 1 mM EDTA pH 8 and transfer to a 1 mL Dounce glass homogenizer (*see* **Note 20**).
7. Aliquot sample into five separate tubes.
8. Determine protein concentration using a standard method.
9. Adjust protein concentration of the five aliquots to 1 μg/μL by dilution with 330/5 buffer supplemented with 50 mM KCl and 1 mM EDTA pH 8; flash freeze in liquid nitrogen and store at −80 °C.

3.5 Cuvette H^+-Pumping Assay

This assay has been optimized for a 1 mL volume (*see* **Note 21**), to be measured in a cuvette placed in a spectrofluorometer. The spectrofluorometer has the advantage that H^+-pumping can be read and recorded continuously. The disadvantages are the rather large assay volume that requires large amounts of plasma membrane vesicles to detect activity, and that only few recordings can be made each time, making multi-replication time-consuming. Alternatively, the assay can be performed in a microplate-based format in a fluorescence microplate reader (*see* Subheading 3.6). The assay can be initiated by the addition of ATP or $MgSO_4$ (Mg^{2+} for the ATP complex), here we use $MgSO_4$.

1. Adjust the spectrofluorometer wavelengths for emission (412 nm) and excitation (480 nm) for ACMA (*see* **Note 22**).
2. Add 10 μL (a total of 10 μg) of plasma membrane vesicles, from a concentrated stock adjusted to 1 μg/μL (**step 3.4.9**), at one side of the cuvette (*see* **Note 23**).
3. Add 20 μL of 0.1 M ATP-KOH at the opposite side of the cuvette.
4. Fill the cuvette with H^+-pumping buffer to a final volume of 1 mL.
5. Transfer the cuvette to the spectrofluorometer (*see* **Note 24**) and wait for baseline stabilization, approximately 60–120 s (*see* **Note 25**).
6. Initiate the assay by adding 4 μL 0.5 M $MgSO_4$ and record fluorescence for 600 s (see **Note 26**).
7. Optional: The assay can be stopped by adding 2 μL of 40 μM nigericin, dissipating the membrane potential. Continue reading for 120 s or until signal is stable.
8. Plot data normalized relative to baseline fluorescence, or the level at assay start, as shown in Fig. 3a.

3.6 Microtiter Plate H^+-Pumping Assay

H^+-pumping activity can also be determined using a fluorescence reader supporting 96-well plates. The advantage of using plates rather than cuvettes is a reduction of the assay volume and thereby the amount of plasma membrane vesicles required. Also, more replications can run in the same settings, saving time. It may be a disadvantage that some plate readers have a delay when initiating recoding and the datapoints from the beginning of the assay might be lost.

1. Adjust emission (412 nm) and excitation (480 nm) wavelengths of the microplate reader for ACMA (*see* **Note 22**).
2. Add 2.5 μL from a plasma membrane stock adjusted to a concentration of 1 μg/μL protein at the bottom of the well (*see* **Notes 27** and **28**).

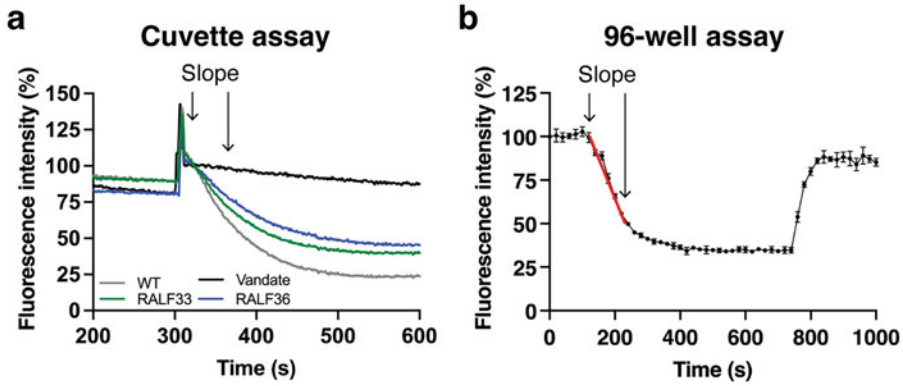


Fig. 3 H^+-pumping activity of plant plasma membrane H^+-ATPase. (**a**) Cuvette assay, with initial baseline until 300 s, H^+-pumping is started at 300 s and quenching of ACMA plateaued at 600 s. Activity of the enzyme can be calculated from the linear slope rate of the initial activity, which is indicated with arrows. Cuvette assays are performed in triplicates, where activity is shown in fluorescence intensity (%) normalized to the beginning of H^+-pumping. Activity is shown from in vivo treatment with RALF33 and RALF36 peptides, and the P-type ATPase inhibitor, vanadate. (**b**) 96-well plate assay, with initial baseline until 120 s, when H^+-pumping is started, and activity measured for 600 s until quenching of ACMA plateaued. Nigericin is added to the assays at 720 s, to dissipate membrane potential and stop the assay. H^+-pumping activity of the plasma membrane H^+-ATPase can be calculated from the linear slope (rate) of the initial activity, indicated by the red line and arrows. 96-well assays were performed in triplicates, and activity is shown as fluorescence intensity (%), where data has been normalized to the initial baseline

3. Add 3 μL of 0.1 M ATP-KOH.
4. Add H^+-pumping buffer to a final volume of 145 μL.
5. Start baseline reading and let signal stabilize for 120 s (*see* **Notes 29** and **30**).
6. Add 5 μL of 60 mM $MgSO_4$ to start assay (*see* **Note 31**).
7. Initiate reading immediately after the addition of $MgSO_4$ and continue for 600 s (*see* **Note 32**).
8. Optional: Stop assay by adding 2 μL of 6 μM nigericin. Continue reading for 120 s or until signal is stable.
9. Plot data normalized relative to the baseline fluorescence, or the level at assay start, as seen in Fig. 3b.

4 Notes

1. Partitioning is greatly influenced by the molecular weight and the ratio of the two polymers. This protocol is optimized for Arabidopsis seedlings/leaf material. If different plant sources are used, it might be required to optimize the ratio between PEG and dextran.

2. The plasma membrane preparations obtained are of high purity and consist mainly of sealed right-side-out (apoplastic side-out) vesicles. The vesicles can be turned inside-out (cytoplasmic side-out) by addition of the detergent Brij58 [16]. Preparations of inside-out vesicles are ideal for studies on properties on the plasma membrane that require a tight vesicle, such as transport, signal transduction mechanisms, and enzyme topology. Biochemical measurement of ion fluxes across membranes requires tight vesicles, meaning no ions are leaking out unless transported via a membrane transporter.
3. In the experiment described here, we used the Arabidopsis Col-0 wild type. However, you also can compare different genotypes with respect to their performance in the H^+-pumping assay, for example, a peptide receptor knock-out mutant compared to the wild-type control. Likewise, you can compare the effect of different peptide treatments in a single genotype. In either case, make sure you generate enough plant material. You will need at least 10 g plant material per sample, which is achievable from 50 mg seeds.
4. Sow 50 mg seeds in horizontal rows, there can be 2–3 rows per plate (Fig. 1). Distribute the seeds on 4 large plates.
5. Higher germination rates are obtained by initial germination on plates instead of directly in liquid media as we have previously observed [[11].
6. Incubation in liquid medium can be shortened or prolonged for optimal growth. The goal is to gain high amounts of plant material without stressing the seedlings. More than 10 g of seedlings is needed for optimal plasma membrane isolation.
7. The polymer solution has been optimized for purification of plasma membranes from *A. thaliana*. Changing the ratio between PEG4000 and Dextran T500 can affect the partitioning of plasma membranes, it is therefore important to be meticulous when preparing the polymer solution.
8. The polymer solutions for aqueous two-phase separation of *A. thaliana* plasma membranes should be prepared 1 day in advance, then stored at 4 °C. The polymer solutions can be used for up to 1 week.
9. The polymer solution must be mixed gently, use a tube roller mixer. The polymer solution will not be able to partition into phases if the polymer solution is mixed too vigorously. Do not vortex!
10. Pre-cooling of pestle and mortar with liquid nitrogen can maintain seedlings frozen for a longer period which will restrict protease activity during tissue homogenization.

11. Adjust weight and volume of the samples with homogenization buffer if needed.
12. If no ultracentrifuge is available, the microsomal fraction can be pelleted at 30,000 × *g* for 1 h at 4 °C in a normal high-speed centrifuge.
13. Resuspend the microsomal fraction in 330/5 buffer by pipetting up and down until pellet is released from ultracentrifuge tube. Transfer the microsomal fraction to a glass Dounce homogenizer, in order to completely homogenize the sample.
14. It is important not to let air into the Dounce glass homogenizer during homogenization, as this will cause air bubbles. Keep the homogenate under vacuum when moving the pestle up and down. Continue until the microsomal fraction is completely solubilized, this can take a while, normally 10–20 strokes.
15. Make sure that you have diluted the microsomal fraction to a little more than 3 mL, this will ensure that there is enough sample to layer 3 g on top of the polymer solution.
16. Important things to notice when working with aqueous two-phase partitioning: Generally, with increasing molecular weight of the polymer, a lower concentration of polymer is required for phase formation. Hydrophobicity is important for protein partitioning due to the phase hydrophobicity and salting out effects. The pH of aqueous two-phase partitioning alters charge and surface properties of the biomolecule. Temperature greatly affects the composition of two phases in an aqueous two-phase partitioning. It is therefore important to always keep samples cold. In general, phase separation is obtained at lower temperature in a polymer–polymer solution.
17. The two-phase solution will not partition into phases if mixed too vigorously with the microsomal fraction (see Movie 1 for correct mixing).
18. The polymers will have separated into two phases, a PEG4000-enriched top-phase and Dextran T500-enriched lower phase. The plasma membranes will separate into the top phase (T1), and other membranes will be retained in the bottom phase (B1). The top phase should be clear and milky colored. If it is still green or slightly greenish it still contains chloroplasts (or parts thereof), and a second aqueous two-phase partitioning is needed (Fig. 2b, c).
19. Optional second extraction: Take a fresh tube with 9 g polymer solution and add 3 g of 330/5 buffer to a final weight of 12 g. Mix and centrifuge the polymer solution with 330/5 buffer as before and remove the top layer with PEG4000 (T2; Fig. 2b). Add the plasma membrane phase from before (T1) to the

newly prepared bottom phase (B2; Fig. 2c). Mix the sample 10 times (Movie 1) and centrifuge as before. The top phase (T3) should be clear at this point and contains the isolated plasma membranes. Proceed with step 5 and transfer the top phase with plasma membranes (T3) to an ultracentrifuge tube.

20. The amount of buffer needed to homogenize the sample depends on the pellet after the last ultracentrifugation step, use small volume of 330/5 buffer if the pellet is less than 1 cm in diameter.
21. Volume of the assay in cuvettes can be adjusted from 200 μL to 1 mL, just keep the concentrations of the assay components fixed.
22. Optional: run an emission scan on 350–450 nm, points/0.1 s and excitation 450–500 nm to ensure buffers are correct.
23. Use cuvettes with black sides for fluorescent readings.
24. If your instrument permits, place a small magnetic stir bar at the bottom of the cuvette, to ensure continuous mixing of the sample.
25. Signal should be around 1×10^5–10^6.
26. Run assay for longer or shorter until the fluorescent quenching has stabilized (Fig. 3a).
27. Adjustment of the amount of plasma membrane vesicles added to the assay may be needed to obtain optimal activity readings.
28. Perform the assay in three technical replicates.
29. Readings/measurements can be done every 10–20 s to get the most datapoints (Fig. 3b).
30. It is important to be ready with all assay components when the initial baseline reading is started since time is of essence during this assay.
31. If your instruments permits, use the automatic injector to start the assay. Alternatively, use a multi-channel pipette to start multiple assays at the same time.
32. The time spent between initiating H^+-pumping with $MgSO_4$ and starting the next reading can lead to loss of initial datapoints. Quickly add in the $MgSO_4$ and start the next reading of 600 s.

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Chapter 8

A Seedling Growth Inhibition Assay to Measure Phytocytokine Activity

Henriette Leicher and Martin Stegmann

Abstract

The study of immunomodulatory peptides, both of exogenous and endogenous origin, attracted increasing attention over the last years. Numerous methods are widely used to study the sensitivity of plants to peptide elicitation, ranging from measuring early to late induced responses. Seedling growth inhibition is a prominent and easy-to-measure output induced by prolonged peptide treatment. Here, we describe a robust *Arabidopsis thaliana* seedling growth inhibition experiment that can be used to measure the direct growth-inhibitory effect of peptides, exemplified by RAPID ALKALINIZATION FACTOR 23 (RALF23) treatment. We also show how the assay can be used to assess the modulatory effect of peptide co-treatment on microbe-associated molecular pattern (MAMP)-triggered seedling growth inhibition, exemplified by GOLVEN 2 (GLV2)'s effect on flagellin (flg22)-induced seedling growth inhibition.

Key words Endogenous peptides, Phytocytokines, Damage-associated molecular pattern (DAMP), Microbe-associated molecular pattern (MAMP), Pattern triggered immunity (PTI), Seedling growth inhibition

1 Introduction

Plant endogenous peptides regulate a wide range of physiological responses, including growth, development, and biotic and abiotic stress [1]. Research in recent years shows that several endogenous peptides have more than one regulatory function, modulating growth, development, and plant immune responses to pathogen infections. In analogy to metazoan systems, these peptides are referred to as phytocytokines [2–4]. The identification of phytocytokines and their physiological characterization is an active field of research that received increasing attention over the last years. In addition to endogenous peptides, plants also perceive exogenous signals derived from potential invaders, so-called microbe-associated molecular patterns (MAMPs), or cell components passively released during infection-induced cellular damage, so-called damage-associated molecular patterns (DAMPs). MAMP, DAMP, and

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phytocytokine perception mechanisms are very similar, involving plasma membrane-localized receptors, which are also known as pattern recognition receptors (PRRs). The best described and characterized example is FLAGELLIN SENSITIVE 2 (FLS2) which detects a 22 amino acid epitope derived from bacterial flagellin (flg22) to activate a robust immune response [5–7]. In many cases, exogenous MAMPs and endogenous phytocytokines activate similar signaling outputs, including the production of reactive oxygen species, the cellular influx of calcium ions, the activation of a MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascade, the production of defense-associated phytohormones and the induction of defense-related gene expression [7, 8]. Importantly, prolonged treatment of plants with MAMPs or with phytocytokines arrests seedling growth, which is likely caused by the growth-defense trade-off [9–16]. In addition, recent research has shown that endogenous peptides may lack the ability to induce defense responses by themselves but rather augment the effect of exogenous MAMPs, including MAMP-induced seedling growth inhibition. For example, the endogenous peptide GLV2, previously associated with the regulation of gravitropic responses, was recently shown to promote flg22-induced seedling growth inhibition without inducing growth arrest by itself [17, 18]. Seedling growth inhibition is a versatile and easily measured parameter to assess the sensitivity of wild type and mutants to endogenous and exogenous peptides without the requirement of sophisticated instrumentation.

Here, we describe this seedling growth inhibition assay (from seed sterilization, plating, transfer to fresh weight measurement) that can be used to test the direct effect of a peptide on seedling growth. This is exemplified by treatment with the RAPID ALKALINIZATION FACTOR 23 (RALF23) peptide, which is perceived by the receptor kinase FERONIA (FER) to induce apoplastic alkalinization and growth arrest, as well as downregulation of plant immunity [19]. This protocol is similarly applicable for testing sensitivity of mutants to exogenous elicitors (including flg22 or other MAMPs), as well as endogenous immune-activating peptides, including PLANT ELICITOR PEPTIDE 1, PAMP-INDUCED PEPTIDE 1, SERINE-RICH ENDOGENOUS PEPTIDEs and SMALL PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS/CTNIPs [9–12, 14, 15]. We also describe a modified form of this assay that was used to characterize the function of GLV2 and its role in promoting flg22-induced immune signaling outputs via ROOT MERISTEM GROWTH FACTOR INSENSITIVE (RGI) receptors [18]. We foresee that the modified assay can be used to assess the activity of other peptides that lack direct immune-eliciting activity but rather promote or inhibit elicitor-induced responses. In summary, the described seedling growth inhibition assay is a fast and easy experiment to reproducibly measure phytocytokine sensitivity of Arabidopsis plants and potential pathway mutants.

2 Materials

1. 13% sodium hypochlorite (NaClO).
2. 32% (concentrated) hydrochloric acid (HCl).
3. Desiccator.
4. Fume hood.
5. Sterile work bench.
6. 250 mL Pyrex beaker.
7. Plant growth cabinet.
8. Arabidopsis seeds: Seeds of the Arabidopsis wild type (Col-0) and any mutant of interest.
9. ½-strength Murashige and Skoog (MS) Medium: Add 2.165 g MS salts including vitamins and 10 g sucrose to 1 L deionized water (*see* **Note 1**). For solid medium, add 10 g/L phyto agar. Autoclave for 12 min, cool down to about 60 °C, then poor into Petri dishes.
10. Round 9-cm Petri dishes.
11. 48-well cell culture plates.
12. Pointy-end precision forceps.
13. 1.5 mL Eppendorf Protein LoBind tubes.
14. 1 mM RALF23 (ATRRYISYGALRRNTIPCSRRGAS YYNCRRGAQANPYSRGCSAITRCRRS): custom-synthesized at 95% purity. Prepare the peptide solution as a 1 mM stock with sterile deionized water in Eppendorf Protein LoBind tubes.
15. 1 mM GLV2 (DMD($TyrSO_3H_2$)NSANKKRHypIHN): custom-synthesized at 95% purity. Prepare the peptide solution as a 10 mM stock, then dilute to 1 mM with sterile deionized water in Eppendorf Protein LoBind tubes.
16. 1 mM flg22 (Ac-QRLSTGSRINSAKDDAAGLQIA): custom-synthesized at 95% purity. Prepare the peptide solution as a 1 mM stock with sterile deionized water in Eppendorf Protein LoBind tubes.
17. Fine balance, precision of 0.1 mg.
18. Micropore tape (e.g., 3 M Micropore surgical tape).
19. 50 mL sterile culture tubes.
20. 1.5 mL microfuge tubes.

3 Methods

3.1 Seed Sterilization and Plating (Fig. 1a)

Seed sterilization has to be performed under a fume hood because of the release of toxic chlorine gas.

1. Transfer about 50–100 dry seeds into 1.5 mL microfuge tubes, use one tube per genotype to be studied (*see* **Note 2**).
2. Place the seeds in open 1.5 mL tubes into the desiccator.
3. Place a beaker containing 50 mL NaClO solution into the desiccator, next to the seeds.
4. Add 15 mL HCl to the NaClO solution and immediately close the desiccator. Incubate seeds for 4 h (*see* **Note 3**).
5. Upon completion, open the desiccator under the fume hood, take out the tubes with the seeds and quickly place them under a sterile bench. Wait for about 30 min (*see* **Note 4**).
6. Sow seeds on solid ½ MS agar plates and try to spread them as widely apart and evenly as possible (see **Note 5**).
7. Seal the plates with one or two layers of micropore tape and place them into a growth cabinet set at long-day conditions (16 h light, 8 h dark, 22 °C, *see* **Note 6**).
8. After 4 days, proceed to Sect. 3.2 for the transfer of seedlings to liquid ½ MS medium.

3.2 Seedling Transfer to Liquid ½ MS Medium (Fig. 1b)

1. Calculate the appropriate amount of liquid ½ MS medium required for the experiment (*see* **Notes 7**). You will need 500 µL per well and 1 well per seedling. Usually, n of 8 seedlings per genotype and treatment is sufficient. In case of weak growth-inhibitory activity, or small phenotypic differences between the tested genotypes, a larger n of 12–16 seedlings is advisable.
2. Prepare the growth medium by adding the respective peptides at the required concentrations to liquid ½ MS in a sterile 50 mL culture tube under a sterile bench. Peptide treatments include 1 µM RALF23, 10 nM flg22 (*see* **Note 8**), 1 µM GLV2, and 1 µM GLV2 + 10 nM flg22. Also include a control with seedlings grown in ½ MS medium without further addition.
3. Pipette 500 µL peptide-supplemented medium for each of n seedlings into the wells of a 48-well culture plate.
4. Transfer individual seedlings into the wells of the 48-well plate using pointy-tip forceps under a sterile bench. Make sure to be gentle in order not to damage the seedlings during transfer (*see* **Notes 9** and **10**).
5. Close the 48-well plate with a lid and seal the plate with micropore tape. Place the completed 48-well plates into the growth cabinet and incubate for 7 days at 16 h light, 8 h dark, 22 °C (*see* **Note 11**).

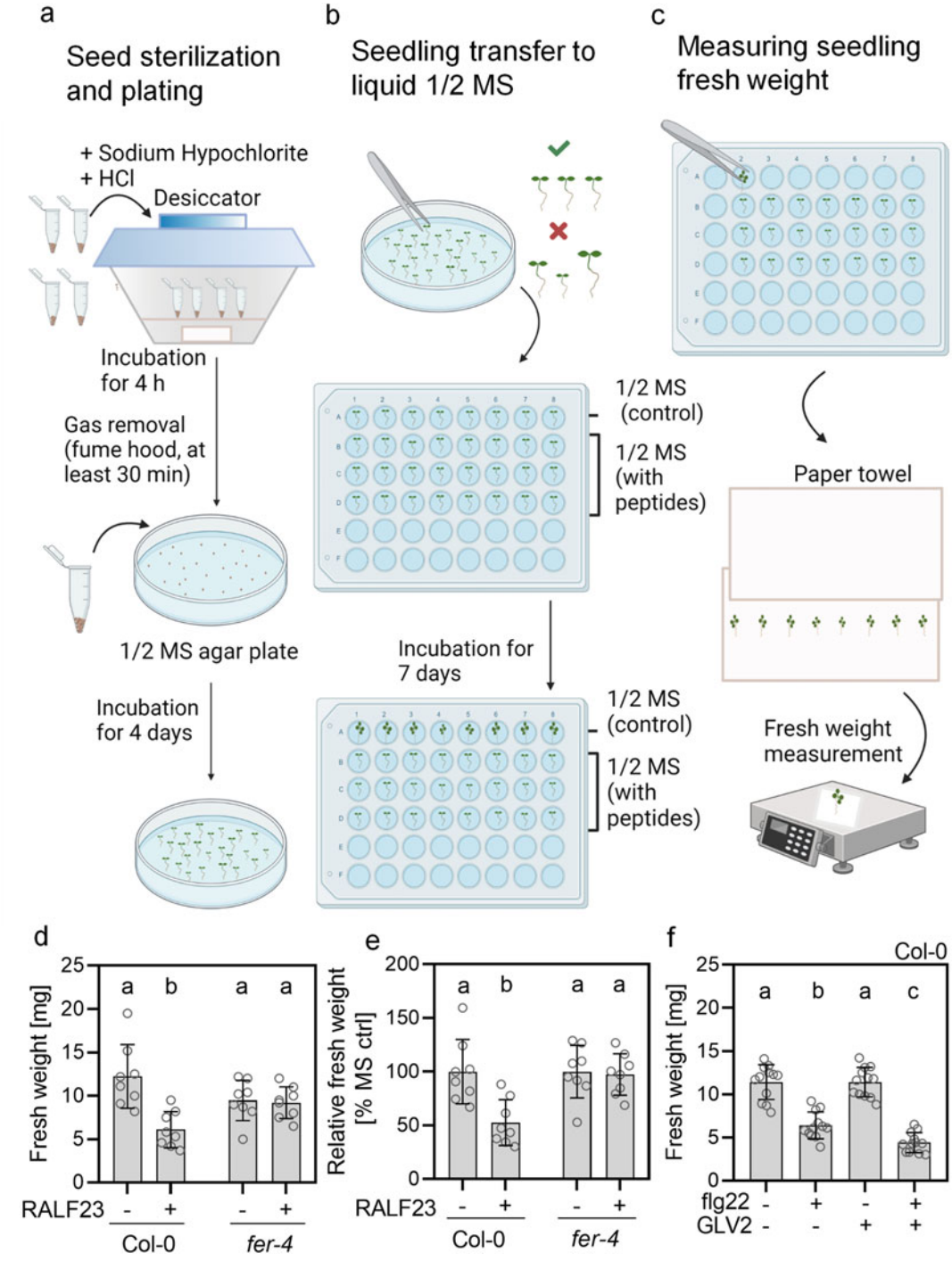


Fig. 1 Graphical summary of the seedling growth inhibition assay. (**a**) Sterilize seeds using chlorine gas sterilization. Subsequently, distribute the seeds equally and disperse on a ½ MS agar plate. (**b**) After 4 days of growth, transfer single seedlings into wells of a 48-well plate filled with ½ MS liquid medium supplemented with the respective peptides. Incubate for 7 days. (**c**) Measure seedling fresh weight by drying seedlings on a paper towel and weighing single seedlings using a fine balance. (**d**) Absolute fresh weight of Col-0 and *fer*-4 seedlings grown in liquid ½ MS medium with and without 1 μM RALF23 (Mean +/− SD, $n = 8$, one way ANOVA, Tukey post-hoc test, a–b: $p < 0.001$). (**e**) Relative fresh weight values of seedlings measured in (**d**)

3.3 Measuring Seedling Fresh Weight (Fig. 1c)

1. Remove individual seedlings from the 48-well plates and place them on a double-layered sheet of paper towel using the pointy-end forceps.
2. Place an additional paper towel on top of the seedlings and gently sweep over to remove as much excess liquid ½ MS medium as possible. Make sure that all seedlings are treated equally.
3. Measure seedling weight using a fine balance and note individual values (*see* **Notes 12** and **13**).
4. Calculate the average seedling weight of the ½ MS medium control, as well as of the peptide-treated samples. In case seedlings derive from genotypes with differences in control fresh weight, it is advisable to calculate relative seedling growth inhibition of each genotype to its respective ½ MS medium control to assess potential differences in their response profile. Figure 1d–f show exemplary results for the growth inhibition of Col-0 and *fer*-4 seedlings treated with RALF23, as well as Col-0 seedings treated with flg22, GLV2, or co-treated with both peptides. The data show that RALF23 inhibits seedling growth in a FER-dependent manner. By contrast, GLV2 does not inhibit seedling growth by itself, but promotes flg22-induced seedling growth inhibition.

4 Notes

1. Do not add buffers to the medium, such as 2-(N-morpholino) ethane sulfonic acid (MES), as this interferes with weaker seedling growth inhibition by less potent inducers.
2. Seeds should be as clean as possible before sterilization to reduce the risk of contaminating fungal growth.
3. Four hours of incubation time is the minimum amount of time required to sterilize the seeds (50–100 seeds); it can be extended to up to 8 h. Longer incubation time will damage the seeds and negatively affect their germination rate on ½ MS agar plates. In case a higher number of seeds need to be sterilized for larger scale experiments, longer sterilization time is advisable (6–7 h) as the chlorine gas may require longer time

Fig. 1 (continued) compared to the ½ MS medium control of Col-0 and *fer*-4 seedlings grown in liquid ½ MS medium with and without 1 μM RALF23 (Mean +/− SD, $n = 8$, one way ANOVA, Tukey post-hoc test, a–b: $p < 0.01$). (**f**) Absolute fresh weight of Col-0 seedlings grown in ½ MS liquid medium supplemented with 10 nM flg22, 1 μM GLV2 or 10 nM flg22 + 1 μM GLV2 (Mean +/− SD, $n = 12$, one way ANOVA, Tukey post-hoc test, a–b: $p < 0.0001$, b–c: $p < 0.05$). (a)–(c) Created with BioRender.com

to penetrate through larger seed volumes for effective sterilization. Also, alternative sterilization procedures may be performed, such as ethanol-based liquid seed sterilization.

4. The 30-min waiting period with open microfuge tubes is essential to allow any remaining chlorine gas to escape from the tubes. If plated too early, chlorine gas will react with the water of ½ MS agar plates to generate hydrochloric acid, which will strongly inhibit seedling germination and growth. After the waiting period, tubes can be closed again for planting on another day. Yet, we advise to not plate seeds too late after sterilization (>3–4 days), as extended storage may affect seedling germination.
5. It is important to spread the seeds widely, as this will facilitate the transfer of the seedlings to 48-well plates. If seedlings are too close, their roots tend to stick to each other, making separation of individual seedings much more difficult, in particular without damaging cotyledons or roots. Yet, separation of seedlings is important. Results will be uninterpretable, if more than one seedling is transferred to each well of the 48-well plate (two seedlings will compete for resources within the well and their fresh weight needs to be excluded from the analysis, reducing the overall n of the experiment).
6. We advise to close the ½ MS agar plates to prevent desiccation during growth incubation time. We usually do not stratify the seeds. It is possible to do so, in order to better synchronize germination, but it is important to note that this will likely result in faster germination and growth of the seedlings. Therefore, the transfer of seedlings from solid ½ MS plates to liquid ½ MS medium may already be necessary after 3 days.
7. Peptide dilutions into ½ MS medium have to be prepared freshly before every experiment. Because of the price tag attached to the synthetic peptides, you do not want to prepare more medium than necessary.
8. Strong elicitors, e.g., flg22, will induce strong seedling growth inhibition at concentrations of 100 nM and a reduced but measurable response at 10 nM. RALF23 is a weaker elicitor and concentrations of 1 μM are required to see reproducible and robust growth inhibition. GLV2 does not induce seedling growth inhibition by itself, but promotes flg22-induced seedling growth inhibition [18]. GLV2's effect on flg22-induced seedling growth inhibition is best measurable when 1 μM GLV2 is added to a low dose of flg22 (10 nM) which will only induce a fairly weak growth inhibition by itself (~50% growth inhibition compared to ½ MS medium control-grown seedlings). GLV2 will also enhance flg22-mediated seedling

growth inhibition at higher concentrations of flg22, but differences will be less pronounced and more difficult to observe in a robust manner.

9. It is important to transfer healthy seedlings with a similar size for each individual genotype. This will reduce biological variability and allow you to obtain more robust and reproducible results.
10. To avoid carryover of medium from wells with peptide-treated seedlings to wells with non-treated control seedlings, it is advisable to transfer the entire batch of seedlings for one particular treatment, and clean the forceps, e.g., by fire sterilization using a Bunsen burner, before continuing with the next treatment.
11. Seedlings of *fer*-2 and *fer*-4 (FERONIA loss of function mutants) tend to be stressed in liquid medium. The seedlings perform better when 48-well plates are incubated in the growth cabinet with slight agitation using an orbital shaker.
12. It is important that seedlings are measured soon after removal from the liquid ½ MS medium. Too much time on the paper towel will result in increased desiccation of seedlings, making fresh weight values less reliable. Usually, we remove one set of seedlings for a certain genotype-treatment combination ($n = 8–16$) and remove excess liquid in one go. This saves a lot of time and does not affect seedling fresh weight when measured quickly afterward.
13. Transfer seedlings gently from the paper towel using pointy-tip forceps. Roots tend to stick to the paper towel. Make sure not to lose the root or parts of the root as this will confound total fresh weight determination.

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Chapter 9

A Trojan Horse Approach Using *Ustilago maydis* to Study Apoplastic Maize (*Zea mays*) Peptides In Situ

Leon Kutzner and Karina van der Linde

Abstract

Plant peptides are important signaling components in many parts of the plant lifecycle, e.g., development, reproduction, environmental stress response, and plant pathogen defenses. Yet, in maize, one of the most grown crops worldwide, only a few peptides have been identified and studied. In general, molecular research is severely impacted by time-consuming and costly maize transformation, and external application of purified peptides does not allow functional analysis in deeper cell layers due to the thickness of the tissue. In an attempt to bypass these problems while studying the function of small secreted proteins in maize, we established the Trojan Horse approach. Here, tagged peptides are delivered into the maize apoplast in a highly localized fashion by using a genetically modified version of the biotrophic pathogen *Ustilago maydis*. This technique offers the possibility of rapid testing of predicted maize peptides for in situ functions.

Key words Peptides, Small secreted proteins, *Zea mays* (maize), *Ustilago maydis* (corn smut fungus)

1 Introduction

In ancient mythology, the Greek besieged the city of Troy, which was protected by strong city walls said to be built by the god Poseidon himself, for 10 years. In order to win the war, the Greek used a ruse, they built a wooden horse with soldiers hidden inside. This Trojan Horse (TH) was placed in front of the gates of Troy as a triumphant gift, while the Greek army pretended to have sailed off. At night, after the Trojans transported the horse into the city, the soldiers sneaked out and opened the gates for the Greek army to conquer the city.

In modern plant biology researchers often face comparable problems when studying plant peptides. Many plant species are reluctant to transformation or, even if transformation is available, it is often time-consuming and costly. External application of the peptide of interest, after chemical synthesis and purification, is frequently used to study the function of plant peptides [1–4]. Yet,

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it remains unclear how deep applied peptides penetrate into the plant tissue. To overcome these obstacles, we established a molecular TH to study the function of plant peptides in situ [5–8]. This approach utilizes the secretory capabilities of the maize pathogen *Ustilago maydis* to deliver maize apoplastic proteins. Since decades *U. maydis* serves as a model organism for genetic studies [9–12] and for biotrophic plant-pathogen interaction, because of its genetic accessibility and the fact that it is easy to cultivate in the laboratory. During infection, which can occur on all aerial tissue of the plant, this fungus naturally secretes an array of different small proteins, so-called effectors (many of which are cysteine-rich), into the apoplast of the infected cell layer to tamper with the plant immune response and to reprogram the hosts' metabolisms in favor of the pathogen [13–15].

For the TH approach, first a plasmid suitable for *U. maydis* transformation based on the p123-vector system is engineered [16]. This plasmid contains the peptide coding sequence of interest fused to an *U. maydis* signal peptide coding sequence for correct secretion into the plant apoplast during infection under control of an *in planta* active promoter, and a *mcherry tag*-coding sequence (Subheading 3.1). After linearization, the construct is integrated into the *ip*-locus of *U. maydis* by homologous recombination [17] (Subheadings 3.2 and 3.3). Correct insertion into the genome is verified by Southern blotting followed by detection with a probe complementary to the *ip*-locus (Subheading 3.4). Once positive *U. maydis* clones have been identified, these clones are tested for progenitor-like behavior in axenic culture (Subheading 3.5.1) and filament formation on solid charcoaled media (Subheading 3.5.2). Secretion of the mCHERRY-fusion protein can be verified by microscopic imaging *in planta* (Subheading 3.5.3). After these quality controls, the TH method can be used to deliver the peptide of interest into the apoplast of various maize tissues and subsequently to analyze the impact/function of the peptide using different readouts, e.g., microscopic imaging, RNA sequencing, or disease ratings (Subheadings 3.6 and 3.7) [5, 6].

We used the TH approach to study the impact of the small secreted protein MULTIPLE ARCHESPORIAL CELLS1 (MAC1) in maize anther development. MAC1 was hypothesized to induce periclinal division of anther lobe cells and to govern cell fate specification of the emerging lobe cell layer [18]. At this stage of anther development, anther lobe cells are already surrounded by multiple layers of flower tissue and deeply buried within the maize leave whorl. Microscopic imaging of anthers after in situ delivery of MAC1 by the TH approach revealed periclinal divisions of anther lobe cells in close vicinity of TH hyphae but not in non-infected anther lobe areas, indicating that MAC1 facilitates periclinal division of anther lobe cells. Molecular analysis of TH-treated tissue by RNA sequencing proved MAC1 function in cell fate specification

[6]. Peptide signals play pivotal roles, not only in plant development but also in plant-pathogen interactions. The *Zea mays* IMMUNE PEPTIDE1 (ZIP1), which activates salicylic acid signaling, was identified as a product of apoplastic papain-like protease activity induced by pathogen attack [5, 19]. Subsequently, TH strains expressing and secreting ZIP1 showed reduced pathogenicity [5]. Hence, the TH approach can be used to deliver peptides and to provide a read-out for peptide function at the same time.

Besides the fact that *U. maydis* transformation and quality control of strains only takes 4 weeks, this method has several other advantages: (1). *U. maydis* invades deep tissue layers allowing peptide delivery to these layers; (2). *U. maydis* infection and therefore cells that receive the peptide can be tracked easily by microscopic imaging; (3). Non-infected cells can be directly compared to treated cells within the same tissue; and (4). Ectopic expression as well as local overdosage of delivered peptides can be studied.

In the following sections we provide step-by-step descriptions for the delivery of peptides into the maize apoplast by using the TH approach (Subheadings 3.1 to 3.5), and how to use disease rating as an example for a possible first analysis of peptide function in situ (Subheadings 3.6 and 3.7). A graphic representation of the workflow is provided in Fig. 1. Figure 2 shows exemplary results of the infection and of a seedling disease rating assay (Subheadings 3.6) that were obtained for two up-to-now uncharacterized maize peptides in maize seedlings.

2 Material

2.1 Cloning of p123 TH Vectors

1. Primers for PCR amplification of the peptide coding sequence: Gene-specific primers are needed to amplify the open reading frame for the peptide of interest without the signal peptide from maize cDNA. Attach the following overhang sequences to the 5′ end of the primers: forward primer: 5′-GCTCTAGA-3′, reverse primer: 5′-CATGCCATGGAGGCGGTGGCGA TCGAGCG-3′. Prepare 100 μM stock and 10 μM working solutions for each of the primers.
2. Proof-reading (high-fidelity) DNA polymerase (e.g., HS VeriFi™ polymerase, PCR Biosystems, London, UK (*see* **Note 1**)).
3. 1× TAE buffer: 4.85 g TRIS, 1.14 mL 99.8% acetic acid, and 100 mL 10 mM EDTA, pH 8.0; make up to 1 L with double-distilled (dd) H_2O.
4. 1% (2%) TAE-agarose gels: Boil 1 g (2 g) of agarose in 100 mL of 1× TAE until agarose is dissolved completely. Let it cool down to approximately 60 °C, add nucleic acid-binding dye (e.g., 5 μL Roti®GelStain, Carl Roth, Karlsruhe, Germany) and pour into a gel cassette with an appropriately sized comb.

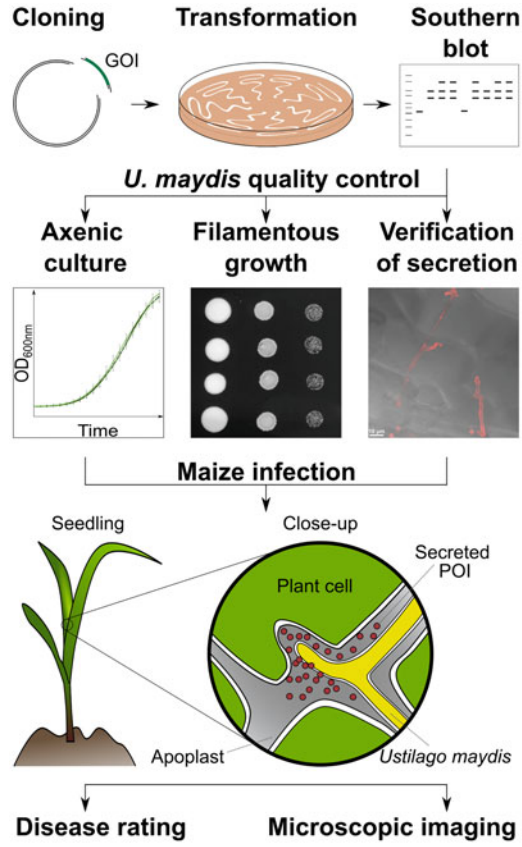


Fig. 1 Workflow for in situ delivery of peptides into the maize apoplast by the Trojan Horse approach. After cloning of the gene of interest (GOI) into the Trojan Horse (TH) vector and integration into the *U. maydis* genome, clones are selected and integration of the construct is verified by Southern blotting. Progenitor-like growth and secretion of the peptide of interest (POI) is verified before the impact of the POI can be studied in situ by various methods including microscopic imaging or disease ratings. The choice of methods will depend on the physiological activity of the POI

5. DNA loading dye (e.g., 6× DNA Loading Dye, Thermo Scientific, Schwerte, Germany).
6. DNA size standard.
7. PCR clean-up and gel extraction kit.
8. Plasmid p123-$P_{Um\mathit{pit2}}$-$Sp_{Um\mathit{pit2}}$-Zm*mac1-mcherry-ha* [6].
9. Restriction enzymes: *Xba*I; *Nco*I, with appropriate buffers.
10. T4 DNA ligase.
11. 100 μM ATP.
12. Chemically competent *Escherichia coli* DH5α cells.

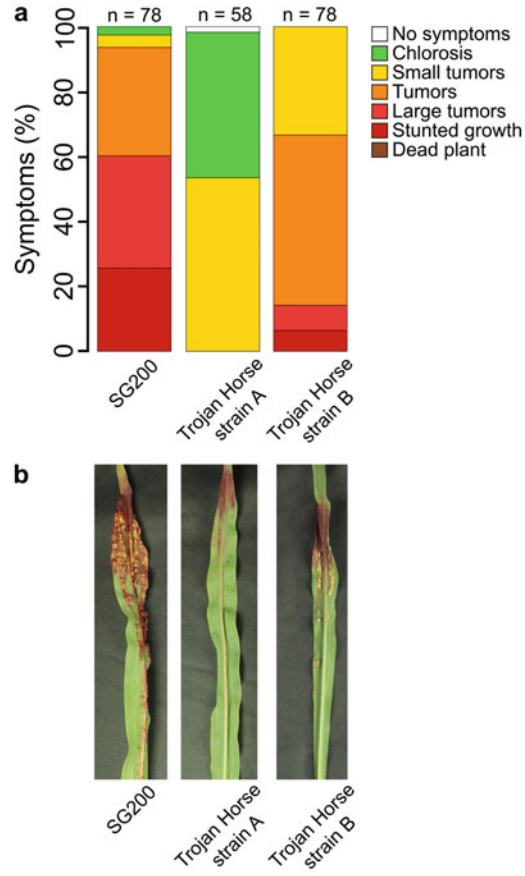


Fig. 2 Disease rating of two Trojan Horse strains and the progenitor *U. maydis* strain SG200. (**a**) Trojan Horse strains A and B were tested, each expressing and secreting a maize peptide of interest. A stacked bar chart is used to display percentage for each symptom category, where darker colors represent more severe symptoms for the two Trojan horse strains and the progenitor strain SG200. Above each bar the total number of plants used in three biological replicates is given. (**b**) For each strain, a representative leaf with average symptoms observed in (a) is shown

13. LB-ampicillin agar: 8 g peptone, 4 g yeast extract, 8 g NaCl, and 8.3 g agar; make up to 800 mL with ddH_2O and autoclave. Let cool to 50 °C, add 800 μL of 100 mg/mL ampicillin and pour into 10 cm petri dishes.
14. LB-ampicillin medium: 8 g peptone, 4 g yeast extract and 8 g NaCl; make up to 800 mL with ddH_2O and autoclave. Let cool to 50 °C and add 800 μL of 100 mg/mL ampicillin.
15. 15 mL culture tubes.

16. Plasmid purification kit (e.g., Nucleospin® Plasmid, Macherey-Nagel, Düren, Germany).
17. Sequencing primer: 5′-CTCCCACGAGAGTCTGTTAC-3′.

2.2 Generation of Ustilago maydis Protoplasts

1. Solopathogenic *Ustilago maydis* strain SG200 (*see* **Note 2**).
2. YEPS-light medium: 10 g yeast extract, 4 g peptone, 4 g sucrose; make up to 1 L with ddH_2O, sterilize by autoclaving.
3. SCS-buffer, pH 5.8: For solution 1, dissolve 5.9 g sodium citrate and 182.2 g sorbitol in 1 L ddH_2O. For solution 2, dissolve 4.2 g citric acid monohydrate and 182.2 g sorbitol in 1 L ddH_2O. Use solution 2 to adjust the pH of solution 1 to pH 5.8.
4. SCS-Lysing enzyme solution: Dissolve 60 mg lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich) in 3 mL SCS-buffer, pH 5.8 (*see* **Note 3**).
5. STC-buffer: 91.09 g sorbitol, 7.35 g $CaCl_2$*$2H_2O$, 5 mL 1 M TRIS-HCl, pH 7.5; make up to 500 mL ddH_2O.

2.3 Transformation of Solopathogenic Ustilago maydis Strain SG200

1. Restriction enzyme: *SSp*I, with the appropriate buffer.
2. 3 M Sodium acetate solution: 24.6 g sodium acetate, in 100 mL ddH_2O.
3. Regeneration-Agar: 10 g yeast extract, 20 g peptone, 20 g sucrose, 182.2 g sorbitol, 15 g agar, make up to 1 L with ddH_2O and sterilize by autoclaving.
4. 10 cm Petri dishes.
5. 10 mL serological pipettes.
6. STC/40% PEG: Dissolve 10 g polyethylene glycol in 15 mL STC buffer and filter sterilize through a 0.22 μm filter.
7. 15 mg/mL heparine: Dissolve 150 mg heparine in 10 mL ddH_2O and filter sterilize through a 0.22 μm filter.
8. 5 mg/mL carboxine solution: Dissolve 50 mg carboxine in 10 mL 99.9% methanol and filter sterilize through a 0.22 μm filter.
9. PD-Agar-cbx plates: 31.2 g potato dextrose agar, 8 mL 1 M Tris-HCl, pH 8.0, make up to 800 mL with ddH_2O and sterilize by autoclaving. Let cool to 50 °C, add 320 μL carboxine solution and pour into sterile 10 cm petri dishes.
10. YEPS-light medium: *see* **2.2**.

2.4 Verification of Genomic Integration by Southern Blotting and Probe Detection

1. YEPS-light medium: *see* **2.2**.
2. 0.4–0.6 mm glass beads.
3. TCL – Tissue and cell lysis solution (Lucigen, Middleton, USA).

4. 50 µg/µL Proteinase K.
5. 5 µg/µL RNase A.
6. MPC – Protein Precipitation Reagent (Lucigen, Middleton, USA).
7. Restriction enzyme: *Nco*I, with appropriate buffer.
8. 0.8% TAE-agarose gel: Boil 0.8 g of agarose in 100 mL of 1× TAE (*see* **2.1.3**) until agarose is dissolved completely. Let it cool down to approximately 60 °C, add nucleic acid-binding dye (e.g., 5 µL Roti®GelStain, Carl Roth, Karlsruhe, Germany) and pour into a gel cassette with an appropriately sized comb.
9. DNA loading dye: *see* **2.1.5**.
10. DNA size standard: *see* **2.1.6**.
11. 0.25 M HCl: Dilute 250 mL 1 M HCl with 750 mL ddH_2O.
12. 0.4 M NaOH: 16 g NaOH in 1 L ddH_2O.
13. Amersham Hybond™-N, pore size: 0.45 µm (GE Healthcare, Chalfont St Giles, UK).
14. Glass bowl and glass plate.
15. Whatman 3MM blotting paper.
16. Parafilm.
17. Disposable paper towels.
18. PCR DIG Probe Synthesis Kit (Roche, Penzberg, Germany).
19. Probe synthesis primers: 5′-CGCTAAGTGGAGTTGTCCG AG-3′ and 5′-CGTACGATTGTGGCGAATCG-3′.
20. 1 M sodium phosphate buffer pH 7.0: Solution 1, 142 g $Na_2HPO_4*2H_2O$ in 1 L ddH_2O. Solution 2, 138 g $NaH_2PO_4*H_2O$ in 1 L ddH_2O. Use solution 2 to adjust pH of solution 1 to pH 7.0.
21. Hybridization buffer: Dissolve 70 g of SDS pellets in 500 mL of 1 M sodium phosphate buffer, pH 7.0; fill up with ddH_2O to a total volume of 1 L.
22. Southern wash buffer: Dissolve 10 g of SDS in 100 mL 1 M sodium phosphate buffer, pH 7.0; fill up with ddH_2O to a total volume of 1 L.
23. DIG buffer: Dissolve 23.21 g maleic acid and 17.53 g NaCl in ddH_2O, adjust pH to 7.5 with NaOH and bring to a total volume of 2 L. Sterilize by autoclaving.
24. DIG wash buffer: Mix 3 mL of Tween® 20 with 997 mL DIG buffer.
25. Blocking solution: Dissolve 0.1 g skim milk powder in 100 mL DIG buffer.

26. Antibody solution: Dilute 2 μL anti-dioxigenin-AP Fab fragments (Roche, Penzberg, Germany) in 20 mL blocking solution.
27. DIG detection buffer: For 1 L, dissolve 12.11 g Tris, 5.84 g NaCl and 4.76 g $MgCl_2$*$7H_2O$ in 800 mL ddH_2O, adjust pH to 9.5 with 1 M HCl, make up to 1 L with ddH_2O.
28. CDP-Star solution: Dilute 10 μL CDP-Star (Roche, Penzberg, Germany) in 90 μL DIG detection buffer.
29. Cling wrap.

2.5 Quality Control of TH Strains

1. YEPS-light medium: *see* **2.2**.
2. 24-well plates.
3. Micropore tape (3 M, Saint Paul, USA).

2.5.1 Analysis of Growth in Axenic Culture

2.5.2 Analysis of Filamentous Growth on Charcoal Media

1. YEPS-light medium: *see* **2.2**.
2. PD-charcoal: 31.2 g potato dextrose agar, 8 g activated charcoal, 8 mL 1 M Tris-HCl, pH 8.0, make up to 800 mL with ddH_2O and sterilize by autoclaving. Let cool to 50 °C and pour into sterile 10 cm Petri dishes.

2.5.3 Verification of Secretion of POI-mCHERRY Fusion Protein on Maize Seedlings

1. *Z. mays* cv. B73 seeds.
2. Seeding substrate.
3. 1 mL plastic syringe.
4. 27-gauge hypodermic needles.
5. Microscope slides.
6. Cover slips, 22 × 22 mm.

2.6 Infection and Disease Rating of Maize Seedlings (3.6) and Tassels (3.7)

1. *Z. mays* cv. B73 seeds.
2. *Z. mays* cv. Gaspe Flint seeds.
3. Seeding substrate.
4. 1 mL plastic syringes.
5. 27-gauge hypodermic needles.

2.7 Equipment

1. Refrigerated centrifuge for microfuge tubes.
2. Benchtop centrifuge for culture tubes.
3. PCR cycler.
4. Vortex mixer with the microtube foam insert attached.
5. Spectrophotometer.
6. Microplate reader (e.g., Spark 10 M, Tecan, Crailsheim, Germany).
7. Incubators for culture plates at 28 °C and 37 °C.

8. Reciprocating shaker.
9. Shaking incubators for microbial cultures at 28 °C and 37 °C.
10. Water bath at 37, 65, and 100 °C.
11. Microwave oven.
12. UV cross-linker (e.g., Stratalinker 1800, Stratagene, La Jolla, USA).
13. Hybridization oven (e.g., HB-1000 Hybridizer, UVP, Upland, CA, USA).
14. Roller mixer.
15. Confocal laser scanning microscope.
16. Light microscope (400× magnification).
17. Chemiluminescence imager.

3 Methods

3.1 Cloning of p123 TH Vectors

1. Use gene-specific primers to amplify the coding sequence of the peptide of interest without the signal peptide from maize cDNA. For the PCR reaction, use a total volume of 50 µL with 1 U proof-reading (high-fidelity) DNA polymerase, 10 µL 5× PCR buffer, 2 µL each of the two primers, and 1 µL 50 ng/µL cDNA template. Run the PCR-cycler at the following conditions: 1 min 95 °C for initial denaturation, followed by 35 cycles: 15 s at 95 °C, 15 s at the annealing temperature specific for your primer pair, 30 s per kilobase at 72 °C.
2. Add 10 µL 6× DNA loading dye to the PCR reaction, load the whole sample on a 2% TAE-agarose gel, run the gel, and cut out the band of the PCR product.
3. Purify the PCR product using the PCR Clean-up and gel extraction kit according to the manufacturer's instructions.
4. Digest the PCR product and 1 µg of the vector p123-P$_{Umpit2}$-Sp$_{Umpit2}$-Zm*mac1-mcherry-ha* each in a total volume of 30 µL with *Xba*I and *Nco*I following the manufacturer's instructions.
5. Separate the digested p123 backbone from the insert by electrophoresis on a 1% agarose gel and extract the upper band of 7259 base pairs.
6. Purify the digested PCR product and the extracted p123 backbone with the PCR Clean-up and gel extraction kit according to the manufacturer's instructions.
7. Ligate 200 ng of the digested p123 backbone with an equimolar amount of digested PCR product in a total volume of 20 µL with 1 µL T4 DNA ligase, 1 µL ATP, and 2 µL 10× T4 DNA ligase buffer at room temperature for 30 min.

8. Transform the whole ligation reaction into 50 μL competent *E. coli* DH5α cells and incubate on LB-ampicillin-agar plates overnight at 37 °C.
9. Inoculate 3 mL of LB-Ampicillin medium with single colonies and incubate overnight at 37 °C, shaking at 200 rpm.
10. Harvest bacterial cells by centrifugation and extract plasmid DNA using the plasmid purification kit according to the manufacturer's instructions.
11. Verify the correct sequence by Sanger sequencing using the sequencing primer.

3.2 Generation of Ustilago maydis Protoplasts

1. Inoculate 2 mL YEPS-light medium with solopathogenic *Ustilago maydis* strain SG200 and incubate at 28 °C, 200 rpm overnight.
2. Inoculate 55 mL YEPS-light medium with 55 μL of the overnight culture and let grow at 28 °C, 200 rpm overnight to an OD_{600} of 0.6–0.8.
3. Centrifuge 50 mL of culture for 10 min at 1080 × *g* and remove supernatant.
4. Resuspend the pellet in 25 mL SCS-buffer at room temperature.
5. Centrifuge 50 mL of culture for 10 min at 1080 × *g* and remove supernatant.
6. Prepare SCS/Lysing Enzyme solution while the centrifuge is running.
7. Resuspend the pellet in 2 mL SCS/Lysing enzyme solution.
8. Incubate for 10–15 min at room temperature.
9. Check the progress using a light microscope: 30–40% of cells should be protoplasts (round shaped) while others should be partly protoplasted (cigar shaped with a round head).
10. Continue all the following steps on ice. Add 10 mL ice-cold SCS-buffer.
11. Centrifuge for 10 min at 690 × *g* at 4 °C.
12. Remove supernatant and carefully resuspend in 10 mL ice-cold SCS-buffer.
13. Centrifuge for 10 min at 690 × *g* at 4 °C.
14. Remove supernatant and carefully resuspend in 10 mL ice-cold SCS-buffer.
15. Centrifuge for 10 min at 690 × *g* at 4 °C.
16. Remove supernatant and carefully resuspend in 10 mL ice-cold STC-buffer.
17. Centrifuge for 10 min at 690 × *g* at 4 °C.

18. Remove supernatant and carefully resuspend in 500 µL ice-cold STC-buffer. Prepare 50 µL aliquots and store at −80 °C or use immediately for transformation.

3.3 Transformation of Solopathogenic Ustilago maydis *Strain SG200*

1. Digest 10 µg of the generated TH vector (*see* step **3.1**) in a total volume of 100 µL with 40 U *SSp*I, overnight at 37 °C.
2. Add 10 µL 3 M sodium acetate and 275 µL 100% ethanol to the linearized vector, vortex briefly and precipitate the DNA at −20 °C overnight.
3. Collect the precipitated DNA in a microfuge at 14,000 × *g* at 4 °C for 20 min.
4. Carefully discard the supernatant. Wash with 1 mL of 70% ethanol and centrifuge again for 1 min.
5. Carefully discard the supernatant and let the residual ethanol evaporate at room temperature.
6. Resuspend the DNA pellet in 15 µL ddH_2O.
7. Liquify regeneration agar in a microwave oven and let cool to 55 °C in a water bath.
8. Prepare the bottom layer of the regeneration media plate by distributing 8 µL of carboxine solution in a 10 cm Petri dish (release the carboxine solution as four to five small droplets from the micro-pipette tip on the bottom of the petri dish; droplets are placed across the whole petri dish; the methanol quickly evaporates while the carboxine remains on the bottom of the petri dish). Use a 10 mL serological pipette to pour 10 mL of regeneration agar on top and let cool down to room temperature.
9. Thaw 50 µL protoplasts of solopathogenic *U. maydis* strain SG200 on ice and add 5 µg of the digested TH vector in a maximum volume of 10 µL. Add 1 µL of 15 mg/mL heparin and incubate for 10 min on ice.
10. During incubation, prepare the regeneration plate top layer by adding 10 mL of regeneration agar on top of the bottom layer of the regeneration plate (*see* **3.3.8**) and let cool down to room temperature.
11. Cut off 1 cm of the tip of a 1000 µL pipette tip; carefully add 500 µL STC/40% PEG drop-by-drop on top of the protoplasts. Slowly pipette everything up and down twice.
12. Incubate on ice for 15 min.
13. Pipette the whole sample on the top plate and distribute the protoplasts evenly by rotating the plate (*see* **Note 4**).
14. Incubate at 28 °C for 4 days.
15. Pick at least 8 single colonies and singularize on PD-Agar-cbx plates. Incubate at 28 °C for 1–2 days. Repeat once. Once

single colonies have been selected, transformed *U. maydis* clones can be cultivated on PD-Agar plates without the addition of carboxine.

3.4 Verification of Genomic Integration by Southern Blotting and Probe Detection

1. Inoculate a minimum of 8 single colonies of newly transformed *U. maydis* in 3 mL YEPS-light medium and incubate overnight at 28 °C, 200 rpm. Also include the SG200 strain as a control.
2. Spin down 2 mL of each overnight culture in a microfuge at 2500 × *g* for 2 minutes. Remove the supernatant.
3. Add 100 µL of 0.4–0.6 mm glass beads, 300 µL "TLC – Tissue and cell lysis solution" and add 1 µL Proteinase K.
4. Incubate for 10 min at 65 °C.
5. Vortex vigorously for 30 min at room temperature.
6. Incubate for 15 min at 65 °C, then chill the samples on ice.
7. Add 1 µL RNase A and incubate for 30 min at 37 °C.
8. Put the samples on ice for 5 min, add 150 µL "MPC – Protein Precipitation Reagent", and vortex briefly.
9. Centrifuge for 10 min at 16,000 × *g* at 4 °C.
10. Transfer 400 µL of the supernatant into a new tube and centrifuge for 10 min at 16,000 × *g* at 4 °C.
11. Transfer 350 µL of the supernatant into a new tube containing 875 µL ice-cold 100% ethanol. Mix by inverting 30 times.
12. Centrifuge for 10 min at 16,000 × *g* at 4 °C.
13. Discard the supernatant and wash the pellet twice with 70% ethanol, then dry the pellet.
14. Set up a restriction digest with 40 U *Nco*I and the appropriate buffer in a total volume of 30 µL. Incubate overnight at 37 °C.
15. Add 5 µL of 6× DNA loading dye, mix and load the whole sample on a 0.8% agarose gel. Add the DNA size standard in a separate gel pocket. Run the gel at 100 V for 2.5 h to obtain a good separation between 3000 and 7000 bp.
16. Inspect the gel under UV-light, take a picture with a ruler placed next to the gel to later mark the respective DNA fragment sizes on the blot.
17. Depurinate the gel in 0.25 M HCl for 15 min with constant agitation on a reciprocating shaker.
18. Neutralize the gel in 0.4 M NaOH for 15 min with constant agitation on a reciprocating shaker.
19. Transfer the DNA to a 0.45 µm Amersham Hybond™-N membrane by setting up the blot as follows: place a glass plate on a glass dish, half filled with 0.4 M NaOH. Add a piece of Whatman paper that is soaked with 0.4 M NaOH.

Make sure that the Whatman paper runs over the edges of the glass plate, to reach into the 0.4 M NaOH in the dish. Place the gel upside down on top of the Whatman paper. Make sure not to include any air bubbles. Use Parafilm to cover the Whatman paper that surrounds the gel. Put a piece of dry Amersham Hybond™-N membrane onto the gel. The membrane should be the same size as the gel. Add another three layers of dry Whatman paper, fitted to the size of the gel, followed by a 15 cm thick layer of disposable paper towels. Put a glass plate on top and blot overnight at room temperature.

20. Disassemble the blot and mark the edges and gel pockets on the membrane with a pencil.
21. Crosslink the DNA to the wet membrane using a UV cross linker (e.g., Stratalinker 1800) at "auto crosslink" settings.
22. For the synthesis of the probe targeting the *ip*-locus in *U. maydis*, use the PCR DIG Probe Synthesis Kit with the "probe synthesis primers" and purified TH plasmid as a template according to the manufacturer's instructions.
23. Place the membrane into a hybridization tube, add 10 mL of hybridization buffer, and rotate the tube in a hybridization oven at 62 °C for 30 min.
24. In the meantime, boil the probe in 50 mL hybridization buffer in a water bath for a minimum of 10 min.
25. Pour off the hybridization buffer and add the boiling probe into the hybridization tube.
26. Rotate the blot in a hybridization tube at 62 °C overnight.
27. Remove the probe and store it at −20 °C for reuse. Wash the membrane twice at 62 °C (in the hybridization oven) for 15 min with Southern wash buffer.
28. For the detection of chemiluminescence signals, all the following steps are carried out at room temperature on a roller mixer.
29. Wash the membrane for 5 min in DIG wash buffer.
30. Block the membrane for 30 min in blocking solution.
31. Incubate the membrane for 30 min in antibody solution.
32. Wash the membrane twice for 15 min in DIG wash buffer.
33. Equilibrate the membrane for 2 min in DIG detection buffer.
34. Place the membrane onto cling wrap, add CDP-Star solution to cover the whole membrane and incubate for 5 min. Place another layer of cling wrap on top and remove excess CDP-Star solution by squeezing it out with a paper towel.
35. Use a chemiluminescence imager to detect the signal according to the manufacturer's instructions. Place a ruler next to the blot as a reference for the DNA fragment sizes. In wildtype

U. maydis, a single 3255 base pair (bp) band should be detected. Single insertions result in two bands of 5860 bp and 4654 bp + the length of the inserted peptide coding sequence, respectively. Multiple insertions show an additional band of 7259 bp + the length of the inserted sequence (*see* **Note 5**). Use only clones that show the correct pattern for single or multiple integrations.

3.5 Quality Control of TH Strains

To rule out the possibility that individual clones suffered from the transformation process, the viability of all strains needs to be tested and compared to the progenitor strain SG200. Only strains that grow comparable to the SG200 can be used for subsequent disease ratings.

3.5.1 Analysis of Growth in Axenic Culture

1. Inoculate 2 mL of YEPS-light medium with your TH strains (as selected and confirmed in Subheading 3.4.). Also include the progenitor strain SG200 as a control and incubate at 200 rpm at 28 °C overnight.
2. Measure the OD_{600} of each culture sample.
3. Use a 24-well plate and inoculate in each well 1 mL of YEPS-light with your *U. maydis* strains in technical triplicates to a final OD_{600} of 0.1.
4. Seal the 24-well plate with Micropore tape and measure OD_{600} in a microplate reader for 24 h with the following settings (*see* **Note 6**): 1. Temperature: 30 °C, 2. Kinetic loop: 500 cycles, 15 minutes interval time, 3. Shaking (orbital): duration = 613 s, position = incubation, amplitude = 6 mm, frequency = 96 rpm, 4. Absorbance: Mode = absorbance, measurement wavelength = 600 nm, number of flashes = 37, settle time = 0 ms, multiple reads per well (circle) = 3 × 3, multiple reads per well (border) = 3000 μm.

3.5.2 Analysis of Filamentous Growth on Charcoal Media

1. Inoculate 2 mL of YEPS-light medium with the selected TH strains. Also include the progenitor strain SG200 as a control and incubate at 200 rpm at 28 °C overnight.
2. Prepare serial dilutions of the cultures to a final OD_{600} of 1.0, 0.1, and 0.01. Spot 5 μL of each dilution and each strain on a PD-charcoal plate and incubate for 12–24 h at 28 °C to verify the capability for filamentous growth of the TH strains.

3.5.3 Verification of Secretion of POI-mCHERRY Fusion Protein on Maize Seedlings

1. Sow 1 seed of *Z. mays* cv. B73 per 6x6 cm pot and grow for 9 days at 28 °C (15 h) and 22 °C (9 h), at least 30,000 lux during daytime, and a relative humidity of 65–75%.
2. Inoculate 2 mL of YEPS-light medium with the TH strains that passed quality control and incubate at 200 rpm at 28 °C overnight.

3. Inoculate 10 mL of YEPS-light with the overnight culture to a final OD_{600} of 0.2 and incubate at 200 rpm at 28 °C for 4–5 h to reach an OD_{600} of 0.8–1.0.
4. Collect the cells by centrifugation at 1080 × *g* for 5 min in a benchtop centrifuge.
5. Discard the supernatant and resuspend the cells in ddH_2O to an OD_{600} of 3.0.
6. Use a 1 mL syringe with a 27-gauge hypodermic needle to infect 9 days old maize seedlings by injecting 0.3–0.5 mL of the *U. maydis* suspension approximately 1 cm above the ground into the center of the leave whorl (*see* **Note 7**).
7. Cultivate the infected seedlings for 3 days under the above-mentioned conditions.
8. Cut a 0.5 × 0.5 cm piece of leaf approximately 1 cm below the puncture holes of the infection. Place the cut leaf on a microscope slide, add a drop of water and place a cover slip on top.
9. Inspect the infected tissue with a fluorescent confocal microscope. Excite mCHERRY with a laser at 561 nm. The mCHERRY signal can then be collected between 578 and 650 nm. Secretion is confirmed if you observe the mCHERRY fluorescence signal surrounding the fungal hyphae and accumulating in the apoplast at maize cell-cell borders. Fungal hyphae growing in the Z-axis show a ring-shaped fluorescence signal.

3.6 Infection and Disease Rating of Maize Seedlings

1. Sow 1 seed of *Z. mays* cv. B73 per 6 × 6 cm pot and grow for 9 days at 28 °C (16 h) 30,000 lux, and 22 °C (8 h) 0 lux, and a relative humidity of 65–75%. Perform the experiment in triplicates with a minimum of 30 infected seedlings per replicate and condition.
2. Inoculate 2 mL of YEPS-light medium with the selected strains and incubate at 200 rpm at 28 °C overnight.
3. Inoculate 20 mL of YEPS-light with the overnight culture to a final OD_{600} of 0.2 and incubate at 200 rpm at 28 °C for 4 to 5 hours to reach an OD_{600} of 0.8–1.0.
4. Collect the cells by centrifugation at 1080 × *g* for 5 min in a benchtop centrifuge.
5. Discard the supernatant and resuspend the cells in ddH_2O to an OD_{600} of 1.0.
6. Use a 1 mL syringe with a 27-gauge hypodermic needle to infect 9-day-old maize seedlings by injecting 0.3–0.5 mL of the *U. maydis* suspension approximately 1 cm above the ground into the center of the leaf whorl.

7. Cultivate the infected seedlings for 12 days under standard conditions (*see* **Note 7**).
8. Score the disease symptoms 6- and 12-days post infection by the heaviest visible symptom as follows: no symptoms, chlorosis (yellow leaf, no tumors visible), small tumors (tumors of <1 mm diameter), tumors (tumors between 1 and 5 mm diameter), large tumors (tumors bigger than 5 mm diameter), stunted growth (shape of the plant growth is affected due to tumor formation), dead plants (*see* **Note 8**).
9. Plot the results as shown in Fig. 2.

3.7 Infection and Disease Rating of Maize Tassels

1. Sow 3 seeds of *Z. mays* cv. Gaspe Flint per 10 ×10 cm pot and grow for 16 days at 28 °C (16 h) 30,000 lux, and 22 °C (8 h) 0 lux, and a relative humidity of 65–75%. Perform the experiment in triplicates with a minimum of 30 infected tassels per replicate and condition.
2. Inoculate 2 mL of YEPS-light medium with your transformed strains and incubate at 200 rpm at 28 °C overnight.
3. Inoculate 20 mL of YEPS-light with the overnight culture to a final OD_{600} of 0.2 and incubate at 200 rpm at 28 °C for 4 to 5 hours to reach an OD_{600} of 0.8–1.0.
4. Harvest the cells by centrifugation at 1080 × *g* for 5 min in a benchtop centrifuge.
5. Discard the supernatant and resuspend the cells in ddH_2O to an OD_{600} of 1.0.
6. Use a 1 mL syringe with a 27-gauge hypodermic needle to infect 16 days old maize plants (*see* **Note 9**) by injecting 0.5 mL of the *U. maydis* suspension into the airspace between leaves and tassel (*see* **Note 10**).
7. Cultivate the infected maize plants for 10 days under standard conditions.
8. Score the disease symptoms 10-days post infection by the heaviest visible symptom as follows: no symptoms, <50% small tumors (tumors of <5 mm diameter), >50% small tumors (tumors of <5 mm diameter), <50% large tumors (tumors of >5 mm diameter), >50% large tumors (tumors of >5 mm diameter), stunted growth (shape of the tassel growth is affected due to tumor formation).

4 Notes

1. Maize is GC-rich. By our experience, not all proof-reading polymerases amplify equally well using maize cDNA as template. In our hands HS VeriFi™ works well.

2. When working with *U. maydis* check for bacterial contamination before starting an experiment by inspecting the culture at 400× magnification with a light microscope. Also check for bacterial contamination after performing growth assays.
3. SCS-lysing enzyme solution always needs to be prepared freshly.
4. When distributing *U. maydis* protoplasts on the plate, it is important not to use glass beads or a spatula, as the mechanical force would destroy the protoplasts.
5. The sizes of fragments detected by southern blotting are only correct as long as no additional *Nco*I cleavage sites are introduced into the vector.
6. These settings are used on the Tecan Spark™ 10 M microplate reader. They may have to be adjusted for other microplate readers.
7. Maize plants infected with *U. maydis* should be kept in a separate growth chamber to avoid cross-contamination. Furthermore, all equipment used should be decontaminated with 70% ethanol.
8. Small tumors can be tricky to see by eye. Therefore, stroking gently over the infected leaf can help to distinguish between chlorosis and small tumors.
9. Depending on the conditions in the greenhouse, the development of tassels might start sooner or later. Therefore, it is recommended to frequently dissect a plant to identify the ideal date of infection (in our case, we typically infect tassels at 1–2 cm size).
10. The position of the tassel can be localized by gently pressing the stalk.

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Chapter 10

Feeding Assay to Study the Effect of Phytocytokines on Direct and Indirect Defense in Maize

Lei Wang and Matthias Erb

Abstract

Phytocytokines mediate defense against pests and pathogens. Many methods have been developed to study the physiological responses triggered by phytocytokines in dicot plants. Here, we describe a detailed peptide feeding protocol to study the effect of phytocytokines on direct and indirect anti-herbivore defense in maize. This method relies on peptide uptake by the excised maize seedling or leaves via the transpiration stream. The headspace volatiles from plant samples are then analyzed by proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS), or by gas chromatography–mass spectrometry (GC-MS). The samples can also be further processed to evaluate phytocytokine-induced defense gene expression or phytohormone production.

Key words Maize, Peptide feeding, Phytocytokine, Plant volatile, Anti-herbivore defense, Indirect defense, Plant-insect interactions, Proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS)

1 Introduction

Plants respond to insect herbivory by sensing various signals that are either derived from the herbivore, or produced in the plant as a result of herbivory [1, 2]. One group of such signals are small peptides called phytocytokines [3, 4]. Phytocytokines, such as systemin and ZmPep3, are induced by insect herbivory and mediate anti-herbivore defense [5, 6]. The defense can function directly by deterring the insect (antixenosis) or via interfering with insect growth and development (antibiosis), or indirectly by recruiting natural enemies [5, 7–9]. Jasmonate signaling is important for both direct and indirect anti-herbivore defense [10–12]. The increased expression of jasmonate biosynthesis and signaling genes, and increased level of jasmonates have been widely used as markers of anti-herbivore defense [1, 5, 11, 12]. In addition, the increased expression of proteinase inhibitors (PIs) and the production/

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emission of herbivore induced plant volatiles (HIPVs) have also been well established as hallmark events of direct and indirect defense, respectively [5, 8, 13–17]. In maize, HIPVs typically include green leaf volatiles (GLVs), indole, monoterpenes, sesquiterpenes and the homoterpenes (E)-4,8–dimethyl–1,3,7-nonatriene (DMNT) and (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) [18]. Synthesis and emission of these volatiles except GLVs have been shown to be induced by ZmPep3 [5, 13] (Figs. 1 and 2).

The development of modern sequencing technologies led to the assembly and annotation of many high-quality plant genomes, including that from maize, raising great opportunities to identify novel phytocytokines [19, 20]. Many assays have been developed to evaluate the role of these potential phytocytokines, such as ethylene induction and burst of reactive oxygen species assays for *Arabidopsis thaliana* and other dicot plants [21]. Here, we provide a detailed protocol to evaluate the ability of plant peptides to mediate direct and indirect defense in maize. This protocol (Subheading 3.1) relies on peptide uptake through the transpiration stream in detached maize seedlings or leaves. Peptide activity can be monitored by quantification of transcripts of defense-related genes via qRT-PCR, measurement of phytohormone concentrations, and profiling of HIPVs (Fig. 2). A similar protocol has been applied to study peptide-mediated anti-herbivore defense in tomato [22]. For comparison, we also provide a peptide treatment protocol modified from simulate herbivory [13, 23]. This protocol (Subheading 3.2) relies on mechanical wounding of leaf tissue from intact plants before applying peptide solutions into the wounds.

2 Materials

1. Maize seeds (Inbred line KN5585, B73).
2. Potting soil.
3. 9 cm × 9 cm × 10 cm plant pots.
4. 120 mL plastic tube (Ø × H 44 × 114 mm; e.g., Boettger, catalog number 08-033-0000).
5. Razor blades.
6. Milli-Q water.
7. 12 mL plastic centrifuge tubes.
8. 250 mL glass beakers.
9. 12 cm × 12 cm square Petri dishes.
10. Drill, Ø 6 mm.
11. Hemostatic forceps.

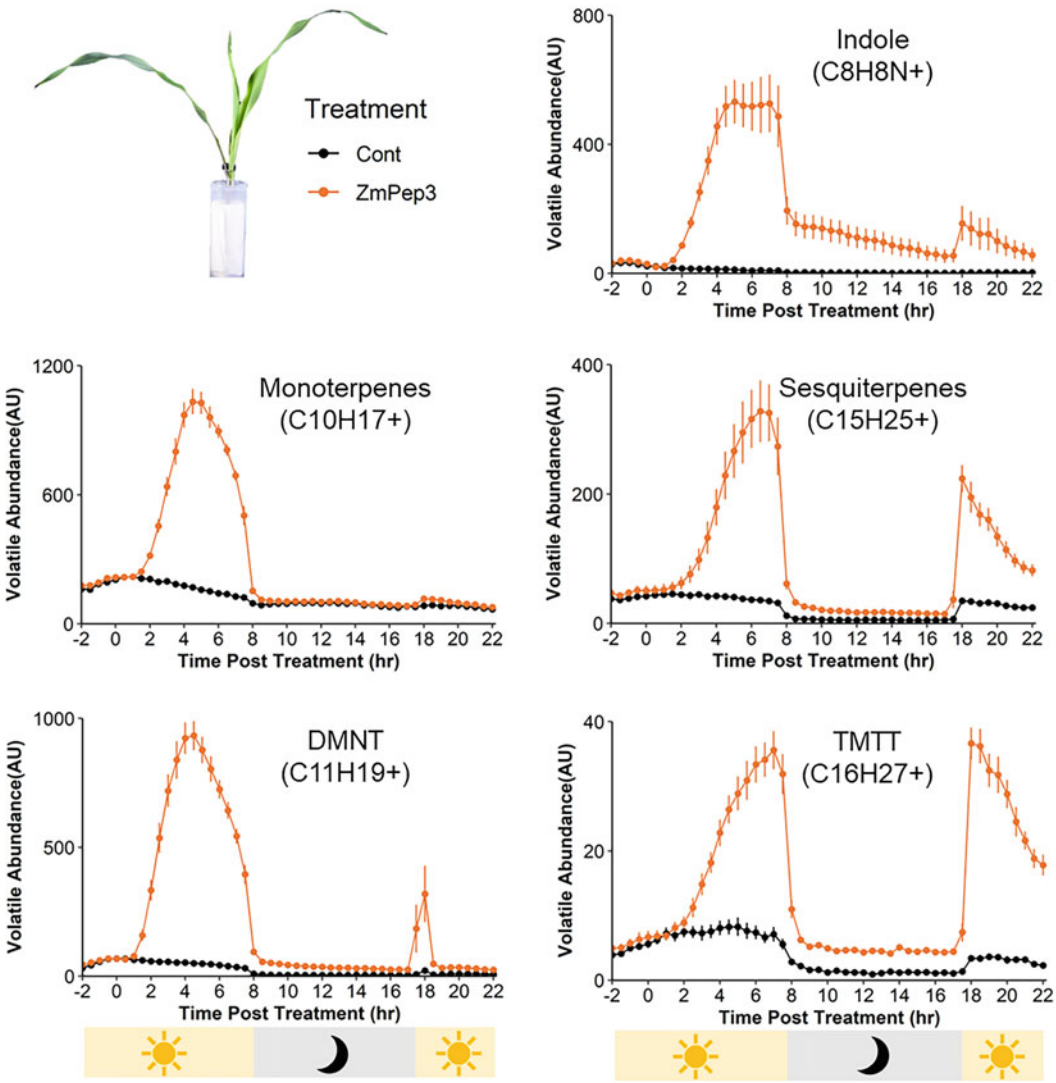


Fig. 1 ZmPep3-induced volatile emission. Volatile emission of excised KN5585 V3 seedlings fed with 1 μM ZmPep3 via the transpiration stream (protocol 3.2). Water treated seedlings were used as controls (Cont). Distinguished by their exact mass, relative emission rates of indole, monoterpenes, DMNT, sesquiterpenes, and TMTT were measured by PTR-MS. Light and dark periods are indicated below the X-axis. Data are shown as mean ± s. e. ($n = 6$)

12. ZmPep3 (TRTPPWPPCPPEEGSGGNGGSHN): The peptide was custom synthesized at >90% purity. Other peptides can be used, depending on the specific research question.
13. Customized plant volatile profiling system coupled to a proton transfer reaction time-of-flight mass spectrometer (Tofwerk, Switzerland). The system consists of transparent glass cylinders (Ø × H 12 × 45 cm, with transparent lids) to house the plants,

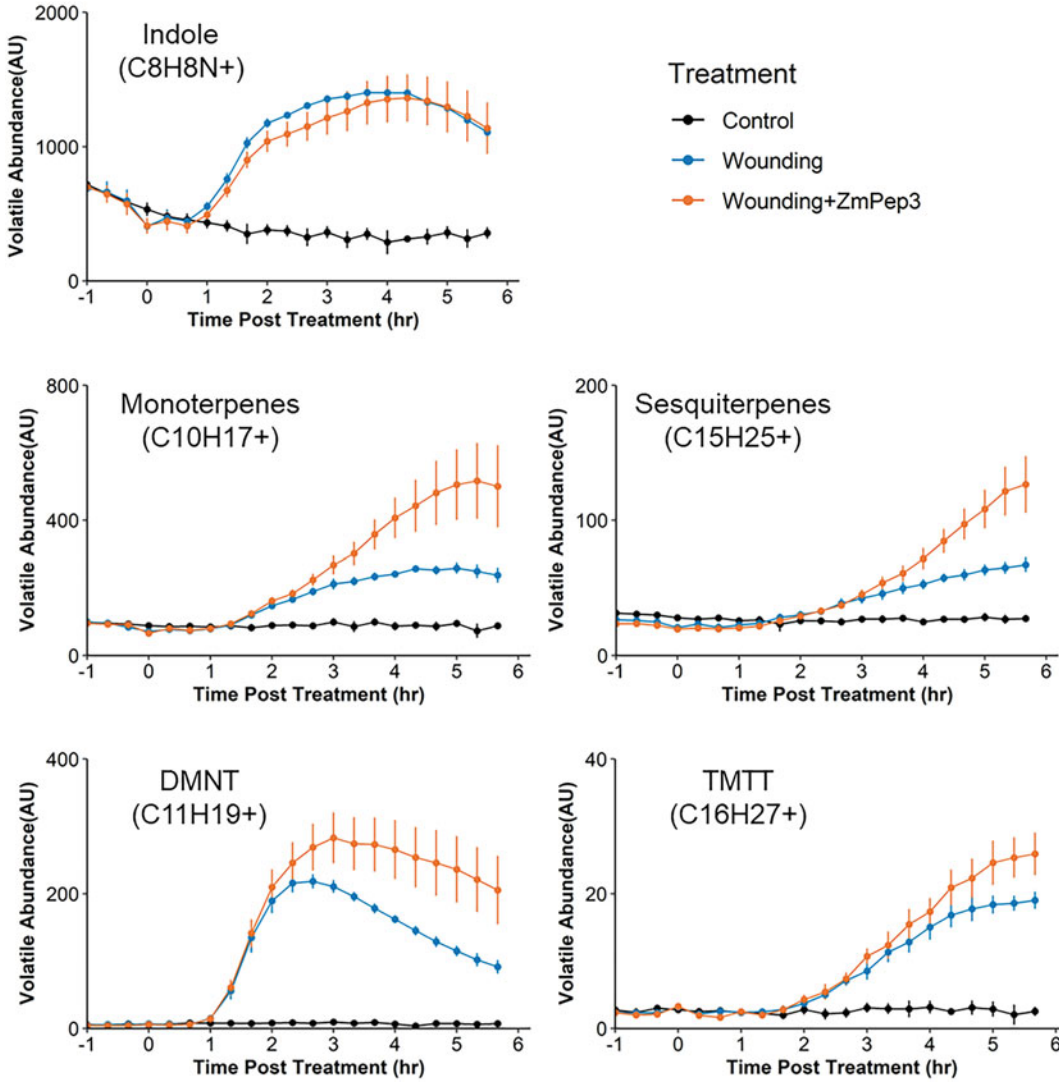


Fig. 2 Wounding+ZmPep3-induced volatile emission. Volatile emission of wounded B73 V2 seedlings with application of ZmPep3 into the wound sites (orange). Untreated (black) and wounding+water-treated seedlings (blue) were used as controls. Distinguished by their exact mass, relative emission rates of indole, monoterpenes, DMNT, sesquiterpenes and TMTT were measured by PTR-MS. Data are shown as mean ± s. e. ($n = 4$)

clean airflow (0.8 L min^{-1}) supplied through an air inlet and an outlet on the glass cylinders, and an automated headspace sampling system (Abon Life Sciences, Switzerland). A customized software allows the autosampler (connected with the PTR-TOF-MS) to switch from one air outlet to the next for real-time volatile profiling. Illumination during the experiment (7:00–21:00) is provided by LED lights (DYNA, Heliospectra) at ~300 μmol m^{-2} s^{-1}.

3 Methods

3.1 Peptide Feeding Via the Transpiration Stream

1. Sow maize seeds directly into 9 cm × 9 cm × 10 cm pots filled with potting soil. Cover the seeds with ~1 cm of soil (*see* **Note 1**). Grow maize seedlings in a glasshouse until the third leaf is fully mature (V3 stage, Fig. 3) (*see* **Note 2**). Glasshouse condition: 14 h/10 h light/dark cycle, ~300 μmol m^{-2} s^{-1} illumination; ~22 °C/18 °C day/night temperature; 40–60% relative humidity.
2. Dissolve ZmPep3 powder in Milli-Q water to make 10 mM stock. Dilute a fraction to 1 mM with Milli-Q water for treatment.
3. Cut V3 maize seedling at the position of first leaf with a razor blade (Fig. 3) (*see* **Note 3**).
4. Remove the second leaf carefully. Place the cutting site of the excised seedling into a square Petri dish filled with Milli-Q water. Further remove ~0.5 cm from the seedling under water (*see* **Note 4**). Transfer the excised seedling into a 250 mL glass beaker filled with ~150 mL Milli-Q water.
5. Incubate the excised seedlings in dim light (< 50 μmol m^{-2} s^{-1}) for 2 h to allow recovery from cutting (*see* **Note 5**).
6. Transfer the excised seedling into a 12 mL plastic centrifuge tube filled with 10 mL Milli-Q water (*see* **Note 6**). Place the centrifuge tube into a 120 mL plastic transport tube or any container with similar shape to keep the centrifuge tube upright.
7. Transfer the excised seedlings in the centrifuge tubes into glass cylinders for acclimation and background volatile measurement via PTR-MS (*see* **Note 7**).
8. One hour after acclimation, take the centrifuge tubes with excised seedlings out from the glass cylinders. Pipette 10 μL ZmPep3 (1 mM) solution or water as control in the plastic tube with seedlings, mix by pipetting. Place the seedlings back to the glass cylinders for continuous volatile profiling via PTR-MS (*see* **Note 8**) (Fig. 1).
9. After volatile profiling, weigh the seedlings and remaining water/peptide solution to normalize volatile emission to plant fresh weight, and to assess any effects on water uptake or transpiration (*see* **Note 9**).
10. Alternatively, 2 h after peptide treatment, harvest the leaves, wrap them in aluminum foil and freeze them immediately in liquid nitrogen. Store the samples at −80 °C for subsequent measurement of transcripts and phytohormone profiling (*see* **Note 10**).



Fig. 3 V3 stage KN5585 seedling. Yellow line indicates the cutting site

3.2 Peptide Treatment Via Wounding

1. Drill 2 holes (Ø 6 mm) into the bottom of the 120 mL plastic tubes. Fill the tubes with potting soil to ~1 cm below the rim. Sow maize seeds directly into the tubes (1 seed per tube), cover with ~1 cm potting soil. Grow maize seedlings in a glasshouse until the second leaf is fully mature (V2 stage) (*see* **Note 11**). Glasshouse condition: 14 h/10 h light/dark cycle, ~300 μmol m^{-2} s^{-1} illumination; ~22 °C/18 °C Day/night temperature; 40–60% relative humidity.
2. The day before experiment, transfer the tubes with V2 maize seedlings to 250 mL glass beakers with 20 mL tap water. Acclimate these plants overnight in the glass cylinders or other transparent glass containers (*see* **Note 12**).
3. Before peptide treatment, check the background volatile emission for 1 h (*see* **Note 12**).
4. Five minutes before headspace sampling, take the maize seedling out of the glass cylinder, wound the third leaf (developing leaf) with hemostatic forceps 3 times (~ 0.5 cm each time, ~ 2 mm between 2 wounds) on each side of the midrib. Pipette 5 μL ZmPep3 (10 μM) into the wounds on each side of the midrib (10 μL in total per leaf), spread the peptide solution evenly with a pipette tip (*see* **Note 13**).
5. Transfer the seedling back to the glass cylinder for volatile profiling (Fig. 2).
6. Continue to treat the remaining plants. Allow sufficient time for wounding and peptide treatment, and make sure that the time interval between treatment and headspace collection is the same for all plants/cylinders (*see* **Note 14**).

7. Alternatively, 2 h after peptide treatment, harvest the wounded leaves and freeze them immediately in liquid nitrogen (*see* **Note 15**). Store the samples at −80 °C for later measurement of transcripts and phytohormone profiling.

4 Notes

1. A ~ 1 L pot filled with nutrient-rich soil is sufficient for a maize seedling to grow till V4 stage without additional fertilization. Smaller pots and low soil quality may lead to malnutritional and stressed plants that are not suitable for this experiment.
2. V3 seedlings are ideally suited for peptide treatment and volatile profiling. V2 seedlings are sensitive to cutting and do not produce large amounts of volatiles after cutting. V4 or even bigger seedlings do respond well to ZmPep3 treatment, but they require much bigger glass containers for volatile profiling. Depending on the greenhouse condition and maize inbred line, plant growth time may vary. The plant developmental stage rather than absolute growth time matters for this experiment.
3. In case V4 or V5 seedlings are needed, cut the seedlings at the position of the second or third leaves, respectively. This protocol can also be used for a single leaf. In this case, cut the fourth leaf of a V4 seedling at the leaf collar position. Prepare at least 20% more excised seedlings or leaves than needed.
4. A second cut under water helps to remove air-filled vasculature, thus facilitates water uptake, and avoids seedling wilting. For experiments with single leaves, a second cut under water is also needed.
5. 2 h incubation in weak light helps the excised plant material to recover from cutting. In addition, excised seedlings or leaves that are not properly handled will start to wilt and can be easily excluded from further experiments. Directly using freshly cut plant material will lead to high volatile background, quickly wilting seedlings/leaves and poor defense induction.
6. 10 mL water is sufficient for a V3 seedling for a 24 h experiment in our setup (light/dark: 14 h/10 h). Depending on the plant material and experiment duration, this volume can be scaled down or up for most efficient peptide usage.
7. Our glass cylinders are supplied with airflow (0.8 L min^{-1}) and illuminated with LED lights. At least 1 h acclimation helps with getting clean volatile background. For labs without similar automated volatile sampling system, the background measurement can be skipped, but the acclimation is still recommended.

8. Our customized plant volatile profiling system allows high-throughput, real-time volatile profiling. For labs without a similar system, the headspace volatiles can also be collected via porapak Q column or super Q filter and subsequently analyzed with gas chromatography–mass spectrometry [13, 16]. ZmPep3-induced volatile emission peaks 4–6 h post treatment. This period is thus the best time window for volatile collection. Other peptides may induce volatile emission with different kinetics, a time series analysis can be performed to find the best volatile collecting time point.
9. Determining the plant fresh weight helps to standardize a plant's ability to produce volatile, especially when comparing mutants with wild-type plants. Determining the water uptake provides information if the peptide assayed affects stomatal aperture.
10. 2 h after peptide treatment is a good time point for determining jasmonate content and the expression of jasmonate biosynthesis/signaling genes. For volatile biosynthesis and proteinase inhibitor (PI) gene expression, a later time point may be more appropriate. For sample harvesting, cut the seedling at the position of the third leaf. Collect either all of the leaf material or just the fourth leaf. This leaf generates enough material for both phytohormone profiling and RNA extraction. The third leaf is hard to grind and yields low RNA quantity, it's thus not recommended.
11. We use these tubes because they fit well into 250 mL glass beakers. They are good for plants up to early V3 stage. Bigger seedlings may get stressed in these pots.
12. Adding water in the glass beakers prevents the plants from drought stress and helps to humidify the air in the glass cylinder.
13. Hemostatic forceps generate a more reproducible wounding pattern than scratching with a razor blade.
14. Wounding and peptide treatments take time. Calculate the time needed well before you start the experiments. Wounding itself also induces defense. Therefore, it is important to include wounding plus water treatment as control, instead of using untreated control solely (Fig. 2).
15. The wounded leaf here may not be enough material for multiple analyses. In case both transcript analysis and phytohormone measurement are desired, use late V2 seedlings as starting material.

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Chapter 11

A Quick Method to Analyze Peptide-Regulated Anthocyanin Biosynthesis

Eric Bühler, Andreas Schaller, and Nils Stührwohldt

Abstract

Post-translationally modified peptides are now recognized as important regulators of plant stress responses. We recently identified the sulfated CLE-LIKE6 (CLEL6) peptide as a negative regulator of anthocyanin biosynthesis in dark-grown and in light-stressed Arabidopsis seedlings. The function of CLEL6 depends on proteolytic processing by subtilisin-like serine proteinase SBT6.1, and on tyrosine sulfation by tyrosylprotein sulfotransferase (TPST), and CLEL6 signaling relies on the ROOT MERISTEM GROWTH FACTOR 1 INSENSITIVE (RGI) receptor family. In this chapter, we describe in detail how to quantify peptide-regulated and stress-induced anthocyanin biosynthesis. We include protocols for peptide treatment of Arabidopsis seedlings and growth under different stress conditions, for the extraction and quantification of anthocyanins, and for the expression analysis of anthocyanin biosynthetic genes.

Key words Anthocyanins, Light-stress, Stress response, CLEL peptides, Gene expression, RNA extraction, cDNA synthesis, qPCR, Photometric analysis

1 Introduction

Anthocyanins are secondary metabolites belonging to the class of flavonoids, and they are prominent pigments coloring flowers and fruits to attract pollinators as well as frugivore animals for seed dispersal. Over 600 different anthocyanins have been described that range in color from orange to red, blue, and purple [1, 2]. At the molecular level, anthocyanins are important stress metabolites produced in response to heat, cold, drought, light, and nutrition stress. A common feature of these stresses is the accumulation of reactive oxygen species (ROS). Due to their electron deficiency, anthocyanins are highly reactive against ROS, thereby protecting the plant from oxidative damage [3, 4]. Additional protection from light stress is provided by the fact that anthocyanins absorb photosynthetically active radiation, thereby protecting the photosynthetic apparatus from photoinhibition [5, 6].

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Anthocyanin biosynthesis is controlled by many transcription factors including MYB11 for the regulation of early biosynthesis genes *CHALCONE SYNTHASE* (CHS), *CHALCONE ISOMERASE* (CHI), and *FLAVANONE 3-HYDROXYLASE* (F3H), and PAP1 controlling *DIHYDROFLAVONOL 4-REDUCTASE* (DFR), *LEUCOANTHOCYANIDIN OXYGENASE* (LDOX) and *UDP-GLUCOSE:FLAVONOID 3-O-GLUCOSYLTRANSFERASE* (UF3GT) that code for enzymes late in the biosynthetic pathway [7]. Light-dependent regulation is provided by the E3 (ubiquitin ligase) complex CONSTITUTIVE PHOTOMORPHOGENESIS1/SUPPRESSOR OF PHYA-105 (COP1/SPA), and the transcription factors PHYTOCHROME-INTERACTING FACTOR3 (PIF3) and ELONGATED HYPOCOTYL 5 (HY5) [8, 9]. Hormonal regulators of anthocyanin biosynthesis include ethylene, gibberellins, abscisic acid, and jasmonates [10]. Furthermore, we recently identified the small post-translationally modified peptide CLE-LIKE6 (CLEL6) as a negative regulator of anthocyanin biosynthesis [11]. The activity of CLEL6 as an inhibitor of anthocyanin biosynthesis depends on post-translational tyrosine sulfation by tyrosylprotein sulfotransferase (TPST), proteolytic cleavage by the subtilisin-like serine proteinase SBT6.1, and perception by the ROOT MERISTEM GROWTH FACTOR 1 (RGI) receptor family. CLEL6 downregulates the expression of the MYB11 and PAP1 transcription factors and of anthocyanin biosynthesis genes to restrain anthocyanin formation [11].

To assess the effect of signaling peptides on the plant stress response, we analyze anthocyanin biosynthesis and accumulation in peptide-treated vs. control seedlings. Here we include protocols for the treatment of Arabidopsis seedlings, the extraction of pigments, and the photometric quantification of anthocyanins in seedling extracts. The effect of peptide treatment on anthocyanin biosynthesis is analyzed by reverse transcriptase (RT) qPCR. Protocols are included for the isolation of total RNA from peptide-treated and control seedlings, for cDNA synthesis, and for gene expression analysis using oligonucleotide primers specific for anthocyanin biosynthesis genes and their transcriptional regulators. Finally, a comparison between the expression level of the genes involved and anthocyanin content yields important information on peptide-regulated anthocyanin biosynthesis.

2 Material

2.1 Growth of Plants

1. 94 mm Petri dishes.
2. ½ MS Plates: Weigh in 1.075 g/L MS salts and 10 g/L sucrose. Dissolve in water. Add 3.8 g/L gelrite, make up to 1 L with water. Sterilize by autoclaving. Cool down to 60 °C.

For peptide treatment, include 1 μM of the peptide from sterile stock solution. Pour into Petri dishes. Store at 4 °C for max of 1 week. Optional for osmotic stress treatment: include 30 mM mannitol.

3. 70% (v/v) ethanol.
4. Sterile ddH_2O.
5. CLEL6 peptide (Sequence: DY(SO_3H)PQPHRKPPIHNE). Take up the lyophilized peptide in autoclaved ddH_2O to result in a 1 mM stock solution (*see* **Note 1**). Store at −20 °C. Avoid repeated freeze-thaw cycles.
6. Rotator.
7. Temperature-controlled plant growth cabinet.

2.2 Anthocyanin Extraction and Measurement

1. Bead beater, with 2 mL reaction tubes and steel beads. We recommend the bead beater for tissue homogenization. Alternatively, frozen tissue sample can be homogenized with mortar and pestle.
2. Liquid nitrogen.
3. Anthocyanin extraction solvent: 45% (v/v) methanol, 5% (v/v) acetic acid.
4. Photometer: We recommend to perform the assay in a 96-well plate format. This requires a microplate reader (e.g., TECAN SPARK). Alternatively, measure the samples in cuvettes using a conventional photometer.
5. 96-well microtiter plates (e.g., flat-bottom Greiner bio-one), or 1.6 mL disposable half micro cuvettes.
6. Microcentrifuge for 1.5 mL/2 mL microfuge tubes.

2.3 RNA Extraction

1. RNAse-free 1.5 mL reaction tubes. To avoid RNA degradation by RNAses we recommend using RNAse-free reaction tubes. Alternatively, you can use standard reaction tubes autoclaved for 45 min.
2. Micropestles (DNase/RNase-free) fitting the reaction tubes.
3. TRIzol reagent.
4. Chloroform.
5. Isopropanol.
6. 70% (v/v) ethanol.
7. RNAse-free water.

2.4 cDNA Synthesis

1. DNAseI (1 U/μL, e.g., Thermo Scientific).
2. 5× RT buffer: 250 mM Tris/HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT.

3. 25 mM EDTA: Weigh in 0.9306 g disodium EDTA dihydrate and dissolve in 80 mL of water. Adjust pH to 8.0 with NaOH while stirring until completely dissolved. Add water up to 100 mL. Store at room temperature.
4. 10 mM dNTPs.
5. 0.5 μg/μL Oligo(dT) primers.
6. Reverse Transcriptase (200 U/μL, e.g., RevertAid, Thermo Scientific).
7. Thermocycler.

2.5 qPCR Analysis

1. qPCR Primers (*see* Table 1): obtain primers custom-synthesized on a 0.01 μmol synthesis scale, purified and desalted. Take up in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) to result in 100 μM stock solutions. Dilute in water to result in 1 μM working solutions. Store at −20 °C.
2. 10,000× SYBR Green solution in DMSO (e.g., SYBR Green I nucleic acid gel stain, Invitrogen). Dilute 1/7000 to obtain the SYBR Green working solution.
3. DNaseI (e.g., DNaseI, RNase-free, 1 U/μL, ThermoFisher).
4. *Taq* DNA-polymerase: We use recombinant *Taq* DNA-polymerase expressed and purified in our lab. Alternatively, any of many commercial sources can be used.
5. 10 mM dNTPs.
6. 5× PCR puffer: 15 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 0.08% (v/v) Triton ×100, 10% (v/v) DMSO, 250 mM KCl, 50 mM Tris/HCl, pH 8.3, 0.4% (v/v) Tween 20.
7. Thermocycler.
8. Thermocycler for qPCR (e.g., CFX Connect Realtime System, Biorad).

3 Methods

3.1 Growth of Plants

1. Surface sterilization of seeds: Put approximately 60 seeds into a 1.5 mL reaction tube and add 1 mL of 70% ethanol. Prepare one tube for each treatment you intend to do (e.g., control vs stress treatment, or control vs peptide treatment at different concentrations).
2. Incubate on rotator for 15 min at room temperature.
3. Perform **steps 3–5** under a clean bench: Let the seeds settle to the bottom of the reaction tube and use a pipet to discard the supernatant ethanol.

Table 1
qPCR primer list. ACTIN, TUB4, and CHS primers were previously published in [11]. F3'H, DFR, LDOX, UF3GT, UGT78D2, PAP1, and MYB11 primers were previously published in [15]

Name	Sequence (5′-3′)
ACTIN2-F	GTGGATATCAGGAAGGATCTGTACGGT
ACTIN2-R	TTCTGTGAACGATTCCTGGACCTGC
Tub4-F	TGAGGAAGGAAGCTGAGAACAG
Tub4-F	ATACACTGGTCAGCGTTTTC
CHS-F	CCTGACTACTACTTCCGCATCACC
CHS-R	CGCACATGCGCTTGAACTTCTC
CHI-F	TCATGTAGACTCCGTCACGTTTG
CHI-R	TGACAGATAGAGAAGGAACGGCG
F3′H-F	CGGTGGACTGGGCTATAGCTGA
F3′H-R	CGAGAGTGGTGTTGGTGGATG
DFR-F	CTTTGTTCGTGCCACCGTTCG
DFR-R	AAAATCCATGGGTGTTGCCAC
LDOX-F	GTTTGCAGCTTTTCTACGAGGGC
LDOX -R	ATGTTGAGCAAAAGTCCGTGGAG
UF3GT-F	TTGTCAGATCGTTTTGGTTCCGC
UF3GT-R	TCTTCCTCACTTTCTCACCGATC
UGT78D2-F	CGGTGTTGGAGAGTGTATCGG
UGT78D2 -R	CCAATCTCCCACACAACCTCC
PAP1-F	TGGTTCCTGAAGCGACGACAAC
PAP1-R	CGCAAACAAATGTTCGAAACAC
MYB11-F	GATGGCGATTGTAACCCAAGC
MYB11-R	ACATGAGGACACGTGGACAGC

4. Wash the seeds by adding 1 mL sterilized ddH_2O, let them settle again and remove the supernatant. Repeat this step two more times.
5. Pipet 45 seeds onto ½ MS plates, one tube per plate (see Subheading 3.1, **step 1**) and distribute them evenly. We recommend pipetting the seeds in three lines, approximately 15 seeds each. Place them as far apart as possible.
6. Stratify seeds for 2 days at 4 °C.

7. Transfer the plates into a growth cabinet to grow seedlings for 5 days at 22 °C. For light-stress experiments, keep them in darkness. Alternatively, grow seedlings at 100 μmol m^{-2} s^{-1} white light under (12 h/12 h) short-day conditions.
8. Induce stress to the Arabidopsis seedlings by specific treatments: for osmotic stress, transfer the seedlings on media supplemented with 300 mM mannitol. To induce light stress, transfer the plates with etiolated seedlings into continuous strong light (150 μmol m^{-2} s^{-1}). To induce heat stress, raise the temperature to 30 °C, or transfer the plates into a 30 °C growth cabinet at the same light regime. For peptide treatment see Subheading 2.1, **step 2**. Seedlings can either be grown on media including peptide, or be transferred on plates including peptide after 5 days.

3.2 Anthocyanin Extraction and Measurement

1. At the desired time point after stress treatment (*see* **Note 2**), collect plant material, about 30 entire seedlings as one sample, and determine the fresh weight (*see* **Note 3**).
2. Freeze samples in liquid nitrogen.
3. Homogenize the samples in a 2 mL reaction tube. Use either a bead beater or mortar and pestle pre-cooled with liquid nitrogen for tissue disruption.
4. Add 200 μL anthocyanin extraction solvent to each sample (*see* **Note 4**).
5. Mix thoroughly using a vortexer.
6. Centrifuge for 10 min at 13,000 × *g* and 4 °C.
7. Transfer supernatant into new reaction tube.
8. Repeat **steps 6 and 7** to remove any insoluble remnants (*see* **Note 5**).
9. Transfer 100 μL of the supernatant into a 96-well plate for measurement in a microplate reader. Alternatively, add 100 μL of the supernatant to 900 μL of anthocyanin extraction solvent in a semi-micro cuvette for measurement in a conventional photometer (*see* **Note 6**).
10. Measure absorption at 534 nm and 657 nm for each sample. For calibration, use a blank including only the extraction solvent.
11. Calculate the anthocyanin content by using the following formula:

$$[\mathrm{Abs}_{534} - (0.25 \times \mathrm{Abs}_{657})] \times 2/\mathrm{g\ fresh\ weight}.$$

12. Plot the results as shown in Fig. 1.

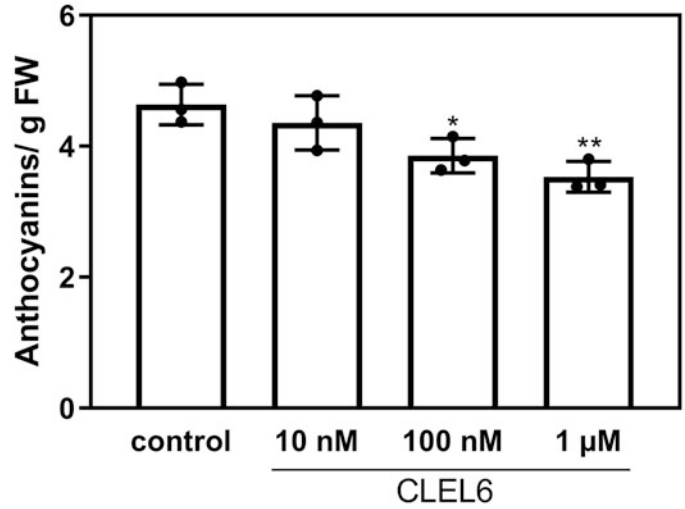


Fig. 1 CLEL6 treatment reduces the anthocyanin levels in wild-type seedlings in a dose-dependent manner. Quantification of the anthocyanin content in wild-type (wt) seedlings that were grown for 5 days in darkness followed by 16 h light stress (150 µmol m^{-2} s^{-1}). The growth medium was supplemented with 10 nM, 100 nM, or 1 µM CLEL6 as indicated. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$; two-tailed t-test, $n = 3$)

3.3 Gene Expression Analysis

Use RNAse-free reaction tubes and pipet tips for all steps in this protocol (Subheading 3.3).

3.3.1 RNA Extraction

1. Collect all entire seedlings of one plate into one reaction tube and freeze them in liquid nitrogen.
2. Homogenize samples using either a bead beater or micropestles for tissue disruption (*see* **Note 7**).
3. Directly add 1 mL of TRIzol, mix thoroughly by vortexing and incubate at room temperature for 10 min.
4. Centrifugation for 10 min at 4 °C, 13,000 × *g*.
5. Transfer supernatant into a new reaction tube.
6. Add 200 µL chloroform and incubate for 5 min at room temperature and shaking at 1500 rpm.
7. Centrifuge for 5 min at 4 °C, 13,000 × *g*.
8. Transfer upper aqueous phase into a new reaction tube.
9. Add 600 µL isopropanol and mix thoroughly.
10. Incubate at 4 °C for 30 min to precipitate RNA.
11. Centrifuge for 10 min at 4 °C, 13,000 × *g*.
12. Use a pipet to carefully discard the supernatant.
13. Add 1 mL of 70% ethanol and incubate for 5 min to wash the RNA pellet.

14. Centrifuge for 5 min at 4 °C, 13,000 × *g*.
15. Use a pipet to carefully remove as much of the supernatant as possible.
16. Dry the pellet by placing the open tubes under a clean bench for approximately 30 min.
17. Resuspend the pellet in 20 µL RNase-free water. Store at −20 °C.
18. Determine RNA concentration in a photometer. We recommend to use a photometer for very small volumes (e.g., Nanodrop, or TECAN microplate reader with NanoQuant Plate). Measure absorption at 260 nm and 280 nm. The ratio 260/280 is a measure for the purity of the RNA. A value between 1.8 and 2 indicates purity, lower values result from contaminating proteins. Calculate RNA concentration by:
    C [µg/mL] = (OD260/pathlength) *dilution factor*40.
    The pathlength depends on the instrument used; for standard cuvettes it is 1 cm.

3.3.2 First-Strand cDNA Synthesis

We recommend to perform the following steps in 0.5 mL PCR-reaction tubes in a thermocycler.

1. Use 1–10 µg of RNA (from Subheading 3.3.1, **step 17**) and add RNase-free water up to a final volume of 15 µL.
2. Add 4 µL of 5× RT-buffer and 1 µL (1 U) DNaseI (*see* **Note 8**).
3. Incubate for 30 min at 37 °C.
4. Add 2 µL 25 mM EDTA.
5. Incubate for 15 min at 65 °C to inactivate DNaseI.
6. Add 2 µL of 0.5 µg/µL oligo(dT) primers.
7. Incubate for 5 min at 70 °C.
8. Add 8 µL 5× RT-buffer, 4 µL dNTPs and 4 µL RNase-free water.
9. Incubate for 5 min at 37 °C.
10. Add 2 µL reverse transcriptase.
11. Incubate for 1 h at 42 °C.
12. Incubate for 15 min at 70 °C to inactivate the reverse transcriptase. The resulting cDNA can be used directly for qPCR analysis, or stored at −20 °C. Depending on the amount of RNA used for cDNA synthesis, we recommend diluting the cDNA before qPCR analysis (e.g., dilute 1:10 for cDNA from 5 µg of RNA).

3.3.3 qPCR Analysis

1. Prepare the reaction mix for every primer combination (*see* **Notes 9** and **10**) as shown in Table 2. We recommend to prepare a master mix containing all components but the

Table 2
Reaction mix for qPCR

Reagent	Volume [μL]
ddH_2O	4.5
5× PCR buffer	4
dNTPs	0.5
Sybr green	4
Primer F	2
Primer R	2
Taq polymerase	1
cDNA	2
In total	**20**

cDNA template. It is highly recommended to perform triplicates for every sample of cDNA to avoid technical errors (*see* **Notes 11** and **12**).

2. Perform the qPCR with the following program:

$$\begin{array}{rl} 2 & \text{min at } 95\,^{\circ}\text{C} \\ 10 & \text{sec at } 95\,^{\circ}\text{C} \\ 10 & \text{sec at } 56\,^{\circ}\text{C} \\ 45 & \text{sec at } 72\,^{\circ}\text{C} \end{array} \times 44$$

3. To check for the specificity of amplification include a melting curve at the end of the qPCR program (*see* **Note 13**).
4. When the qPCR is finished, check the Ct values for the reference genes (*ACTIN2* and *TUB4*) obtained for the different cDNA samples. They should not exceed 35 (*see* **Note 14**) and they should not differ by more than 2 (*see* **Note 15**).
5. Calculate the treatment (peptide or stress)-induced change in relative expression of the target gene using the $2^{-\Delta\Delta Ct}$ method [12, 13] (*see* **Note 16**). We recommend to use two reference ("house keeping") genes (*ACTIN2* and *TUB4*) for normalization. Calculate relative expression changes for both, and use the mean.
6. Plot the results as shown in Fig. 2.

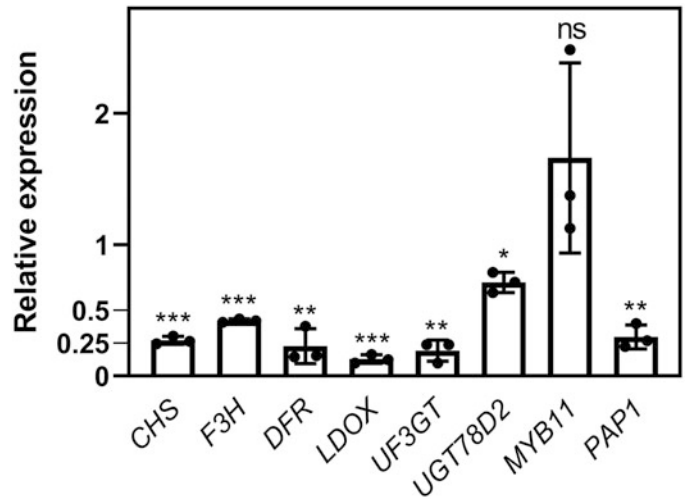


Fig. 2 CLEL6 treatment downregulates the expression of anthocyanin biosynthesis genes. Relative expression of *CHS*, *F3H*, *DFR*, *LDOX*, *UF3GT*, *UGT78D2*, *MYB11*, and *PAP1* in five-day-old etiolated seedlings treated with CLEL6 relative to the untreated control. 1 μM CLEL6 was added to the growth medium. Gene expression was normalized to *ACTIN2* and *TUB4* ($n = 3$). ns indicates no significant difference; asterisks indicate significant differences at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one sample t-test)

4 Notes

1. Peptides should be custom-synthesized at >90% purity or higher.
2. We recommend 1–2 days of stress treatment before the sampling for anthocyanin measurement. For the analysis of stress-induced induction of anthocyanin biosynthesis genes, we recommend a shorter time of stress exposure of approximately 2 h before sampling. Alternatively, a time-course analysis can be performed.
3. For accurate fresh weight determination, use a tissue to cautiously clear plant samples of adhering water or medium.
4. The volume may have to be adjusted depending on the fresh weight of the samples. We recommend to use × μL per × mg of fresh weight. Be aware that you need to adapt the formula for the calculation of the anthocyanin content (Subheading 3.2, **step 11**) if extraction volumes are changed.
5. It is very important that the anthocyanin extract is clear and does not contain any insoluble material that would affect photometric measurement. If it is not, repeat **steps 6** and 7 as often as required.

6. We recommend to measure each sample in technical duplicates and calculate the mean.
7. Make sure that the plant material does not thaw during homogenization before you add the TRIzol reagent. Otherwise RNA will be degraded.
8. DNAseI is added to degrade any contaminating genomic DNA [14]. It is important that the product used is free of RNase activity.
9. In this protocol we included the primers recommended for the analysis of anthocyanin biosynthesis genes. If you want to analyze the expression of other genes, you will need different primers. For the design of primers, aim at an amplicon length of approximately 100–200 base pairs. The primers should be 20–25 nucleotides in length, and the melting points should be in the range of 55–63 °C. It is crucial that the primers bind specifically to the gene of interest and to no other targets in the genome. If possible, choose primers that span an exon-intron boundary to avoid the amplification of any residual genomic DNA. We recommend using a tool like QuantPrime (https://quantprime.mpimp-golm.mpg.de/) for primer design.
10. Primer efficiency needs to be determined experimentally before you use them for qRT-PCR analysis. To create a standard curve, prepare a serial dilution of your cDNA (e.g., 0.5, 0.25, 0.125, 0.0625, 0.03125) and amplify these samples with the primer pair of interest in a standard qPCR. Plot the log of dilution against the measured Ct values and calculate the slope of regression. Calculate primer efficiency using the following formula: primer efficiency (%) $= (10^{-1/\text{slope}} - 1) \times 100$.

    A pair of primers is suitable for qPCR analysis if efficiency is between 90 and 110%. We recommend to use the Bio-Rad CFX Maestro tool to analyze and calculate primer efficiency easily.
11. Don't forget to include a non-template control (NTC) for every master mix. This can be used to evaluate contaminations.
12. To accurately compare different cDNA samples for a specific primer pair, they have to be amplified with the same master mix and run on the same qPCR plate.
13. The melting temperature depends on the length of the amplicon, which depends on the primer pair used. Therefore, the melting curve should be the same, with a specific peak for all samples amplified with the same pair of primers. If there is more than one peak, or no clear peak detectable, there are more than one PCR product, or primer dimers present. Additional amplicons may result from contamination with genomic DNA, or the amplification of off-targets. In this case the primers are not suitable and should be re-designed.

14. At Ct values above 35, results are not reliable. Repeat the analysis with an increased amount of cDNA template.
15. The Ct value of the house keeping genes (*ACTIN2* or *TUB4*) should not differ by more than 2 between the different samples. Otherwise they are not comparable. If the difference is too big, one of the cDNA samples should be diluted and the qPCR analysis repeated.
16. First calculate the relative expression of the reference gene (R; *ACTIN2* or *TUB4*) between treated and control sample. In this step, the difference in the Ct of house keeping genes between the control and treated sample is calculated to later normalize the values of the target gene.

$$R = 2\ \Delta\text{Ct (Ct control sample} - \text{Ct treated sample)}$$

Second calculate the relative expression of the target gene (T). In this step, the difference in the Ct of the target gene between the control and target sample is calculated. Thereby the fold change of the target gene between control and treated sample is calculated.

$$T = 2\ \Delta\text{Ct (Ct control sample} - \text{Ct treated sample)}$$

To normalize the fold change of the target gene, divide relative expression of target by that of the reference genes.

$$N = T/R$$

If primer efficiency ≠ 100%, modify the formulas for $T$and R by substituting '2' with 1 + 1*(primer efficiency(%)/100.

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Chapter 12

Quantitative Measurement of Pattern-Triggered ROS Burst as an Early Immune Response in Tomato

Rong Li, Andreas Schaller, and Annick Stintzi

Abstract

The rapid accumulation of extracellular "reactive oxygen species" (ROS), also known as the "oxidative burst", is an early plant immune response triggered by pathogen-derived microbe-associated molecular patterns and by endogenous plant signaling molecules. The oxidative burst is often used as a readout for the activation of defense signaling. Here, we present a detailed protocol for the continuous measurement of ROS production in leaf discs of tomato plants, using a chemiluminescence-based assay in a microtiter plate format. We also include recommendations for data analysis and for the quantitative assessment of differences in ROS burst dynamics, as caused by different types of elicitors, or in different tomato genotypes.

Key words Chemiluminescence, Reactive oxygen species (ROS), Oxidative burst, Pattern-triggered immunity (PTI), Elicitor, Phytocytokine, Systemin, flg22, Chitin, *Solanum lycopersicum*

1 Introduction

Plants are able to sense danger by using plasma membrane-localized pattern recognition receptors [1, 2] that detect molecular signatures of potential microbial pathogens, so-called microbe-associated molecular patterns (MAMPs, e.g., flg22 and chitin) [3–5], as well as molecular signatures of injury, also known as damage-associated molecular patterns (DAMPs, e.g., pectic cell wall fragments, and plant elicitor peptides, PEPs) [6–8]. When MAMPs and DAMPs are perceived by cognate PRRs, a sequence of early signaling responses is initiated, including plasma membrane depolarization, increase in cytosolic $[Ca^{2+}]$, and the production of reactive oxygen species (ROS), which ultimately lead to the activation of pattern-triggered or innate immunity [1, 9–13]. In addition, plants produce peptide signals known as phytocytokines to modulate early defense signaling and pattern-triggered immune responses (e.g., Golven peptides and systemins; [14–17]).

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The rapid production of extracellular ROS, i.e., the ROS burst, is mediated by plasma membrane-localized Respiratory Burst Oxidase Homologs (RBOHs), catalyzing the NADPH-dependent reduction of molecular oxygen (O_2) to superoxide radicals (O_2^-), and by their subsequent conversion to H_2O_2 by superoxide dismutase [18, 19]. Extracellular ROS are toxic to potential invaders, they promote the oxidative crosslinking and reinforcement of the cell wall, and they serve as signaling molecules for the activation of defense gene expression, thereby contributing to plant resistance in multiple ways [13]. The ROS burst thus provides a convenient readout for the activation of pattern-triggered immune responses.

Chemiluminescence assays have been used frequently to quantify ROS [20–22]. In these assays, the oxidation of a chemiluminescent probe results in the emission of photons that can be quantified as relative light units (RLUs) or photon counts (Counts) using a microplate reader or luminescence imager. A variety of probes (e.g., fluorescent and chemiluminescent dyes) have been developed to monitor ROS dynamics in plants [23, 24]. Here, we present a detailed protocol for the detection of pattern-triggered ROS production in tomato leaf discs using the luminol-derived probe L-012. The peroxidase-catalyzed oxidation of L-012 by H_2O_2 results in the formation of an unstable endoperoxide that decomposes to emit luminescence. Our assay is performed in a 96-well microtiter plate format allowing for efficient screening of mutants for quantitative differences in pattern-triggered ROS production.

2 Materials

2.1 Plant Maintenance

1. Sturdy seedling propagation trays (35 × 21.5 × 5 cm) with 77 cells (3 × 3 × 5 cm).
2. Round plastic pots (Ø10 × 9 cm).
3. Soil: 1:1 mixture of seedling substrate (e.g., Klasmann Seedling Substrate) and cultivation soil (e.g., ASB Greenworld Premium Anzuchterde).
4. Plant growth chamber (26 °C, 16 h light, 100 $\mu mol\ m^{-2}\ s^{-1}$ light intensity).
5. 70 °C incubator.

2.2 ROS Assay Setup

1. Disposable biopsy punch with plunger (Ø4 mm; e.g., Miltex™ biopsy punch), a small cutting mat (approx. 6 × 7 cm), and a spatula.
2. ddH_2O, autoclaved.
3. Tip-Tub autoclavable reagent reservoir, 50–60 mL.
4. 8-Channel pipette, 10–100 µL.

5. White 96-well microtiter plates, flat bottom wells, for chemiluminescence detection (e.g., LUMITRAC 200; Greiner Bio-One).
6. 20 mM luminol (L-012): Dissolve 6.2 mg L-012 in 1 mL sterile ddH_2O. Make sure pH is around 7 ~ 8. Prepare 40 μL aliquots and store at −20 °C in the dark (*see* **Note 1**).
7. 5 mg/mL horseradish peroxidase (HRP): Weigh 5 mg HRP into 1 mL sterile ddH_2O. Prepare 20 μL aliquots and store at −20 °C.
8. 1 mM peptide elicitors: Obtain systemin (AVQSKPPSKRDPPKMQTD) and flg22 (QRLSTGSRINSAKDDAAGLQIA) peptides, custom-synthesized at >90% purity. Dissolve lyophilized peptides in ddH_2O to prepare 1 mM aqueous stock solutions, divide into 10 μL aliquots and store at −20 °C (*see* **Note 2**).
9. 10 mg/mL colloidal chitin: slowly add 500 mg chitin power from shrimp shells into 10 mL 1 N HCl on a magnetic stirrer; stir at high speed overnight at room temperature. The next day, add 100 mL ddH_2O (10 volumes) under continuous stirring, keep stirring for 2 h. Centrifuge the colloid for 10 min at 16,000 × *g*, wash the acidic pellet, once with 1 N NaOH then with ddH_2O until the pH of the supernatant after centrifugation is 7. Spin down once more, resuspend the chitin pellet in 50 mL ddH_2O, autoclave the suspension for long-term storage (*see* **Note 3**).
10. Prepare aliquots of elicitor solutions diluted to working concentration (0.6 μM systemin, 0.6 μM flg22, 1 mg/mL chitin) and store at −20 °C.

3 Methods

3.1 Plant Growth

1. Dry tomato (*S. lycopersicum*) seeds in the 70 °C incubator overnight.
2. Incubate the tomato seeds in 23% (w/v) trisodium phosphate (TNP) to inactivate potentially contaminating tobacco mosaic virus (TMV).
3. Sow the seeds into moist soil in seedling propagation trays. Keep under transparent plastic cover for gemination under long-day conditions (26 °C, 16 h light/8 h dark cycle).
4. After 2 weeks, select seedlings identical in size, and transplant them individually into round plastic pots (*see* **Note 4**).
5. Continue to grow tomato plants until they are 4–5 weeks old (Fig. 1a) (*see* **Note 4**).

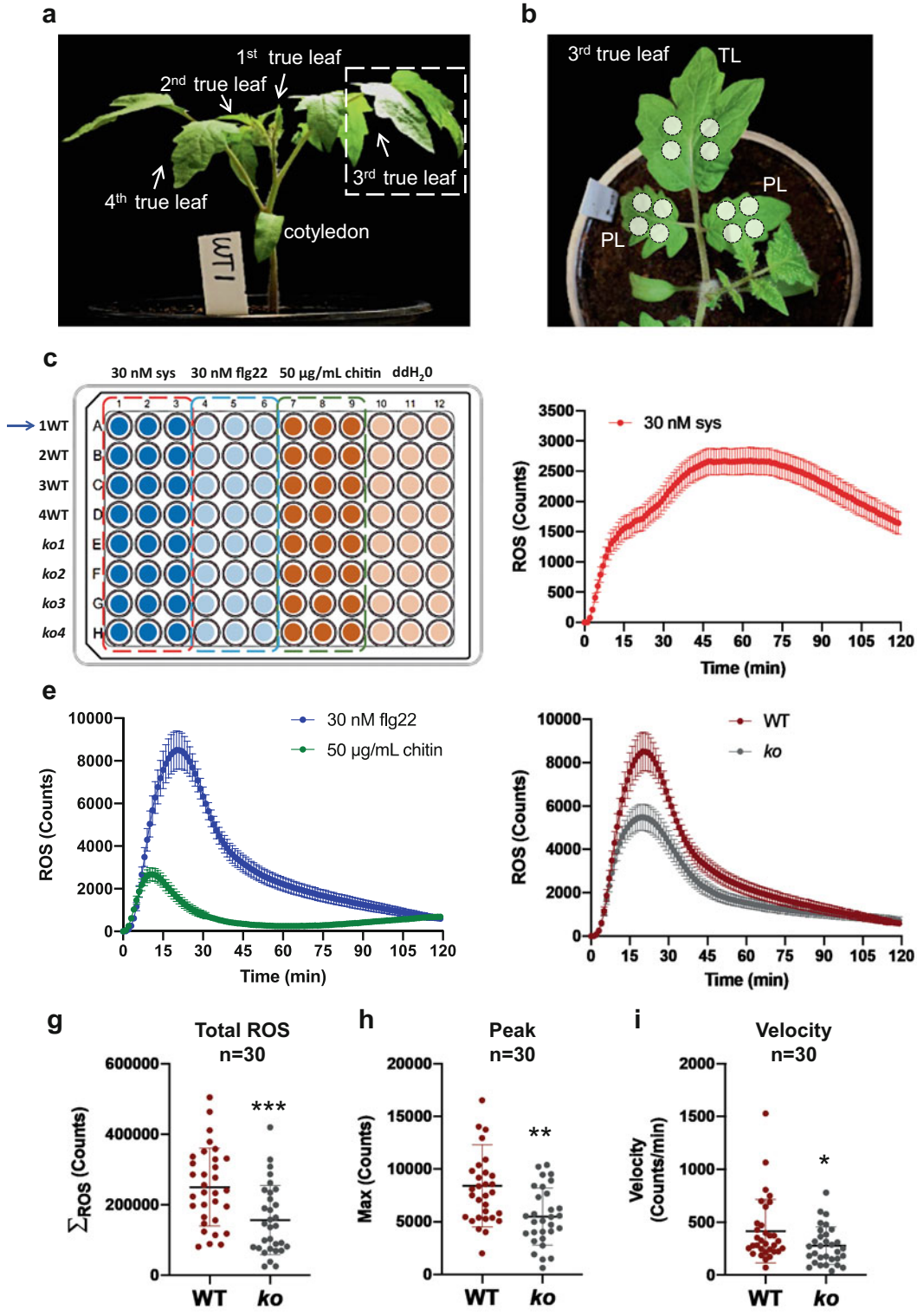


Fig. 1 The establishment and application of a L-012-based ROS assay in tomato. (**a**) Representative picture of a 4-week-old tomato plant. Dashed rectangle highlights the region of the third leaf from the top selected for ROS assays. (**b**) Top view of the region boxed in (**a**) with the terminal (TL) and a pair of primary leaflets (PL).

3.2 Collection of Leaf Discs

1. Plan the experiment well ahead: print out the layout of a 96-well microplate and draw the experimental setup (Fig. 1c) (*see* **Note 5**). Arrange leaf discs of the individual plants in rows (e.g., four wild-type individuals in rows A-D, a different mutant genotype in rows E-H) Arrange different elicitor treatments in columns (e.g., systemin treatment in columns 1–3, flg22 treatment in columns 4–6, chitin treatment in columns 7–9, and mock (water) treatment in columns 10–12 (Fig. 1c) (*see* **Note 6**). Thereby, you will have 3 leaf discs per individual and treatment, and 4 individuals for each of the 2 genotypes per plate.
2. Right before leaf disc collection, use a multi-channel pipette to fill 200 μL ddH_2O into each well of the 96-well microtiter plate.
3. Choose fully expanded leaves at positions 3 or 4 from the top of a 4- to 5-week-old plant (Fig. 1a). Collect leaf discs from the terminal leaflet (TL) and from the two primary leaflets (PL) of the selected leaf (*see* **Note 7**) (Fig. 1b).
4. Use the biopsy punch to punch out a leaf disc and use the plunger to eject it, one well after the other, onto the surface of the water (*see* **Notes 8** and **9**).
5. Cover microplates with a transparent lid to reduce evaporation.
6. Incubate the microplate overnight at room temperature.

3.3 Elicitor Treatment and ROS Measurement

The following steps are performed in this particular sequence in order to reduce the risk of "accidental" ROS production caused by unintended stresses (e.g., mechanical damage, desiccation).

Fig. 1 (continued) Dashed circles indicate the symmetric positions from where leaf discs were collected. (**c**) The experimental design of the ROS assay in a 96-well microplate format. Leaf discs of different individuals/genotypes are arranged in rows, treatments in columns. (**d**, **e**) Progress curves showing the ROS burst elicited by 30 nM systemin (**d**), 30 nM flg22 and 50 μg/mL chitin (**e**) over 120 min in WT tomato plants. (**f**) Comparison of flg22-triggered ROS burst in wild-type (WT) and PP2C loss-of-function (*ko*) plants. (**d**–**f**) Data points represent the mean of ROS counts for 30 biological replicates ($n = 30$). Error bars represent standard errors of the mean. (**g-i**) Quantitative features of ROS curves in (**f**) are compared between wild type (WT) and mutant (*ko*) (n = 30) showing total ROS accumulation (**g**), peak ROS production (**h**), and the speed of the ROS burst (Velocity = peak ROS counts/time to reach the maximum) (**i**). Error bars represent the mean +/− standard deviation of $n = 30$ biological replicates

1. Turn on and program the microplate reader in advance (*see* **Note 10**). You need two programs that will be executed in sequence, named "background ROS" and "elicitor ROS". Both have the same actions in their scripts: Plate - Kinetic Loop - Luminescence (*see* **Note 11**).
   - Plate: [GRE96fw_chimney] – Greiner 96 Flat White [GRE96fw_chimney].
     - Lid lifter: No lid.
     - Humidity Cassette: No humidity cassette.
     - Smooth mode: select.
     - Plate layout: click wells in use.
   - Kinetic Loop.
     - Loop type: Number of cycles 15 (for "background ROS"), 120 (for "elicitor ROS").
     - Interval type: Fixed – [hh:mm:ss]: 00:01:00.
   - Luminescence.
     - Name: "background ROS", or "elicitor ROS".
     - Type: Filter settings.
     - Wavelength [nm]: 415–530 nm, central wavelength: 473 nm, bandwidth: 115 nm.
     - Integration time [ms]: 300 ms.
     - Settle time [ms]: 0.
     - Output: Counts.
2. Prepare 10× luminol master mix in a 2 mL microfuge tube covered with aluminum foil: add 20 μL L-012 and 8 μL HRP to 1972 μL ddH_2O for one 96-well microtiter plate.
3. Add 2 mL 10× luminol master mix into a Tip-tub with 17 mL ddH_2O to make 1× luminol working solution. Mix gently and cover with aluminum foil to reduce light exposure (*see* **Note 1**).
4. Meanwhile thaw elicitors (see Subheading 2.2, **step 10**) on ice.
5. Open the interface of the first program "ROS background".
6. Swiftly remove ddH_2O from each well using a one-channel pipette (100–1000 μL) (*see* **Note 12**).
7. Use a multi-channel pipette to dispense 190 μL 1× luminol reaction buffer into each of the wells. Make sure no leaf discs are drowned in the reagent before you start the program (*see* **Note 9**).
8. Place the microplate into the reader and start the "ROS background" measurement.
9. Stop the background measurement as soon as ROS production remains constant; this may take around 5 min (*see* **Note 13**).

10. When background measurement is done, use a multiple-channel pipette to apply 10 μL of the elicitors into the wells according to your experimental design (**step 3.3.1**) (*see* **Note 14**).
11. Start the second program "elicitor ROS" to begin recording the elicitor-induced ROS burst.

3.4 Data Analysis

Each program will automatically export one Excel spreadsheet. Therefore, after completion of Subheading 2, you will have two Excel tables for each plate measured: one from program "background ROS", and the second one from program "elicitor ROS". These tables include information on the program settings and on ROS production per well over time.

1. Data cleaning: For each well in the "elicitor ROS" table, subtract the background ROS level averaged over 5 min (*see* **Note 15**).
2. Data cleaning (optional): Subtract the ROS values of water-treated control discs in "background ROS" from the corresponding cells in the "elicitor ROS" table (*see* **Note 16**).
3. Data processing: Firstly, average three technical replicates (i.e., 3 values in a row in Fig. 1c) for each treatment per plant (e.g., for systemin treatment, average A1-A3 of the first plant, average B1-B3 of the second plant…). This results in one set of time-coursed ROS value per plant per treatment. In Fig. 1d–f, each progress curve represents the mean values and standard errors (SE) of ROS counts over time for 30 wild-type or mutant plants, respectively ($n = 30$) (*see* **Note 17**).
4. Plot the data as ROS counts (y-axis) vs. time of treatment (x-axis) to visualize differences in the kinetics and amplitude of ROS production in response to different elicitors (Fig. 1d, e), or in different genotypes (Fig. 1f).
5. Extract and compare specific features of the ROS burst, in order to describe differences between genotypes/elicitors in a quantitative manner.
   - Cumulative ROS production, reflecting total ROS counts after elicitation (Fig. 1g): add up ROS counts of one leaf disc over time (e.g., 60 min), then average the values obtained for technical replicates (the different leaf discs of one individual plant). Plot the ROS accumulations of individual plants.
   - Peak of ROS production (Fig. 1h): for each leaf disc, extract the maximum ROS count value. Plot the mean of technical replicates (different leaf discs of one individual plant).
   - Speed of ROS production (Fig. 1i): For each leaf disc, divide the peak value by the time to reach this peak. Average

technical replicates (leaf discs of the same individual) and plot the mean.

4 Notes

1. L-012 is pH-sensitive (the optimal pH is 7.5 according to the manual book) and light-sensitive. Therefore, avoid exposure to direct light. Prepare solutions in dim light and cover vials with aluminum foil.
2. Peptides should be kept as a concentrated stock (e.g., millimolar or mg/mL level) to maintain activity at −20 °C for at least 1 year. Avoid frequent freeze-thaw cycles. Diluted peptide solutions (low μM range) are stable for up to 1 week at +4 °C.
3. Colloidal chitin is not soluble in water. It is necessary to always vortex the suspension right before application.
4. Plants always produce ROS, particularly when growing under stress. Therefore, it is important to minimize variation wherever possible, for example, variation in size, age, developmental stage, or growth conditions. Therefore, be careful to select plants similar in size and appearance for transplantation into larger pots. Finally, use similar looking 4–5-week-old plants that do not show any signs of stress (anthocyanin accumulation) for leaf disc collection and ROS assays.
5. You can setup the ROS assay in three ways: (1) test multiple elicitors in one genotype; (2) test multiple genotypes by one treatment; (3) combination of the first two settings as shown in Fig. 1c.
6. To reduce the delay between technical replicates (multiple treatments of one plant with the same elicitor), these replicates need to be planned along the reading direction of the microplate reader you use. Biological replicates (different plants/genotypes), on the other hand, are arranged perpendicular to the reading direction. The microplate reader we use (Tecan Spark) scans the wells horizontally (1–12), one row after the other (A-H).
7. Try to get leaf discs as uniform as possible. Do not collect tissue from the mid-vein, from the edges, or from the leaf tip. Punch leaflets symmetrically to limit the variation between leaf discs. At least four discs can be harvested from each leaflet (one terminal leaflet and two primary leaflets). Thereby you obtain at least 12 leaf discs (Ø4 mm) from the 3rd and from the 4th leaf. We highly recommend to use at least 3 leaf discs, collected from the three different leaflets, for each treatment of an individual plant.

8. A new biopsy punch with sharp edge works best. The biopsy punch can be reused several times unless it is used improperly and damaged. A punch with worn-down edge causes extra wounding during harvest resulting in irreproducible results. In order to keep punches in good working order, and also to protect your fingers, always use a self-healing cutting mat.
9. When leaf discs adhere to the cutting mat, use a spatula to transfer them into the wells. When you transfer the leaf discs, it does not matter which side faces up and which down. No significant differences are found comparing results for leaf discs facing abaxial or adaxial side down [22]. However, make sure that each leaf disc is floating on the water. Discard leaf discs that submerge, either during overnight incubation, or after addition of the luminol reaction solution.
10. We used the Tecan Spark™ microplate reader. The scripts may have to be adjusted for other models.
11. **Kinetic loop** defines how often the selected area is read, and how long each cycle takes (15 cycles of 1 min in the case of "background ROS"). The **wavelength** filter depends on the chemiluminescent probe. L-012 has maximal signal strength at 473 nm. Here, an arbitrary luminescence pass filter is selected, from 415 to 530 nm centered at 473 nm. **Integration time** specifies for how long each well is read. This duration time/well/cycle should be as short as possible to minimize the variation in ROS detection caused by the time shift between the first and the last well. **Settle time** specifies the lag time before the measurement starts.
12. We recommend to use a one-channel "blue" pipette to remove water. Be careful not to damage the leaf discs. A trick for fast pipetting without stabbing the leaf discs is to assemble a white tip on a blue tip. Thereby you take advantage of the more pointed white tip and the larger blue tip volume. Note that leaf discs without water will experience stress very soon. Therefore, replace the water with 1× luminol reaction solution as quickly as possible.
13. Only after leaf discs have "calmed down" to release low and stable ROS, can the elicitor-induced ROS burst be recorded reliably. The "ROS background" program, allows for a maximum of 15 min to measure the background ROS prior to elicitor treatment. 15 min are usually not needed for stabilization of ROS values.
14. A multichannel pipet needs to be used in order to quickly apply the elicitors. Tip-tubs are typically used as a reservoir when using a multichannel pipet. However, considering the dead volume of Tip-tubs and the price tag attached to the peptide elicitors, we recommend to use 0.5 mL PCR tubes or strips

instead. Arrange the PCR tubes/strips with 80 μL of the elicitor solutions in a 96-well rack according to your experimental design (e.g., Fig. 1c). Then add the elicitors row-by-row using the multichannel pipet. In the assay, elicitors are diluted 1:20 resulting in a final concentration of 30 nM systemin, 30 nM flg22, and 50 μg/mL chitin.

15. The subtraction of background ROS values is not a critical step in data processing. In most cases, the elicitor-induced ROS burst is much higher than background ROS. Therefore, the subtraction of background ROS levels may not even be visible in the final graph.
16. The addition of water will not induce massive ROS production let alone a ROS burst. However, it is important to include the water (or solvent) control in a ROS assay. For the results shown in Fig. 1d–f, the ROS counts of water-treated control discs were averaged in each row (i.e., for each plant) and subtracted from the ROS counts of elicitor-treated discs (e.g., A1-mean (A10 + A11 + A12), A2-mean(A10 + A11 + A12), A3-mean (A10 + A11 + A12),...). Alternatively, you can include the curve for the water-treated control and show it together with the curves for elicitor treatments in the same figure.
17. Exclude values of "non-responsive" leaf discs (those whose elicitor-induced ROS values do not differ from the mock (water)-treated control).

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Chapter 13

Automated Real-Time Monitoring of Extracellular pH to Assess Early Plant Defense Signaling

Xu Wang, Rong Li, Annick Stintzi, and Andreas Schaller

Abstract

Extracellular alkalinization mediated by the inhibition of plasma membrane-located proton pumping ATPases hallmarks the initiation of defense signaling in plant cells. Early defense responses also include depolarization of the plasma membrane, increase in cytosolic Ca^{2+} concentration, and an oxidative burst. Together these early signaling events lead to the activation of plant immunity. The transient alkalinization response is triggered by well-studied pathogen-derived and plant endogenous elicitors, including, for example, bacterial flagellin, fungal chitin, and tomato systemin in both model and agronomic species. Employing cell suspension cultures, extracellular alkalinization can be easily assessed by measuring the elicitor-induced pH changes of the cultivating medium. Here, we provide a protocol for an improved alkalinization assay in a system which is able to simultaneously monitor multiple samples, and fully automatically transfer customizable real-time pH records. In this system flagellin, chitin and systemin elicit robust time- and dose-dependent responses, proving a powerful tool for assessing plant early defense signaling.

Key words Extracellular alkalinization, Apoplastic pH, Systemin, flg22, Chitin, Cell suspension culture, Early defense response

1 Introduction

Plants are constantly challenged by various environmental factors, particularly by biotic stress. To respond to and cope, plant cells employ diverse arrays of cell surface pattern-recognition receptors (PRRs), which recognize conserved microbe- or herbivore-associated molecular patterns (MAMPs or HAMPs, respectively), and phytocytokines as endogenous regulators of immune responses. Upon ligand perception, cognate PRRs activate cellular responses via dynamic association/dissociation with co-receptors and/or receptor-like cytoplasmic kinases [1–3]. These PRR complexes signal through phosphorylation cascades to trigger a common set of early responses, including plasma membrane depolarization, extracellular alkalinization, cytosolic calcium

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increase, oxidative burst, and ethylene production [4–9]. Extracellular alkalinization, i.e., the rapid increase in apoplastic pH, can be readily measured as one of the earliest defense reactions triggered by endogenous and pathogen-derived elicitors. Extracellular alkalinization ensues when plasma membrane-bound H^+-ATPases are inhibited via changes in their phosphorylation status [7, 10–13]. In cell suspension cultures, transient inhibition of plasma membrane proton pumps and the concomitant increase in apoplastic pH results in alkalinization of the growth medium. In the early 1990s, the plant cell suspension-culture-based alkalinization assay was established to demonstrate bioactivity of chitin fragments from fungal cell walls, of flg22 derived from bacterial flagella, and of the wound response peptide systemin [4–6, 14]. It has since been used to characterize many newly identified phytocytokines and MAMPs, for example, AtPep1, elf18, and RALFs [8, 15–20]. Thanks to the high growth rate and homogeneity of suspension cell cultures, the alkalinization assay delivers fast and highly reproducible results. It is one of the easiest ways to assess early plant defense signaling output. Compared to other defense reactions such as oxidative burst, calcium influx, activation of mitogen-activated protein kinase cascades, and marker gene expression, extracellular alkalinization is also cheaper and less laborious to measure, thus providing an accessible tool for evaluating early plant defense responses to various pathogenic elicitors or defense signals.

In this method chapter, we describe a customized real-time pH monitoring system for robust and quantitative measurement of extracellular alkalinization in response to systemin, flg22, and chitin using a tomato (*Solanum peruvianum*) cell suspension. Combining multi-channel pH meters with an automated data recording and transfer system, we assay all three elicitors together with the mock treatment (H_2O) simultaneously (Fig. 1a). At their respective working concentrations, systemin (10 nM) flg22 (20 nM), and colloidal chitin (15 μg/mL) induced rapid medium alkalinization within 3–5 min after application. Amplitude of the pH changes and kinetics of time-dependent curves are comparable for these three elicitors (Fig. 1b, c). To further characterize the sensitivity of the systemin perception system, we performed the alkalinization assay with systemin at different concentrations. The resulting dose-response curve shows characteristic sigmoidal concentration dependence. The cell suspension responded to subnanomolar concentrations of systemin, resulting in half-maximal stimulation (EC_{50}) at around 0.6 nM (Fig. 1d).

Our real-time pH-monitoring system is not limited to assaying tomato cells. It rather is applicable to any plant species, for which suspension-cultured cell lines are available, such as *Arabidopsis*, tobacco, maize, potato, sweet potato, poplar, and grape [9, 16, 17, 21–23]. In fact, irrespective of the plant species under investigation, a well-maintained cell suspension culture is vital to the

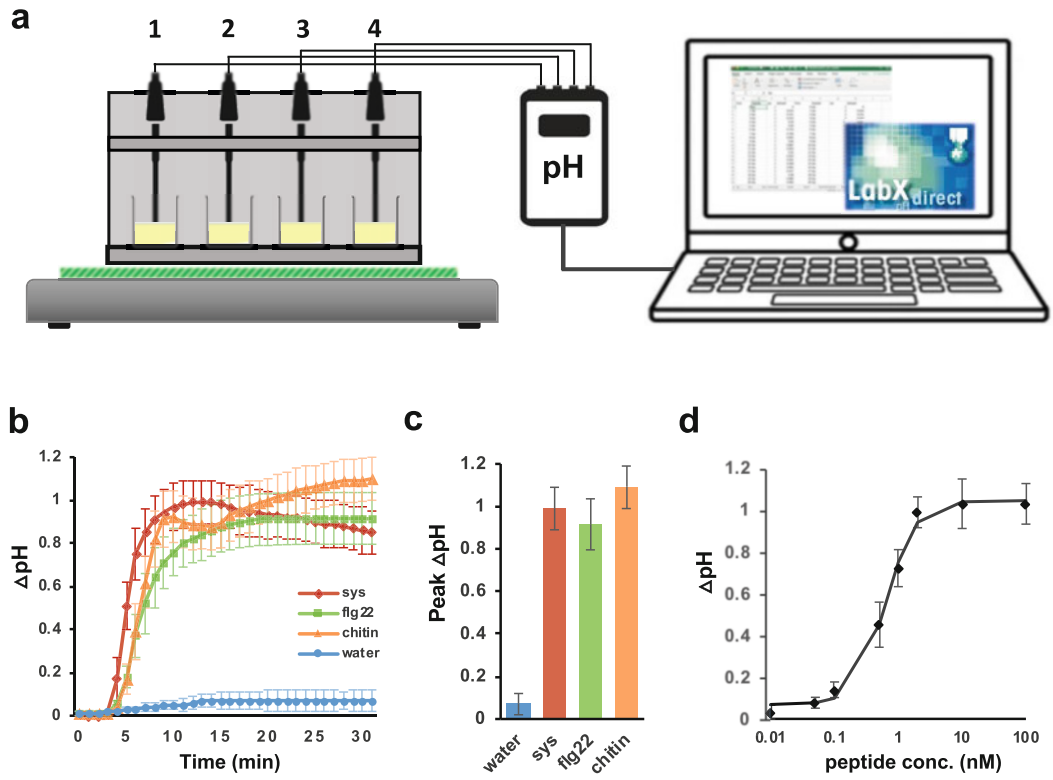


Fig. 1 Extracellular alkalinization assay of pathogenic and plant endogenous elicitors in tomato cell suspension culture. (**a**) Schematic diagram showing the setup of extracellular alkalinization system. Four pH sensors and testing beakers are placed on the docking station (light gray), and the docking station is fixed on an orbital shaker (dark gray, bottom) via an adhesive mat (green). The pH meter connects all sensors for real-time reading, and it links to a personal computer for data recording. Software and program configurations are described in Subheadings 2.3 and 3.2. (**b**) Time course of pH changes of cell suspensions treated with 10 nM systemin, 20 nM flg22, 15 μg/mL chitin, and solvent (water) control ($n = 6$). Zero time point is when the elicitors are applied. (**c**) Peak ΔpH values of cell suspensions treated with elicitor and water ($n = 6$). (**d**) Dose-response curves showing ΔpH induced by systemin at concentrations from 10 pM to 100 nM. Error bars = standard deviations

success of the alkalinization assay. Therefore, in addition to the setup of our system, we also describe key procedures and critical notes for propagation and maintenance of the cell cultures.

2 Materials

2.1 Equipment

1. Incubation shaker for cell cultures (e.g., INFORS HT Multitron Pro) (*see* **Note 1**).
2. Downflow (vertical laminar flow) biological safety cabinet.
3. Orbital shaker, 25 mm shaking throw.

4. Multi-channel benchtop pH meter (e.g., METTLER TOLEDO, SevenMulti).
5. Customized pH sensor docking station and plastic beakers (45 mL, ∅ 3.5 cm) (*see* **Note 2**).
6. Microplate reader.
7. Personal computer with vendor software package for pH value recording; equipped with USB (or other) port that can be used to connect to pH meters.
8. Suction pipette controller.
9. 10 mL serological pipettes (sterile, individually wrapped, non-cytotoxic, non-pyrogenic).
10. Spray bottle with 70% ethanol.
11. Standard biochemistry equipment including Eppendorf tubes, Erlenmeyer flasks (250 mL), 96-well microplates, measuring cylinders, and pipette tips.

2.2 Cell Suspensions, Elicitors, and Reagents

1. *Solanum peruvianum* cell suspension, cultivated in incubation shaker for 6–8 days (*see* **Note 3**).
2. Custom-synthesized peptide elicitors systemin (AVQSKPPSKRDPPKMQTD) and flg22 (QRLSTGSRINSAKDDAAGLQIA) (*see* **Note 4**). Prepare 1 mM stock solutions of synthetic systemin and flg22 peptides in sterilized double-distilled water. Confirm/determine the concentrations of the peptide solutions using the *DC* protein quantification kit in a 96-well plate format following the manufacturer's instructions. Read absorbance at 750 nm in a microplate reader, and determine peptide concentration using bovine serum albumin as a reference (*see* **Note 5**).
3. Chitin powder from shrimp shells (e.g., Sigma-Aldrich). Suspend 0.5 g of chitin in 10 mL 37% HCl. Slowly add 1 L of distilled water while stirring. Collect the chitin fragments by centrifugation for 10 min at 16,000 × *g*. Wash the pellet extensively with double-distilled water, and resuspend colloidal chitin in 50 mL water to reach a final concentration of 10 mg/mL. Sterilize by autoclaving.
4. For cell culture medium: Murashige-Skoog (MS) salts containing Nitsch vitamins (e.g., Duchefa), sucrose, KH_2PO_4, 2 M KOH.
5. NAA (1-naphthaleneacetic acid): 40 mg/mL in 2 M NaOH.
6. BAP (benzylaminopurine): 10 mg/mL in 96% ethanol.
7. Commercial protein assay reagents (e.g., *DC* Protein Assay, Bio-Rad).

2.3 Software and Data Analysis

1. LabX® direct data transfer software (version 2.1, compatible with METTLER TOLEDO pH meters).
2. Microsoft Excel.

3 Methods

3.1 Medium Preparation and Cell Suspension Culture

1. To prepare cell suspension culture medium dissolve 4.41 g/L Murashige-Skoog (MS) salts containing Nitsch vitamins, 30 g/L sucrose, 0.34 g/L KH_2PO_4. Add 5 mg/L NAA and 2 mg/L BAP from stock solutions. Adjust pH to 5.5 with 2 M KOH.
2. Aliquot into 250 mL Erlenmeyer flasks with 70 mL each. Close flasks with a foam stopper, cover with aluminum foil, steam autoclave at 121 °C for 20 min. Store sterilized medium for up to 1 month at 4 °C.
3. On the day of subculture, transfer the required number of Erlenmeyer flasks (*see* **Note 6**) with culture medium to the UV-treated downflow biological safety cabinet and remove the aluminum foil. Allow them to acquire room temperature.
4. Place the flask with the old culture (the one from the previous week that needs to be sub-cultured, *see* **Note 7**) into the safety cabinet, then surface-sterilize all flasks and the suction pipette controller by spraying with 70% ethanol.
5. Remove the foam stopper to open the flasks. Set them aside. Be careful not to contaminate the stoppers and do not move any material over the now-open flasks.
6. Break off the tip of a 10 mL serological pipette (*see* **Note 8**), and transfer 8 mL of old cell suspension into a new flask (*see* **Note 9**).
7. Briefly flame the neck of the flask for sterilization and seal by re-inserting the foam stopper.
8. Put the flask with the sub-cultured cell suspension into the incubation shaker. Set growth condition to 26 °C with 120 rpm (*see* **Note 10**).

3.2 Setting-up the Alkalinization System

1. Connect all four pH sensors with pH meters via cable cords, and connect pH meters with computer via USB cables.
2. Turn on the computer and launch the pH recording software LabX® direct. Set up the program as follows:
   - Select a desired language interface.
   - Configure pH meter instrument: select “SevenMulti” with default settings.
   - Select “Excel” as target program for data transfer. Alternatively, other applications can be selected as destination file

(e.g., Word, Notepad). The selected program should open automatically in a separate window.

- Configure the data format for the opened application. Here you have the chance to customize data input for each column. Otherwise, use the default layout.
- Configure sample IDs. Enter sample names/IDs specifying, for example, name and concentration of the elicitor used with the respective pH sensor.

3. Get one of the culture flasks from the incubation shaker, and transfer a 10 mL aliquot of the culture into each of the plastic beakers using a serological pipette without tip (*see* **Notes 8, 9,** and **11**).
4. Fit the plastic beakers with cell suspensions into the pH sensor docking station, which is fixed on the benchtop orbital shaker with adhesive mat.
5. Briefly rinse the pH electrode with autoclaved double-distilled water. Use a Kimwipe to gently remove excessive liquid. Fit the pH sensors back into the docking station with electrodes submerged in cell suspension. Both, the pH sensors and the beakers need to be tightly secured in their positions.
6. Turn on the benchtop orbital shaker (*see* **Note 12**) and set the speed at 120 rpm (25 mm shaking throw).
7. Press the "Read" button to view real-time pH values for each of the pH sensors, and to record these values in the target program. We typically record pH values at 10-second intervals. The recording interval can be adjusted from 3 to 2400 s (Menu - > Interval Time for Serial Measurement −> ON −> 10 s).

3.3 Elicitor Preparation and Application

1. From the 1 mM stock solutions, prepare 200-fold concentrated peptide working solutions (2 μM) in 50 μL aliquots for the alkalinization assay (*see* **Note 13**).
2. From the stock of colloidal chitin, prepare 200-fold concentrated chitin working solution (3 mg/mL) in 50 μL aliquots for the alkalinization assay.
3. Wait until extracellular pH values from all four samples have stabilized for at least 15 min before adding elicitors. Once stabilized (*see* **Note 14**), add the peptide/elicitor directly into each cell suspension without stopping the shaker.
4. Keep recording real-time pH values till the desired end point, then stop recording by pressing the "Read" button on the pH meter.

3.4 Data Analysis

1. Save the file containing the pH values measured by all four pH sensors over the entire assay period (data from the four electrodes are stored in a single file).
2. From the original file, copy the data for each of the electrodes (corresponding to one elicitor treatment or one replicate) and store them as individual files, or as separate sheets in the same file.
3. Re-assign time points to each measurement so that all time-coursed pH values follow the same timeline, with 0 as the time point of elicitor addition.
4. Collect the data from the different replicates (*see* **Note 15**) with the same elicitor treatment into one excel sheet side-by-side. For further analysis, normalize each data set by subtracting the starting pH value (*see* Subheading 3.3, **step 4**, and **Note 14**).
5. For each data point calculate the mean and standard deviation (or standard error).
6. Plot the data as shown in Fig. 1b–d.

4 Notes

1. Similar incubation shaker models with temperature control can be used. To generate a homogenous cell suspension, to ensure sufficient aeration with minimal stress for the cells, and to deliver reproducible cellular responses, we recommend orbital shakers with a shaking throw (diameter of the orbit) of 50 mm or greater.
2. Depending on experimental need, multiple pH electrodes can be mounted in the docking rack, which we fix on the orbital shaker using an adhesive mat. Our customized system holds four pH electrodes, for the simultaneous recording of up to four biological replicates, or different elicitor treatments. Plastic beakers are filled with the cell suspension, and the minimal volume to be used should ensure full submersion of the pH electrode.
3. Exact cultivation time is subject to change depending on the growth rate, and this can be affected by many factors (e.g., initial cell density, fresh media-to-old culture ratio during sub-culture, shaker speed, genetic background of the cell culture). In our hands, the wild-type *S. peruvianum* cell suspension reaches its original cell density at day 7 after sub-culture and at this age, it performs consistently during alkalinization assays. When different species or genetic backgrounds are used, the optimal time point (cell density) that delivers reproducible data needs to be tested out.

4. To achieve accurate concentration and more specific cellular responses, high-quality and purity peptides should be used (>95% purity is recommended). Our peptide elicitors were obtained from PepMic (Suzhou, China).
5. If your peptide of interest contains aromatic amino acids (Trp or Tyr), read absorbance at 280 nm (OD_{280}) and use the molar extinction coefficient to determine protein concentration. This is faster and more accurate as compared to the protein assay kit. Calculate the molar extinction coefficient for your peptide as ϵ_{280} = (nTrp * 5500) + (nTyr * 1490) + (nCys * 125) [M^{-1} cm^{-1}]. Use Beer's law, $OD_{280} = \epsilon_{280} \times c$ [concentration, M] × d [pathlength, cm], to derive the peptide concentration.
6. For routine maintenance of the cell culture, we recommend to inoculate two new flasks for each cell line to be maintained. One of the flasks will provide the inoculum for the next round of subculture 1 week later. The second flask serves as a backup. In case of accidental contamination, the second flask that was not opened will help to re-establish the cell line. In addition to the two flasks that are required for routine maintenance, inoculate as many additional flasks as you need for alkalinization assays to be performed 6–8 days later.
7. Cells are transferred when the culture is in the stationary phase. Time until transfer depends on the volume of the inoculum and on the growth rate of the culture. To maintain the *S. peruvianum* cell suspension culture that was used in the experiment described here, we transfer 8 mL of 'old' culture into 70 mL of fresh medium on a weekly basis. The cells are used for alkalinization assays when the culture is in the stationary phase, at 6–8 days after subculture.
8. To maintain aseptic conditions, we recommend to spray the tip of the still-wrapped serological pipette from all sides, then wear gloves to break off the tip, peel off the wrapper, and insert the pipette into the suction controller.
9. The cells settle rapidly. Therefore, you need to swirl the culture flasks to get cells back into homogenous suspension before you pipet out the 8 mL inoculum that is to be transferred.
10. The remainder of the "old" culture can be used for alkalinization assays.
11. When aliquoting cells into individual testing beakers, gently swirl the culture flask to maintain the cells in homogenous suspension. Cell density within each of the testing beakers needs to be identical to yield comparable and reproducible results. We always use 10 mL of cell culture in each beaker. However, this volume can be adjusted depending on your specific application, the cell culture you use, and the size/volume of testing beaker.

12. Temperature control is usually not needed. We perform the assay at room temperature, ranging from 22 to 26 °C. We have noticed that the cell suspension is less responsive, or pH responses may fluctuate, when the temperature is out of this range.
13. Use freshly prepared elicitor solutions, or frozen aliquots.
14. Even if cell samples are derived from the same flask, some variation in initial pH values is expected. For our wild-type S. *peruvianum* cell suspension, for example, extracellular pH typically ranges between 5.2 and 5.6 at the beginning of the assay. These values tend to decrease by 0.2–0.4 pH units within 45 min – 1 h. until they stabilize. If initial pH is oddly high or low, and if it fails to self-adjust during a 2 h. acclimation period, this cell culture should not be used for assays.
15. Based on variations we have observed, we recommend to perform six biological replicates for each treatment to generate reliable data.

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Chapter 14

Peptide-Mediated Cyclic Nucleotide Signaling in Plants: Identification and Characterization of Interactor Proteins with Nucleotide Cyclase Activity

Ilona Turek and Chris Gehring

Abstract

During the last decades, an increasing number of plant signaling peptides have been discovered and it appears that many of them are specific ligands for interacting receptor molecules. These receptors can enable the formation of second messengers which in turn transmit the ligand-induced stimuli into complex and tunable downstream responses. In order to perform such complex tasks, receptor proteins often contain several distinct domains such as a kinase and/or adenylate cyclase (AC) or guanylate cyclase (GC) domains. ACs catalyze the conversion of ATP to 3′,5′-cyclic adenosine monophosphate (cAMP) while GCs catalyze the reaction of GTP to 3′,5′-cyclic guanosine monophosphate (cGMP). Both cAMP and cGMP are now recognized as essential components of many plant responses, including responses to peptidic hormones. Here we describe the approach that led to the discovery of the Plant Natriuretic Peptide Receptor (PNP receptor), including a protocol for the identification of currently undiscovered peptidic interactions, and the subsequent application of computational methods for the identification of AC and/or GC domains in such interacting receptor candidates.

Key words Peptide hormones, Peptide hormone receptors, Cyclic nucleotides, Nucleotide cyclases, Adenylate cyclase, Guanylate cyclase, cAMP, cGMP

1 Introduction

Specific recognition of primary stimuli and/or stimulus-dependent ligands and subsequent modulated temporal and spatial responses are key properties of living systems. The ligands are often peptidic molecules [1–5] that bind specifically, but mostly not covalently, to interactors, many of which show structural and functional characteristics typical for receptors [4]. These interactors can enable complex downstream signaling cascades that orchestrate complex systemic responses and some, but not all of the interactors are canonical receptors [4], others are enzymes [6] that are specifically modified upon interacting with the peptidic ligand. It is

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noteworthy that an increasing number of canonical plant receptors are leucine-rich repeat receptor-like kinases (LRR-RLKs) that contain functional AC or GC domains embedded in their kinase domains [1, 7–9]. Recent evidence suggests that a functional GC catalytic center nested in the cytosolic kinase domain of LRR-RLKs can enable intramolecular crosstalk [10], where the GC-generated cGMP modulates kinase activity and hence phosphorylation [11]. Additionally, cGMP can also directly interact with enzymes to modulate their activities [12] and affect the transcriptome [13].

Given that cyclic nucleotides are now established as signaling components in plant responses, the search for cyclic nucleotide-producing enzymes, chiefly ACs and GCs, is essential for our understanding of their biological roles. This makes the discovery of functional AC and GC catalytic centers in receptors, including receptors for peptidic ligands, of particular interest. Functional catalytic centers in GCs were first discovered using motif searches, employing motifs derived from the alignments of functionally annotated amino acid residues of known GCs of prokaryotes and eukaryotes [14]. This search method was later modified to identify hitherto undiscovered ACs in complex plant proteins [15–17]. The rationale and methodologies for these motif-based searches for candidate ACs and GCs are detailed elsewhere [18].

Here we provide a detailed protocol for the discovery of receptors and, more generally, peptidic plant interactors that contain functional AC or GC domains and are therefore candidates for cAMP- respectively cGMP-dependent downstream signaling (Fig. 1). The example we present is the identification and characterization of the Plant Natriuretic Peptide Receptor (PNP receptor) [4] that specifically binds the peptidic ligand PNP [19, 20]. We also detail how the GC domain in the receptor was discovered and outline experimental methods to determine GC function of the receptor in vitro. The methods described can be readily applied to the discovery of other PNP interactors and/or PNP receptor candidates and their potential functionality as ACs or GCs.

2 Materials

2.1 Equipment

1. Mortar and pestle.
2. Liquid nitrogen.
3. Rotary tube mixer at 4 °C.
4. Heating block.
5. Magnetic tube holder (e.g., DynaMag-2 magnetic tube holder, Invitrogen).

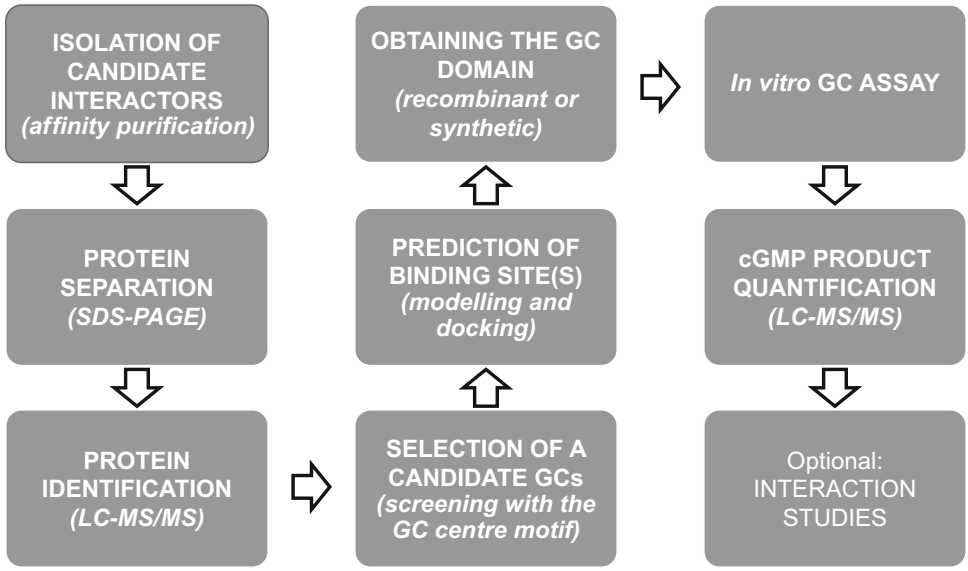


Fig. 1 Flow diagram of the major steps taken to identify peptide receptor candidates with GC activity. Analogous processes can be used to identify proteins with AC activity

6. Equipment for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (e.g., Mini-Protean Tetra Cell, Bio-Rad).
7. Ultrasonic bath.
8. Glass plates.
9. Scalpel blades.
10. SpeedVac vacuum concentrator (e.g., Savant SPD1010 SpeedVac Concentrator, Thermo Fisher) (*see* **Note 1**).
11. High-performance liquid chromatography (HPLC) system coupled to a tandem mass spectrometer capable of multistage mass spectrometric (MS) analysis (see below). We used the Accela HPLC system (Thermo Scientific) equipped with a quaternary pump, an autosampler-column compartment module, and Sepax SFC Cyano column (150 mm × 4.6 mm, 5 μm) (*see* **Note 2**).
12. Tandem mass spectrometer: we used the Linear Trap Quadrupole (LTQ) – Orbitrap Velos mass spectrometer (Thermo Scientific) (*see* **Note 3**) coupled with a nanoelectrospray ion source (Proxeon Biosystems), equipped with 0.3 × 50 mm Magic C18 (octadecylsilane) AQ (Michrom) pre-column and 0.1 × 150 mm Magic C18 AQ (Michrom) column, and Xcalibur software, version 2.1 or later (Thermo Fisher Scientific) for data recording.
13. Autosampler vials and vial closures (e.g., National Scientific Company).

2.2 Affinity-Based Isolation of the Plant Peptide Interactors

1. Plant material from a sequenced organism, in this case leaf tissue of five-week-old *Arabidopsis thaliana* (Col-0) plants (*see* **Note 4**).
2. Biologically active (*see* **Note 5**) N- or C-terminally biotinylated peptide containing the active region of *A. thaliana* plant natriuretic peptide (AtPNP-A) and a corresponding scrambled peptide. Peptides should be synthesized at high purity and their dilution to the desired stock/final concentration needs to be made accurately as they should be used at equimolar concentration (*see* **Note 6**).
3. Extraction buffer (*see* **Note 7**): 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 10% (w/v) glycerol, 1% (v/v) nonyl phenoxypolyethoxylethanol (NP-40), 0.5% (w/v) sodium deoxycholate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% (v/v) Triton X-100, 0.1% (v/v) Tween 20, protease inhibitor cocktail for plant cell and tissue extracts (Sigma), pH 7.4. Weigh 1.19 g HEPES, 877 mg NaCl, 0.5 g sodium deoxycholate, and resuspend in approximately 70 mL of Milli-Q water in a glass beaker. Add 10 mL glycerol, 10 mL of 10% NP-40, 1 mL of 10% Triton X-100, 1 mL of 10% Tween 20, 1 mL protease inhibitor cocktail for plants and tissue extracts, and mix well avoiding foaming. Add 1 mL of 100 mM PMSF stock solution (*see* **Note 8**), adjust pH to 7.4, transfer the solution to a cylinder and top up with Milli-Q water to the 100 mL mark.
4. Streptavidin-coupled Dynabeads M-280 Streptavidin magnetic beads (Invitrogen).
5. Washing buffer 1 (*see* **Note 7**): 50 mM Tris(hydroxymethyl)-aminomethane (Tris)-HCl, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1% (v/v) NP-40, 0.1% (v/v) Triton X-100, 0.1% (v/v) Tween 20, 0.5% (w/v) sodium deoxycholate, 1 mM PMSF, cOmplete Mini protease inhibitor cocktail (Roche), pH 7.4. Weigh 606 mg Tris, 877 mg NaCl, 0.5 g sodium deoxycholate, and resuspend in approximately 70 mL of Milli-Q water in a glass beaker. Add 10 mL of 10% NP-40, 1 mL of 10% Triton X-100, 1 mL of 10% Tween 20, 10 tablets of cOmplete Mini protease inhibitor tablets (*see* **Note 9**), and mix well. Add 1 mL of 100 mM PMSF stock solution (*see* **Note 8**), adjust pH to 7.4, transfer the solution to a cylinder and top up with Milli-Q water to the 100 mL mark.
6. Washing buffer 2 (*see* **Note 7**): 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.1% (v/v) Triton X-100, 0.1% (v/v) Tween 20, 0.5% (w/v) sodium deoxycholate, 1 mM PMSF, cOmplete Mini protease inhibitor cocktail (Roche), pH 7.4. Weigh 606 mg Tris, 292 mg NaCl, 0.5 g

sodium deoxycholate, and resuspend in approximately 70 mL of Milli-Q water in a glass beaker. Add 10 mL of 10% NP-40, 1 mL of 10% Triton X-100, 1 mL of 10% Tween 20, 10 tablets of cOmplete Mini protease inhibitor cocktail (*see* **Note 9**), and mix well. Add 1 mL of 100 mM PMSF stock solution (*see* **Note 8**), adjust pH to 7.4, transfer the solution to a cylinder and top up with Milli-Q water to the 100 mL mark.

7. Elution buffer 1 (*see* **Note 7**): 10 μM AtPNP-A peptide, cOmplete Mini protease inhibitor cocktail (Roche) (*see* **Note 9**), in 50 mM Tris-HCl, pH 7.4 (*see* **Note 10**).
8. Elution buffer 2 (*see* **Note 7**): 100 μM AtPNP-A peptide, cOmplete Mini protease inhibitor cocktail (Roche) (*see* **Note 9**) in 50 mM Tris-HCl, pH 7.4 (*see* **Note 10**).
9. Elution buffer 3 (*see* **Note 7**): 10 μM biotin, cOmplete Mini protease inhibitor cocktail (Roche) (*see* **Note 9**) in 50 mM Tris-HCl, pH 7.4 (*see* **Note 10**).
10. Elution buffer 4 (*see* **Note 7**): 100 μM biotin, cOmplete Mini protease inhibitor cocktail (Roche) (*see* **Note 9**) in 50 mM Tris-HCl, pH 7.4 (*see* **Note 10**).
11. Elution buffer 5 – SDS-PAGE sample loading buffer (1×): 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS, electrophoresis-grade), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.005% (w/v) bromophenol blue. For 10 mL, add to a plastic tube 5 mL Milli-Q water, 1.25 mL Tris-HCl, pH 6.8 (Resolving gel buffer (4×), see below), 1 mL glycerol, 1 mL 20% (w/v) SDS, 5 mg bromophenol blue, and 500 μL 2-mercaptoethanol (*see* **Notes 11** and **12**). Mix well, avoiding foaming, and top up with Milli-Q water to 10 mL. Optionally: aliquot the buffer to microcentrifuge tubes.

2.3 Protein Separation Using SDS-PAGE

1. Resolving gel buffer (4×): 1.5 M Tris-HCl, pH 8.8. Weigh 18.17 g of Tris and add it to about 70 mL of Milli-Q water in a 100 mL glass beaker. Mix well, adjust pH to 8.8 with HCl, and top up with Milli-Q water to 100 mL in a graduated cylinder.
2. Stacking gel buffer (4×): 0.5 M Tris-HCl, pH 6.8. Weigh 6.06 g of Tris and add it to about 70 mL of Milli-Q water in a 100 mL glass beaker. Mix well, adjust pH to 6.8 with HCl, and top up with Milli-Q water to 100 mL in a graduated cylinder.
3. 40% (w/v) acrylamide:Bis solution (37.5:1 acrylamide:Bis, 2.6% C), stored at 4 °C (*see* **Note 13**).
4. 10% (w/v) ammonium persulfate (APS): weigh 0.1 g APS into a microcentrifuge tube (*see* **Note 14**). Dilute completely in 1 mL Milli-Q water by vortex-mixing. Prepare fresh.

5. *N,N,N′,N′*-tetramethylethylene-diamine (TEMED), stored at 4 °C (*see* **Note 15**).
6. 4× Laemmli sample buffer (Bio-Rad) supplemented with 10% (v/v) 2-mercaptoethanol to obtain a final 1× concentration of 355 mM of 2-mercaptoethanol (*see* **Note 12**).
7. Protein molecular ladder (e.g., PageRuler prestained protein ladder, Fermentas).
8. Tris-glycine SDS running buffer (10×): 25 mM Tris, 1.92 M glycine, 0.1% (w/v) SDS. For 1 L buffer, pour about 700 mL of Milli-Q water to a glass beaker on a stirring plate set to 30 °C, weigh 30.2 g Tris, 144 g glycine, and 10 g SDS and transfer them to the beaker. Mix well until all components are dissolved and make up to 1 L with dH_2O in a graduated cylinder. To make 1× running buffer, dilute 100 mL of 10× running buffer stock with 900 mL Milli-Q water (*see* **Note 11**).
9. Staining solution: Coomassie Brilliant Blue (Bio-Rad).
10. Destaining solution: 10% (v/v) acetic acid, 50% (v/v) methanol, 40% (v/v) H_2O. Add 500 mL of methanol to 400 mL of deionized H_2O in a graduated cylinder, and then add 100 mL acetic acid and mix (*see* **Note 16**).

2.4 Excision of Protein Bands from SDS-PAGE and in-Gel Protein Digestion

All solutions should be prepared using high-quality HPLC-grade reagents, including solvents. All reagents should be prepared and stored in sterile plastic or glass ware.

1. Bovine sequencing-grade modified trypsin (Promega), stored at −20 °C.
2. 100 mM ammonium bicarbonate (NH_4HCO_3) stock solution. Weigh 395 mg NH_4HCO_3, transfer it to 50 mL plastic tube, top up with HPLC-grade water to the 50 mL mark, and mix.
3. Reducing solution: 10 mM dithiothreitol (DTT), 100 mM NH_4HCO_3. Weigh 15.4 mg DTT, transfer to 15 mL plastic tube, resuspend in 10 mL 100 mM NH_4HCO_3 stock solution, and mix (*see* **Note 17**).
4. 50 mM NH_4HCO_3: obtained by diluting the 100 mM NH_4HCO_3 stock 1:1 with HPLC-grade water.
5. Alkylation solution: 50 mM iodoacetamide (IOA), 50 mM NH_4HCO_3. Weigh 15.4 mg IOA, transfer to 15 mL plastic tube, resuspend in 10 mL 50 mM NH_4HCO_3 stock solution, and mix (*see* **Note 18**).
6. Trypsin solution. Reconstitute bovine sequencing-grade modified trypsin (20 μg vial) in 100 μL trypsin solubilization buffer provided by the manufacturer (Promega). Dilute this solution to 10 ng/μL trypsin in 50 mM NH_4HCO_3 by mixing 50 μL

trypsin in trypsin solubilization buffer with 950 μL of 50 mM NH_4HCO_3 (*see* **Note 19**).

7. Dehydration solution: 25 mM NH_4HCO_3, 50% (v/v) ACN in HPLC-grade water. To prepare 40 mL solution, add 10 mL of 100 mM NH_4HCO_3 stock to 10 mL HPLC-grade water and 20 mL 100% (v/v) ACN in a 50 mL plastic tube, and mix.
8. 20 mM NH_4HCO_3: obtained by diluting the 100 mM NH_4HCO_3 stock 5× with HPLC-grade water.
9. Extraction solution: 5% (v/v) FA, 50% (v/v) ACN in HPLC-grade water. Add 505 μL of 99% (v/v) FA and 5 mL of 100% (v/v) ACN to 4 mL HPLC-grade water in a glass cylinder. Top up with HPLC-grade water to 10 mL and mix.

2.5 Mass Spectrometric Identification of the Candidate Interactors

All solutions should be prepared using high-quality HPLC-grade reagents, including solvents. All reagents should be prepared and stored in sterile plastic or glass ware.

1. Resuspension solution: 0.1% (v/v) FA, 5% (v/v) ACN. Add 10.1 μL of 99% (v/v) FA and 0.5 mL of 100% (v/v) ACN to 7 mL HPLC-grade water in a glass cylinder. Top up with HPLC-grade water to 10 mL and mix.
2. Mobile phase A: 0.1% (v/v) FA, 5% (v/v) ACN. Add 1.01 mL of 99% (v/v) FA and 50 mL of 100% (v/v) ACN to 900 mL HPLC-grade water in a glass cylinder. Top up with HPLC-grade water to 1 L and mix. Degas the solution in an ultrasonic bath (e.g., FB15047, Fisher Scientific) for 15 min (*see* **Note 20**).
3. Mobile phase B: 0.1% (v/v) FA, 90% (v/v) ACN. Fill glass cylinder with 900 mL of 100% (v/v) ACN, add 1.01 mL of 99% (v/v) FA, top up with HPLC-grade water to 1 L and mix. Sonicate the solution in an ultrasonic bath (e.g., FB15047, Fisher Scientific) for 15 min (*see* **Note 20**).
4. Software for files conversion: MSConvert (open-source software), Proteome Discoverer, version 1.2.0 or later (Thermo Fisher Scientific).
5. Software for peptide and protein identification: Mascot, version 2.4.0 or later (Matrix Science).
6. Software for data compilation and relative protein quantification: Scaffold Q+, version 4.10.0 or later (Proteome Software).

All computational tools listed here and used for amino acid sequence screening with AC/GC search motifs, three-dimensional (3D) protein structure prediction, and docking of the ligand or nucleotide substrate to the protein are freely available in the public domain.

2.6 Selection of the Candidate GCs Based on the Protein Amino Acid Sequence Screening Using the GC Catalytic Center Search Motif, Followed by in Silico Prediction of the Substrate/Ligand Binding

1. NCBI website (www.ncbi.nlm.nih.gov) and UniProt (www.uniprot.org).
2. TAIR website (www.arabidopsis.org) to use the PatMatch function (https://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) (*see* **Note 21**).
3. 3D model of the putative GC downloaded from the Protein Data Bank (PDB) website (www.rcsb.org) or, if the 3D model of the protein of interest is not available in PDB, online tools for 3D structure prediction, such as HHPred (www.toolkit.tuebingen.mpg.de/tools/hhpred), or I-TASSER (www.zhanggroup.org/I-TASSER/) (*see* **Note 22**).
4. Software for interactive analysis of molecular structures and related data: UCSF Chimera software version 1.10.2 or later (www.cgl.ucsf.edu). Optional: ClusPro LigTBM server (https://ligtbm.cluspro.org) for automatic small molecule docking.
5. ClusPro protein – protein docking server version 2.0 (www.cluspro.bu.edu).

2.7 In Vitro GC Assay and cGMP Quantification Using LC-MS/MS

All reagents should be prepared and stored in sterile plastic ware.

1. GC protein (either recombinant or synthesized; *see* **Note 23**): the protein concentration needs to be determined accurately (*see* **Note 24**).
2. 50 mM Tris pH 7.4: for a 50 mL solution, dissolve 302 mg Tris in 30 mL of the HPLC-grade water and mix the solution on a magnetic stirrer. Adjust the pH of the solution to 7.4 and then make up with HPLC-grade water to a final volume of 50 mL (*see* **Note 25**).
3. 100 mM GTP stock solution: add 523 mg of GTP (if you are using the sodium salt hydrate form) into 10 mL of HPLC-grade water in a 15 mL plastic tube and mix by vortexing. Store at 4 °C until further use.
4. 1 μg/μL cGMP stock solution: weigh out 1 mg of cGMP and dissolve in 1 mL of HPLC-grade water in a microcentrifuge tube and store at 4 °C until further use.
5. cGMP internal calibration standards: 0.5–100 pg/μL cGMP in HPLC-grade water. Just before LC-MS/MS analysis dilute the cGMP stock solution 1:1000 to get 1 ng/μL cGMP in HPLC-grade water. Use the 1 ng/μL cGMP solution to prepare a total of eight cGMP calibration standards by performing subsequent serial dilutions using HPLC-grade water. This will result in the 0.5, 1, 2, 5, 10, 20, and 100 pg/μL cGMP calibration standards (*see* **Note 26**).

6. 1 M stock solution of $MgCl_2$: dissolve 2.03 g $MgCl_2 \cdot 6H_2O$ in 10 mL of HPLC-grade water in a 15 mL sterile plastic tube and mix by vortexing. Store at 4 °C until further use.
7. 1 M stock solution of $MnCl_2$: dissolve 1.98 g $MnCl_2 \cdot 4H_2O$ in 10 mL of HPLC-grade water in a 15 mL sterile plastic tube and mix by vortexing. Store at 4 °C until further use.
8. Optional: Calcium stock solutions of various concentrations. For 10 mL of 1 M $CaCl_2$, weigh 1.47 g of $CaCl_2 \cdot 2H_2O$ and dissolve in 10 mL of HPLC-grade water in a 15 mL sterile plastic tube, and mix by vortexing. Store at 4 °C until further use (*see* **Note 27**).
9. Optional: 50 mM stock solution of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX). Weigh out 22.2 mg of IBMX into a 2 mL microfuge tube and add 2 mL of dimethyl sulfoxide (DMSO), and then mix the solution by vortexing (*see* **Note 28**).
10. Mobile phase C: 10 mM ammonium acetate ($NH_4CH_3CO_2$) in HPLC-grade water. For 2 L of mobile phase C, weigh 1.54 g of $NH_4CH_3CO_2$ and add to 1.8 L of HPLC-grade water in a glass beaker on a stirring plate. Top up to the 2 L mark in the graduated cylinder with HPLC-grade water, and filter. Sonicate the solutions in an ultrasonic bath (*see* **Note 20**).
11. Mobile phase D: 100% (v/v) acetonitrile, LC-MS-grade.
12. Nitrogen gas from liquid nitrogen dewar is used as sheath gas as well as auxiliary gas. Helium is used as damping gas in the ion trap as well as the collision gas during MS/MS analysis (*see* **Note 29**).

3 Methods

3.1 Affinity-Based Isolation of the Plant Peptide Interactors

Ensure the biotinylated peptide used as a bait is biologically active (*see* **Note 5**). Perform pull-down experiments with at least two negative control baits (e.g., biotin, biotinylated scrambled peptide, no peptide) to eliminate non-specific preys and decrease the number of false positives (*see* **Note 6**). Protein extraction, affinity-based isolation, and washing steps should be performed at 4 °C. Affinity-based isolation of candidate interactors (and ideally SDS-PAGE separation) should be performed the same day.

1. Collect 2.5 g of *A. thaliana* leaf tissue and flash-freeze it in liquid nitrogen. Grind the frozen tissue to a fine-powder in a pre-cooled mortar and pestle, not allowing any tissue to thaw.
2. Immediately add the ground frozen tissue to the 5 mL extraction buffer and mix well, avoiding foaming.

3. Aliquot the protein extract into three pre-cooled 2 mL microcentrifuge tubes, with 1.6 mL protein extract per tube. Optionally: retain the remaining protein extract for protein quantification to ensure reproducibility between independent experiments.
4. Centrifuge at 14,000 × *g* for 30 min at 4 °C. Carefully transfer the supernatants into three new pre-cooled 2 mL microcentrifuge tubes without disturbing the pellet. Discard the pellet. Keep the tubes with supernatant on ice.
5. To each microcentrifuge tube add 200 pmol biologically active biotinylated peptide bait, or equimolar amount of the negative control bait (e.g., biotinylated scrambled peptide, biotin, or prepare a "no bait" control).
6. Incubate at 4 °C with slow tilt rotation mixing (~40 rpm) for 30 min on a rotary tube mixer.
7. Resuspend Dynabeads M-280 Streptavidin beads by inverting the vial several times. For each pull-down reaction, aliquot 100 μL into a separate microcentrifuge tube (*see* **Note 30**).
8. Allow the beads to settle on the DynaMag-2 magnetic tube holder for 3 min and remove the remaining storage solution, avoiding aspiration of the beads.
9. Equilibrate the beads by adding 1 mL of extraction buffer to 100 μL of beads, mixing the tubes at 4 °C with slow tilt rotation mixing (~40 rpm) for 5 min on a rotary tube mixer, allow the beads to settle on the magnetic tube holder for 3 min, and discard the solution. Repeat a total of 3 times.
10. Transfer the mixture of protein extract supernatant with the bait (Subheading 3.1, **step 7**) to the microcentrifuge tubes containing equilibrated beads. Repeat for each sample and incubate for 15 min at 4 °C with slow tilt rotation mixing (~40 rpm) on a rotary tube mixer.
11. Allow the beads to settle on the magnetic tube holder for 3 min and carefully remove the supernatant, collecting it as the flow-through fraction.
12. Add 1 mL of washing buffer 1 to the protein-bound beads, incubate at 4 °C with slow tilt rotation mixing (~40 rpm) for 5 min on a rotary tube mixer, allow to settle on the magnetic tube holder for 3 min, and carefully remove the supernatant, collecting it as the wash 1 fraction.
13. Repeat **step 12** twice, collecting each wash fraction (wash 2 and 3).
14. Add 1 mL of washing buffer 2 to the protein-bound beads, incubate at 4 °C with slow tilt rotation mixing (~40 rpm) for 5 min on a rotary tube mixer, allow to settle on the magnetic

tube holder for 3 min, and carefully remove the supernatant, collecting it as the wash 4 fraction.

15. Repeat **step 14** twice, collecting each wash fraction (wash 5 and 6).
16. Add 50 μL of elution buffer 1 and transfer the mixtures with protein-bound beads to fresh microcentrifuge tubes. Perform elution steps at room temperature (~20 °C) (*see* **Note 31**).
17. Flick the tubes with your fingers for 1 min taking care to collect the mixtures at the bottom of the tubes, allow to settle on the magnetic tube holder for 3 min, and carefully remove the supernatants, collecting them as the elution 1 fractions.
18. Add 50 μL of elution buffer 2, flick the tubes with your fingers for 1 min taking care to collect the mixtures at the bottom of the tubes, allow to settle on the magnetic tube holder for 3 min, and carefully remove the supernatants, collecting them as the elution 2 fractions (*see* **Note 31**).
19. Repeat **step 18** with elution buffer 3 and 4, and collect elution fractions 3 and 4 (*see* **Note 31**).
20. Add 50 μL of elution buffer 5, flick the tubes with your fingers for 1 min taking care to collect the mixture at the bottom of the tubes, and heat the samples for 10 min at 80 °C in a heating block. Centrifuge in a microfuge at 1000 × *g* for 30 s and collect the supernatants as the elution 5 fractions, which are denatured and ready for SDS-PAGE separation. Optionally: save the protein extract/flow through/washing fractions for SDS-PAGE to monitor the efficiency of the protein extraction and affinity purification process.

3.2 Protein Separation Using SDS-PAGE

Proteins are separated using one-dimensional SDS-PAGE according to the method of Laemmli [21].

1. Clean gel plates (7.25 cm × 10 cm × 1.0 mm) and combs with 70% (v/v) ethanol and allow to dry.
2. Mount the gel cassette on a gel casting stand assembly (e.g., Mini-Protean Tetra Cell handcast system). Make sure that the thin and thick glass plates are well aligned at the bottom and sides to prevent gel solution from leaking.
3. Prepare the resolving gel: For one gel, mix 4.35 mL Milli-Q water, 2.5 mL of resolving gel buffer, 3 mL of acrylamide:Bis stock solution, and 100 μL of 10% (w/v) SDS in a 50 mL plastic tube. Add 50 μL 10% (w/v) APS and 5 μL TEMED (*see* **Notes 13–15**).
4. Close the tube and mix gently by inverting, without incorporating air into the mixture. Pour the resolving gel solution in between the glass plates, leaving about 2.5 cm space between

the top of the solution and the edge of the glass plates for the stacking gel.

5. Immediately gently overlay with 0.5 mL isopropanol and allow the resolving gel to polymerize (approximately 20 min).
6. After polymerization (separate layers of the gel and isopropanol are clearly visible), completely remove the isopropanol with paper wicks without touching the resolving gel.
7. Prepare the stacking gel: For two gels, mix 3.18 mL Milli-Q water, 1.26 mL separating gel buffer, 0.5 mL of acrylamide:Bis stock solution, and 50 μL of 10% (w/v) SDS in a 15 mL plastic tube. Add 25 μL 10% (w/v) APS and 5 μL TEMED (*see* **Notes 13–15**).
8. Close the tube and mix gently by inverting, without incorporating air into the mixture. Pour the stacking gel solution in between the glass plates on top of the resolving gel, and insert the comb (e.g., 15 well). Allow to polymerize (approximately 20 min).
9. Remove the comb and insert the gel into the tank. Fill the tank with a 1× SDS-PAGE running buffer, covering all wells (*see* **Note 11**).
10. Prepare the samples. Samples from elution steps 1–4 should be diluted in the 4× Laemmli sample buffer supplemented with 2-mercaptoethanol, mixed and heated for 5 min at 95 °C in the heating block, while samples from denaturing elution step 5 are ready for SDS-PAGE separation (*see* **Notes 12** and **32**).
11. Briefly spin down the samples and load 25 μL into the wells of the stacking gel. Avoid spill-over from one well to the other. Load 5 μL of the protein ladder. Optional: store remaining sample at −20 °C.
12. Top up the gel apparatus with 1× SDS-PAGE running buffer to the required level and electrophorese at 80 V until the dye front enters the resolving gel, and at 100 V for approximately 15–20 min thereafter. Optional: If the number of samples to be analyzed by mass spectrometry is not limited, increase the duration of separation to improve resolution and decrease potential masking effects due to the presence of highly abundant proteins (*see* **Note 33**).
13. Switch off and dismantle the apparatus. Place the gel into staining solution and incubate for at least 1 h on a horizontal shaker.
14. Pour off the staining solution and replace with destaining solution. Change the destaining solution every 0.5–1 h of shaking until the protein bands can be clearly visualized and the background is reduced (*see* **Note 16**).

3.3 Excision of Protein Bands from SDS-PAGE and in-Gel Protein Digestion

Avoid dust, wear personal protective equipment, change gloves regularly and keep them clean, and keep hair tied back at all times to minimize contamination with keratin (*see* **Note 33**).

1. Re-hydrate the destained SDS-PAGE gel in HPLC-grade water for 30 min twice and place it on a clean glass plate.
2. Using a clean scalpel blade, excise the entire lane for each of the elution fractions, excluding excess (outside the lane) gel.
3. Divide the lane into five gel slices with a clean scalpel blade, changing the blade after each lane to prevent cross-contamination between the samples (*see* **Note 33**).
4. Cut each gel slice into smaller (approximately 1×1 mm^2) cubes and carefully collect them into a microcentrifuge tube.
5. Rinse and rehydrate the gel pieces in each microcentrifuge tube by covering them with 100 μL HPLC-grade water, making sure all gel pieces are covered. Centrifuge at maximum speed in a microcentrifuge for 30 s, if needed. Optional: store at 4 °C prior to in-gel tryptic digestion.
6. Prepare the solutions required for in-gel tryptic digestion (Subheading 2.4).
7. Remove the HPLC-grade water from the gel pieces by aspirating the solution.
8. Destain the gel pieces by incubating them in 100 μL of the destaining solution (25 mM NH_4HCO_3, 50% (v/v) ACN) for 30 min with intermittent vortexing. Spin down in a microcentrifuge at maximum speed for 15 s and discard the destaining solution. Repeat this step to ensure efficient destaining of the gel pieces.
9. Dehydrate the gel pieces by incubating them for 10 min in 200 μL ACN. Spin down in a microcentrifuge at maximum speed for 15 s and discard ACN.
10. Dry the gel pieces in open microcentrifuge tubes in a SpeedVac concentrator for 20 min.
11. Set the heating block to 56 °C.
12. Cover the gel pieces with 50 μL (or a volume sufficient to cover the gel pieces when rehydrated) of the reducing solution to reduce cysteine residues. Incubate for 45 min at 56 °C (*see* **Note 17**).
13. Cool the samples to room temperature, spin down in a microcentrifuge at maximum speed for 15 s and discard the reducing solution.
14. Set the heating block to 37 °C.

15. Cover the gel pieces with 50 μL (an equal volume to that of the reducing solution added in **step 12**) of the alkylation solution to alkylate the cysteine residues. Incubate for 30 min at room temperature (~20 °C) in the dark (*see* **Note 18**).
16. Spin down in a microcentrifuge at maximum speed for 15 s and discard the alkylation solution.
17. Wash the gel pieces with 200 μL of 100 mM NH_4HCO_3 and discard the solution.
18. Add 200 μL ACN, vortex, incubate for 10 min at room temperature (~20 °C), and discard the solution.
19. Add 100 μL of 100 mM NH_4HCO_3, incubate for 10 min at room temperature (~20 °C), and discard the solution.
20. Repeat **steps 18** and **19** twice.
21. Add 200 μL ACN, vortex, incubate for 10 min at room temperature (~20 °C), and discard the solution.
22. Dry the gel pieces in open microcentrifuge tubes for 20 min in a SpeedVac concentrator set to low heat mode to prevent heating of the samples.
23. Cool the samples by incubating them on ice for 30 min.
24. Prepare the trypsin solution (*see* **Note 19**).
25. Cover the gel pieces with 20–40 μL trypsin solution and incubate overnight at 37 °C in the heating block. After 1 h verify if the solution covers the gel pieces, and if required, add 10–30 μL of 50 mM NH_4HCO_3 to ensure full coverage of the gel pieces (*see* **Note 34**).
26. Cool the samples to room temperature, spin down in a microcentrifuge at maximum speed for 15 s and add 20 μL (or a volume sufficient to cover the gel pieces when rehydrated) of 20 mM NH_4HCO_3, vortex, and incubate for 10 min.
27. Centrifuge at maximum speed for 15 s and for each sample collect the solution containing peptides into a fresh microcentrifuge tube.
28. Extract remaining peptides by covering the gel pieces with 20 μL (or a volume sufficient to cover the gel pieces when rehydrated) of the extraction solution (5% (v/v) FA, 50% (v/v) ACN) for 10 min. Then, for each sample, combine the extract with the previous elution. Repeat this step, combining the solutions collected in **steps 27** and **28** into one microcentrifuge tube per each sample processed.
29. Dry the combined fractions in a SpeedVac concentrator. Store the samples at −20 °C prior to subjecting them to tandem mass spectrometric analyses (*see* **Note 35**).

3.4 Mass Spectrometric Identification of the Candidate Interactors

Resuspend the samples and perform tandem mass spectrometric analyses according to the procedure of the proteomics facility of your choice. The method outlined below has been optimized for the Linear Trap Quadrupole (LTQ) – Orbitrap Velos mass spectrometer and for the label-free relative quantification of proteins from *Arabidopsis thaliana*. However, it can be modified to identify the proteins from other (sequenced) species using different LC-MS/MS systems and to semi-quantify proteins using different software and statistical techniques.

1. Dilute the samples in 15 μL of the resuspension solution. Vortex the tubes to ensure complete resuspension, and centrifuge at 20,800 × *g* for 5 min.
2. Transfer 15 μL of the resuspended peptide samples into autosampler vials and close them to avoid evaporation. Briefly centrifuge the vials to ensure the solution is at the bottom of the vial, without air bubbles trapped.
3. Prepare mobile phase solutions to generate the gradient used for peptide elution from the chromatographic column. Equilibrate the chromatographic system in mobile phase A.
4. Subject the samples to tandem mass spectrometric analyses. Load the vials into the autosampler of the LC-MS/MS system. Set up the run program optimized for the equipment used, and run a blank sample between the unknown samples.
5. Inject 5 μL of the resuspended peptide mixture, and elute the peptides from the C18 column at a flow rate of 500 nL/min using a three-step gradient of mobile phases A and B: 0–40% mobile phase B in 20 min, followed by 40–90% mobile phase B in 5 min, and 90% mobile phase B for 15 min.
6. Apply a spray voltage of 1500 V and acquire data in the MS scan range (mass to charge, m/z) between 350 and 1600 with resolution of 60,000 (at m/z of 400). Select the top ten precursor ions for fragmentation in the linear ion trap using collision-induced dissociation; set the normalized collision-induced dissociation to 35 V. A specific ion to be sequenced twice at most and to be excluded from the list for 45 s, with repeat duration of 30 s and exclusion list size of 500. The minimum threshold count set to 200, and the +1 charge state rejected. Record the data using Xcalibur software, version 2.1 or later.
7. Convert the ".raw" files from LTQ – Orbitrap mass spectrometer to ".mzXML" files with MSConvert.
8. Convert the ".mzXML" files to ".mgf" files using Proteome Discoverer, version 1.1.0 or later.
9. Submit the ".mgf" files to Mascot, version 2.4.0 or later, and search against the TAIR database (release 10) (taxonomy:

Arabidopsis) using the following settings: precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of 0.3 Da, strict trypsin specificity allowing up to one missed cleavage, carbamidomethyl modification on cysteine residues as fixed modification, methionine oxidation as variable modification, and both +2- and + 3-charged peptides analyzed in a monoisotopic mode (*see* **Note 36**).

10. Upload the ".dat" files from Mascot to Scaffold Q+, version 4.10.0 or later, for improved identification, results compilation and subsequent relative quantification of proteins.
11. For each experiment, combine the results from the five gel slices from a single SDS-PAGE lane, corresponding to one fraction of elution. Filter the data to remove common contaminant proteins.
12. Set the peptide/protein probability and false discovery rate (FDR) thresholds (*see* **Note 37**).
13. Verify results from the separate elution fractions for each sample by comparing the total spectrum counts of each protein identified at a fixed probability (e.g., greater than 99%), to assess if the protein is identified in one or more samples and how abundant it is in the pull-down sample obtained with the biotinylated AtPNP-A peptide bait, compared to the negative control pull-down samples (*see* **Note 38**).
14. Perform statistical analysis of the abundance of identified proteins based on the total spectrum count (ANOVA test, $P < 0.05$) with peptide and protein discovery rate (FDR) $< 0.1\%$. To be considered a candidate interactor, the total spectrum count of a given protein has to be twice greater (a fold change >2) in the sample containing the biotinylated bait peptide compared to negative control samples (containing biotin, the biotinylated scrambled peptide, or no bait) in at least two out of three independent biological replicates (*see* **Note 39**).

3.5 Selection of Candidate GCs Based on Protein Amino Acid Sequence Screening Using the GC Catalytic Center Search Motif, Followed by in Silico Prediction of Substrate/Ligand Binding

The method detailed below is aimed at identification of candidate GCs in *Arabidopsis thaliana*. However, with some modifications it can be useful in assessing the presence of a putative GC/AC active site in proteins from Arabidopsis and other sequenced species. A step-by-step protocol detailing the workflow to computationally determine candidate cyclic nucleotide cyclases is given elsewhere [22].

1. Download the amino acid sequences of the interactors with potential GC active site from NCBI website (www.ncbi.nlm.nih.gov) or UniProt (www.uniprot.org).

2. Evaluate the final set of candidate interactors using appropriate search motifs for the prediction of catalytic GC (AC) sites [14, 22, 23] with the PatMatch function on TAIR website (https://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) or another pattern matching tool. Alternatively, assess the proteins with potential nucleotide binding sites for putative GC catalytic site with web tools such as the GCPred server (http://gcpred.com/) [24] (*see* **Note 21**).
3. Select the proteins with amino acid sequences that are predicted to contain a GC catalytic center.
4. Retrieve 3D structures of the proteins containing a putative GC catalytic center from the Protein Data Bank (PDB) website (www.rcsb.org).
5. In case the 3D structures of the proteins of interest are not available on PDB, perform a protein-protein BLAST on the NCBI website (www.ncbi.nlm.nih.gov) against the "Protein Data Bank" (PDB) database. Depending on the degree of homology between the putative GC protein and the most similar PDB protein with resolved 3D structure, submit the amino acid sequence of your protein of interest to a homology-based 3D structure prediction server (e.g., HHPred (www.toolkit.tuebingen.mpg.de/tools/hhpred)) or a server using a fold recognition method for 3D model prediction (e.g., iterative threading assembly refinement - I-TASSER (www.zhanggroup.org/I-TASSER/)) (*see* **Note 22**).
6. Visually inspect the predicted GC active center(s) in the 3D model using UCSF Chimera software, version 1.10.2 (www.cgl.ucsf.edu) by analyzing structural properties of the site and the functional domain (e.g., shape, conformation hydrophobic interactions, hydrogen bonds, domain organization) to assess functional compatibility.
7. Using the AutoDock Vina plugin in the UCSF Chimera software, perform docking of the GTP substrate into the predicted GC catalytic site in the 3D model. Optional: for automatic GTP substrate docking, ClusPro LigTBM server (https://ligtbm.cluspro.org) can be used.
8. Upload the (predicted) 3D models of the candidate GC and its putative peptide ligand interactor to the ClusPro docking server (www.cluspro.bu.edu) to predict the site of interaction. Kinetics of the binding should be determined in vitro on purified recombinant or synthetic peptide(s) and protein (e.g., using surface plasmon resonance).

3.6 In Vitro GC Assay and cGMP Quantification Using LC-MS/MS

The method outlined below describes steps required for in vitro cGMP generation and subsequent cGMP detection and quantification using selected reaction monitoring (SRM). However, by changing the reaction substrate to ATP and selecting a different precursor ion for fragmentation, based on the cAMP internal calibration standard, and a different product ion, the methodology can easily be adjusted to investigate cAMP generation by the protein of interest, and its quantification with respect to the calibration curve using cAMP standards.

1. Prepare the reactions for the GC assay (*see* **Note 28**). GC assay mix: 50 mM Tris–HCl, pH 7.4, 1 mM GTP (or 1 mM ATP in case of assessing AC activity), 5 mM Mg^{2+} or 5 mM Mn^{2+} as a cofactor; and 1 μg of the purified recombinant protein containing a putative GC catalytic center, dialyzed into 50 mM Tris–HCl pH 7.4, or a synthetic peptide containing a putative GC catalytic center, resuspended in HPLC-grade water. Optionally: add $CaCl_2$ and/or IBMX. The final volume of reaction is 100 μL.
2. Start the reaction by adding 1 μg of either the purified recombinant protein (dialyzed into 50 mM Tris pH 7.4) or a synthetic peptide in ≤5 μL of HPLC-grade water, to 100 μL of reaction mix (*see* **Note 25**).
3. Incubate for 20 min at room temperature (20 °C) (*see* **Note 40**).
4. Terminate the reactions by heating to 95 °C for 3 min, and then cool the samples down on ice for 2 min, followed by centrifugation at 2300 × *g* for 3 min (*see* **Note 41**).
5. Transfer the supernatants to new tubes. At this stage, the supernatant can be either diluted 7.5-fold (≥ five-fold) in HPLC-grade water and transferred to autosampler vials for LC-MS/MS analysis or it can be dried and stored (*see* **Note 42**).
6. Set up the LC-MS/MS system using the Xcalibur software, version 2.2 or later. Apply an isocratic flow (55% - 45%) of mobile phase C and mobile phase D over 5 min at a flow rate of 600 μL/min. Use electrospray ionization in the positive mode at a cone voltage of 4 kV, and a scan range of 75–400. Use high collision dissociation fragmentation, linear trap precursor analyzer, and Orbitrap as a fragment analyzer. Set normalized collision energy to 30%, activation time to 0.1 ms, with resolution of 30,000, precursor ion of 346.056, and product ion of 152.056 (*see* **Notes 43** and **44**).
7. Inject 10 μL of each of the cGMP internal calibration standards and of the unknown samples at least in technical triplicates,

with 1–2 blank samples run in-between. Avoid introducing air bubbles.

8. Analyze the data using the Xcalibur software, version 2.2 or later.

4 Notes

1. Although not essential for the basic determination of AC/GC activity, additional equipment for the recombinant protein purification using Fast Protein Liquid Chromatography (FPLC) (e.g., Åkta System, GE Healthcare) and for the assessment of protein–protein/peptide interactions using a method of choice, such as surface plasmon resonance (SPR) (e.g., Biacore T100, GE Healthcare), is advisable.
2. Other types of multichannel HPLC pumps can be used. Other types of columns, such as C18 and C8, may be used, but better retention was observed for cGMP under the mobile phase composition used in this method with the Cyano column compared to C18 and C8, which is of particular importance when dealing with multiple compounds that are analogous to cGMP (such as GMP and cAMP).
3. Other mass spectrometers such as ion traps and triple quadrupoles are also suitable. Simultaneous determination and quantitation of multiple compounds are possible when using the multiple reaction monitoring (MRM) mode of analysis in triple quadrupoles even when the compounds are not chromatographically well separated. In ion traps MS/MS mode is best suited for simultaneous quantitation of multiple compounds if the compounds are already chromatographically separated.
4. To ensure plant growth, *A. thaliana* (Col-0) seeds from the European Arabidopsis Stock Centre (uNASC); http://arabidopsis.info were surface-sterilized, vernalized, and sown on soil in a controlled environment growth chamber at the specified long day photoperiod (16 h light per day) at the white light irradiance of 150 μmol quanta m^{-2} s^{-1} at the leaf level, 23 °C, 65% humidity with the CO_2 concentration maintained at 400 μL L^{-1} (air). In case the ligand of interest is expressed at a very low level, consider increasing its abundance (e.g., by treatment with an elicitor known to enhance its expression), thus likely increasing the abundance of its interactors.
5. Peptides containing the active region of AtPNP-A (aa 33–66), with the disulfide bond preserved, were synthesized (GenScript) at the purity level > 85% verified with HPLC. Biological activity of the N- or C-terminally biotinylated peptides needs to be determined using method(s) appropriate for the processes

the peptide is known to modulate. In case of the AtPNP-A, the biological activity of peptides was assessed by measurement of the net water influx into mesophyll cell protoplasts with respect to the activity of the recombinant AtPNP-A protein and biologically inactive scrambled peptide, in which the order of amino acid residues of the initial peptide sequence has been randomly shuffled to form a new amino acid combination, as described in [25].

6. To ensure equal concentration of the peptide solutions, we recommend preparation of serial dilution with constant dilution factor. Highly concentrated (e.g., 1 mM) stock solutions of peptides can often be stored for prolonged periods of time at −20 °C without significant loss in their biological activity. It is advisable to include negative controls in the experiment (e.g., biotinylated scrambled peptide and biotin-based bait). The amino acid sequence of the biologically active N-terminally biotinylated peptide corresponded to the AtPNP-A amino acids 27–66, thus included its active site (aa 33–66) with the disulfide bond (underlined residues) preserved: AVYYDPPYTR SACYGTQRETLVVGVKNNLWQNGRACGRRY . The N-terminally biotinylated biologically inactive scrambled peptide corresponding to the biologically active peptide, consisting of the same amino acids which are randomly rearranged (e.g., PYGGLPCVGKTRYSVWYQGNTANLCTYEVYQDVRNAR RAR) can be used as a negative control to distinguish between the true and false positive hits.
7. All buffers used for affinity-based isolation of proteins, except the elution buffer 5, should be prepared no longer than 20 h before the experiment (ideally, on the day of use), use Milli-Q water, and cool the buffers down. A total of 5 mL extraction buffer is sufficient to extract approximately 2.5 g leaf tissue and to perform affinity-based isolation with the bait of interest and two negative control baits (0.8 g per pull-down), while a total of 10 mL of each washing buffer is sufficient for performing all washing steps of up to four pull-down samples. A total of 1 mL of each of the native elution buffers is sufficient to perform elution in 20 pull-down samples. To eliminate variability, samples should be processed in parallel, with a single preparation of the required buffers and one large-scale protein extraction.
8. PMSF, a serine protease inhibitor, has a short half-life in aqueous solutions. Its stock solution (10 mM) should be prepared fresh and protected from light to ensure maximal activity. Since it is difficult to dissolve in nonorganic solvents and tends to precipitate in most buffers when added, weigh 174 mg PMSF into a 15 mL tube and dissolve it completely in 10 mL of absolute ethanol to make a 10 mM stock for further use.

9. cOmplete Mini protease inhibitor tablets contain EDTA (1 tablet yields a 1 mM EDTA solution in 10 mL), so no addition of EDTA is required.
10. Stepwise elution of protein preys bound to the biotinylated AtPNP-A peptide bait (or a control bait, e.g., biotinylated scrambled peptide or biotin) under non-denaturing conditions is performed using the biologically active AtPNP-A peptide and biotin, allowing for competitive displacement of the bound proteins.
11. Wear personal protective equipment while handling SDS as it is harmful when inhaled and causes skin irritation. To enhance dissolution, warm up the SDS-containing solution to about 40 °C. Handle the solutions containing SDS gently to avoid foaming.
12. Due to its toxicity, add 2-mercaptoethanol in a fume hood. Wear personal protective equipment while handling 2-mercaptoethanol.
13. As a carcinogen, acrylamide should be handled with extreme care. Wear personal protective equipment while handling acrylamide.
14. As an irritant and a strong oxidizer, APS needs to be handled carefully. Wear personal protective equipment while handling APS.
15. TEMED is harmful by inhalation and can cause burns. Wear personal protective equipment and handle TEMED in a fume hood. Because the acrylamide:Bis start to polymerize as soon as TEMED is added, the solution should be mixed and poured in between the two plates immediately upon TEMED addition.
16. Prepare and handle the solution under a fume hood and wearing personal protective equipment.
17. Only freshly made DTT should be used. Wear personal protective equipment while handling DTT as it is hazardous.
18. IOA is unstable and light-sensitive. Solutions containing IOA should be prepared immediately before use, protected from light, and alkylation should be performed in the dark (e.g., by covering the tubes with aluminum foil). Wear personal protective equipment while handling IOA.
19. Reconstitute trypsin in the trypsin solubilization buffer and prepare the trypsin solution immediately before use. A total of 1 mL of the 10 ng/μL trypsin solution is sufficient to digest proteins in 25–50 samples.
20. Removal of dissolved air from the solutions will prevent potential problems with pressure in the LC-MS/MS analyses.

21. If proteins from organisms other than Arabidopsis are investigated, servers dedicated to those organisms should be used for motif search. Alternatively, online motif searches using MOTIF (https://www.genome.jp/tools/motif/) or ScanProsite (https://prosite.expasy.org/scanprosite/) can be used.
22. In case several 3D models of the protein of interest are available in PDB, choosing the model of the full-length protein, or at least the domain in which the predicted AC/GC active site is embedded, resolved at the highest resolution available, is advised. If no 3D model of the protein of interest is available in PDB, and at least 30% sequence identity between the protein of interest and the template with resolved 3D structure exists, homology-based modeling can be performed. Quality estimation of the predicted 3D model can be assessed by the qualitative model energy analysis on the QMEAN server (https://swissmodel.expasy.org/qmean/).
23. Identification of candidate GCs, cloning and expressing of candidate GCs, and the isolation of recombinant GC have been described extensively [7, 26]. Given that receptor kinases containing GC domains are often large transmembrane proteins, it may be desirable and sufficient to clone and express only the (cytosolic) GC (or AC) domain of a plant receptor, which typically spans about 100 amino acids with 50 amino acids on each side of the catalytic center (e.g., [7, 22]). However, using the cytosolic domains only will not allow to study ligand-dependent effects on GC activity. If the ligand dependence of a GC (or AC) will be tested, the entire plant receptor kinase, including the ligand-binding domain, needs to be expressed (e.g., [4]). Subheadings 2.6 (**steps 1–5**) and 3.5 (**steps 1–8**) could be useful in assessing the desirable portion (s) of the protein to focus on. As an alternative to cloning, there are numerous companies that offer peptide synthesis services, and it may be expedient to have a synthetic candidate GC domain synthesized for functional testing.
24. Accurate determination of the protein concentration, essential for the quantification of GC (or AC) activity, can be done using colorimetric Bradford method, for instance with Bradford Protein Assay (Bio-Rad).
25. The pH of the buffer solution should be within 1.0 pH unit of the protein's isoelectric point (pI) to ensure protein stability, but should not overlap with its pI.
26. Using the HPLC-MS/MS setup described, the lowest amount detectable amount of cGMP is 0.1 pg/μL.
27. Addition of calcium to the standard reaction mix is required when dependence of the GC (or AC) on calcium ions is studied. Calcium concentrations should be kept at a physiological

range (≈ 10 nM), and free calcium concentration can be determined using the Ca-EGTA Calculator version 1.3 or later (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/CaEGTA-TS.htm) considering the temperature, pH and the ionic strength of the buffer.

28. To establish background cGMP levels, a series of negative controls where the reaction mixtures contain all combinations of the reaction ingredients with the exception of one ingredient at a time, should be prepared. A total volume of samples and concentrations of the ingredients need to be the same for all samples. Make sure pH of the reaction buffer does not overlap with pI of the protein. IBMX is a potent nonspecific inhibitor of phosphodiesterases and is not essential in in vitro studies.
29. Argon or nitrogen can be used as collision gas.
30. According to the manufacturer, concentration of the stock solution of beads is 10 mg/mL, and 1 mg of beads typically binds ≈ 200 pmol biotinylated peptide or 650–900 pmol free biotin.
31. Addition of 50 μL of elution buffer 1 (10 μM peptide) or elution buffer 2 (100 μM peptide) equates to the amount of 0.5 nmol and 5 nmol of the biotinylated AtPNP-A peptide, respectively; these levels are 2.5 and 25 times greater than those of the bound peptide (≈ 200 pmol). Accordingly, addition of 50 μL of elution buffer 3 (10 μM biotin) or elution buffer 4 (100 μM biotin) equates to the amount of 0.5 nmol and 5 nmol of free biotin, respectively; these levels are 2.5 and 25 times greater than those of the bound peptide (≈ 200 pmol), respectively, and 0.55–0.77 and 5.5–7.7 times greater than those of the free biotin predicted to be bound (650–900 pmol).
32. Efficiency of protein separation and subsequent in-gel tryptic digestion and mass spectrometric analyses can be verified by running SDS-PAGE of a control sample (e.g., 200 ng bovine serum albumin) and performing all steps of the control sample preparation in parallel with the samples of interest.
33. To prevent masking of low-abundance proteins by more abundant ones, which can be preferentially ionized and detected in mass spectrometric analyses, the gel lanes can be cut into more slices, depending on the LC-MS/MS availability. This would ensure the abundant proteins will have the masking effect on lower number of proteins present in the same smaller gel slice, rather than on a greater number of proteins present in a bigger gel slice (or a whole lane).
34. Addition of 200–400 ng of trypsin to each sample, followed by overnight incubation at 37 °C, was sufficient for efficient digestion of the proteins.

35. For best recovery, do not dry the samples completely, but evaporate the samples to approximately 1 μL.
36. Cysteine carbamidomethylation is a modification due to a reaction with iodoacetamide and is a known artifact of over-alkylation. Since the proteins were alkylated prior to trypsin digestion (**step 15** in Subheading 3.3), the assignment of this modification is highly likely and it should be selected as a fixed modification, in contrast to variable modifications which may or may not occur (e.g., oxidation of methionine residues).
37. Peptide identifications were accepted if they could be established at a probability greater than 99% by the Peptide Prophet algorithm [27]. Protein identifications were accepted if they could be established at the probability greater than 99% by the Protein Prophet algorithm [28] and contained at least two identified peptides.
38. Proteins which eluted in the final denaturing elution have high affinity for the bait, while those that eluted at low (10 μM) concentration of peptide have low affinity. Proteins identified in at least three fractions of elution are likely to bind nonspecifically.
39. Rank the most likely interactors, based on comparison of the spectral counts for samples with the peptide bait and negative controls in descending order. Use additional independent methods to confirm candidate interactors (e.g., yeast two-hybrid, coimmunoprecipitation followed by LC-MS/MS).
40. For the endpoint reaction, GC (or AC) activity in measured after 20 min, whereas for kinetic measurements a time series, typically spanning between 30 s and 30 min, needs to be chosen.
41. At this stage the samples can be subjected to cGMP (or cAMP) quantification using colorimetric cGMP (or cAMP) assay, such as the Direct Biotrak enzyme-linked immunoassay (Cytiva). Although less sensitive than mass spectrometry, this alternative method can be used to confirm results obtained from mass spectrometric analysis.
42. Dried samples need to be reconstituted in ≥200 μL of HPLC-grade water by vortexing for 30 s, followed by 10 min centrifugation at 20,000 × *g* at 4 °C. Optionally: spike the samples with internal standards.
43. Detection of cGMP is based on SRM by fragmenting its precursor (a.k.a. parent) ion at m/z 346.05 which yields a product (a.k.a. daughter) ion at 152.05. Quantification of cGMP relies on a standard calibration curve based on the chromatographic

peak areas of each calibration concentration using the extracted chromatogram of the product ion *m/z* 152.05.

44. Collision-energy was optimized to allow at least 85% conversion of the precursor to product. Information on optimized transitions for SRM analyses of small molecules can be accessed in METLIN-MRM (https://metlin.scripps.edu) [29] or on Skyline (https://skyline.ms) [30]. Since monoisotopic molecular weight of cGMP is 345.05, the precursor ion (*m/z* 346.05 [M + 1]+) and the product ion *m/z* 152.05 [M + 1]+) can be detected.

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Chapter 15

Detection of Ligand-Induced Receptor Kinase and Signaling Component Phosphorylation with Mn^{2+}-Phos-Tag SDS-PAGE

Zunyong Liu, Shuguo Hou, and Ping He

Abstract

Plasma membrane-resident receptor kinases (RKs) are crucial for plants to sense endogenous and exogenous signals in regulating growth, development, and stress response. Upon perception of ligands by the extracellular domain, RKs are usually activated by auto- and/or trans-phosphorylation of the cytoplasmic kinase domain, which in turn phosphorylates downstream substrates to relay the signaling. Therefore, monitoring ligand-induced in vivo phosphorylation dynamics of RKs and their associated proteins provides mechanistic insight into RK activation and downstream signal transduction. Phos-tag specifically binds phosphomonoester dianions of phosphorylated serine, threonine, and tyrosine residues, which enables Phos-tag-containing SDS-PAGE gels to separate phosphorylated proteins from non-phosphorylated form. Here, we describe a detailed method of Mn^{2+}-Phos-tag SDS-PAGE analysis to detect the ligand-induced in vivo phosphorylation of RKs and associated proteins.

Key words ABI1, NUT, Protein phosphorylation, Peptide signaling, Protoplast transfection, Receptor activation, Phos-tag, SCREW, SDS-PAGE, Western blot

1 Introduction

Phosphorylation and dephosphorylation of serine, threonine, or tyrosine residues mediated by protein kinases and phosphatases are fundamental post-translational modifications, which control many biological processes, including plant immunity and development [1]. Following an assessment of the phosphorus content of a certain protein, various techniques, such as isotope labeling, electrophoresis, phospho-site-specific antibodies, and mass spectrometry (MS), have been developed for detecting protein phosphorylation status [2, 3]. A dinuclear metal complex, i.e., the 1,3-bis [bis (pyridin-2-ylmethyl) amino] propan-2-olato dizinc (II) complex, as a phosphate-binding molecule in aqueous solution

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at neutral pH was first characterized as a Phos-tag in 2003 [3, 4]. After integrating two metal ions, Phos-tag selectively captures a phosphate monoester dianion with a 10,000-fold higher affinity than other anions present in living organisms [5, 6]. Later, researchers found that the manganese II isoform of Phos-tag (Mn^{2+}-Phos-tag) can capture a phosphor-monoester dianion, such as phosphoserine or phosphotyrosine, at alkaline pH conditions [7–9]. These findings promoted acrylamide-pendant Mn^{2+}-Phos-tag as an additive in normal SDS-PAGE resolving gels for developing phosphate-affinity gel electrophoresis to separate phosphor-proteins from their non-phosphorylated counterparts [3, 7–9]. Compared to other methods for detecting protein phosphorylation, the Mn^{2+}-Phos-tag SDS-PAGE method does not require any radioactive or chemical labels. Meanwhile, it can detect various phosphor-protein isotypes and determine the quantitative ratio of phosphorylated to non-phosphorylated proteins in vivo under different physiological conditions. This method is particularly suitable for monitoring the phosphorylation status of transmembrane proteins, as it is often challenging to obtain a sufficient amount of full-length proteins for kinase assays [3].

Receptor kinases (RKs) regulate diverse signaling processes in plant growth, development, and stress responses [10, 11]. A typical plant RK contains an N-terminal extracellular domain for ligand binding, a single-transmembrane region, a juxtamembrane domain, a cytoplasmic kinase domain, and a C-terminal tail region [11, 12]. Upon the perception of cognate ligands by the extracellular domain, RKs undergo auto- and/or trans-phosphorylation to initiate downstream signaling. In addition to the phosphorylation within the RK cytoplasmic kinase domain for RK activation, the juxtamembrane domain also can be phosphorylated, leading to kinase activation from an off state [13, 14]. Accordingly, phosphorylation-induced kinase activation is essential for the RK-mediated signaling relay [15, 16].

The model plant Arabidopsis possesses a large number of RKs with >600 members [17]. Among them, members with leucine-rich repeat (LRR) ectodomains (LRR-RKs) comprise the largest subfamily of RKs and are primarily peptide receptors [17] in regulating plant growth, development, and stress responses. For example, CLAVATA 1 (CLV1) is an LRR-RK containing 21 LRRs to sense the CLV3 peptide in maintaining stem cell homeostasis in the shoot apical meristem [18]. Recently, LRR-RK MALE DISCOVERER 1-INTERACTING RECEPTOR-LIKE KINASE 2 (MIK2) was found to recognize the conserved signature motif of SERINE-RICH ENDOGENOUS PEPTIDEs (SCOOPs) from *Brassicaceae* plants as well as proteins present in fungal *Fusarium* spp. and bacterial *Comamonadaceae*, and to elicit immune responses [19, 20]. LRR-RK PLANT SCREW UNRESPONSIVE RECEPTOR (NUT) was identified as a receptor of SMALL

PHYTOCYTOKINES REGULATING DEFENSE AND WATER LOSS (SCREWs), a family of immunomodulatory phytocytokines regulating plant immunity and water loss [21, 22]. The kinase activity of NUT was shown to be required for SCREW-induced MAP kinase activation. The activation of the NUT-SCREW signaling pathway is relayed through the phosphorylation of ABA (abscisic acid) INSENSITIVE 1 (ABI1). SCREW peptide-mediated ABI1 phosphorylation promotes its phosphatase activity, inhibiting the phosphorylation of kinase OST1 (OPEN STOMATA 1) to regulate guard cell movement.

Here, we use SCREW1-induced NUT and ABI1 phosphorylation as examples and describe a detailed Mn^{2+}-Phos-tag SDS-PAGE gel method to determine the in vivo phosphorylation of RK NUT and an associated downstream component ABI1. Results are shown in Fig. 1, for SCREW1-dependent phosphorylation of NUT in panel 1a, and of ABI1 in panel 1b.

2 Materials

2.1 Plants

Arabidopsis: Grow *Arabidopsis thaliana* Col-0 plants for 4 weeks on soil (Jolly Gardener C/20 and C/GB Growing mix, 1:1) in a growth room at 20–23 °C, 50% humidity, and 100 $\mu E\, m^{-2}\, s^{-1}$ light with a 12-hour (h) light-12-h dark photoperiod. Stress-free plants are important (*see* **Note 1**).

2.2 Protein Sample Preparation Reagents and Equipment

1. SCREW1 peptide: Obtain SCREW1 (or other SCREW peptides) as custom-synthesized peptides at >95% purity. Dissolve the peptides in ddH_2O at 1 mM concertation. Store at −20 °C as small aliquots to avoid freeze-thaw cycles.
2. *pHBT-NUT-FLAG* and *pMD32-ABI1-FLAG*: High-quality plasmid DNA is required for the epitope-tagged genes of interest in a plant gene expression vector (*see* **Note 2**). Here, we used the constructs of FLAG-tagged NUT1 and ABI1 in the vectors *pHBT* and *pMDC32,* respectively [21].
3. Enzyme solution: 1.5% cellulase R10 (Yakult), 0.4% macerozyme R10 (Yakult), 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7. Prepare immediately before use (**step 3.1.1**).
4. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7.
5. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7.
6. 40% (w/v) polyethylene glycol (PEG) solution: To make 10 mL of PEG solution, add 4 g of PEG4000 into 3 mL of H_2O, 2.5 mL of 0.8 M mannitol, and 1 mL of 1 M $CaCl_2$.

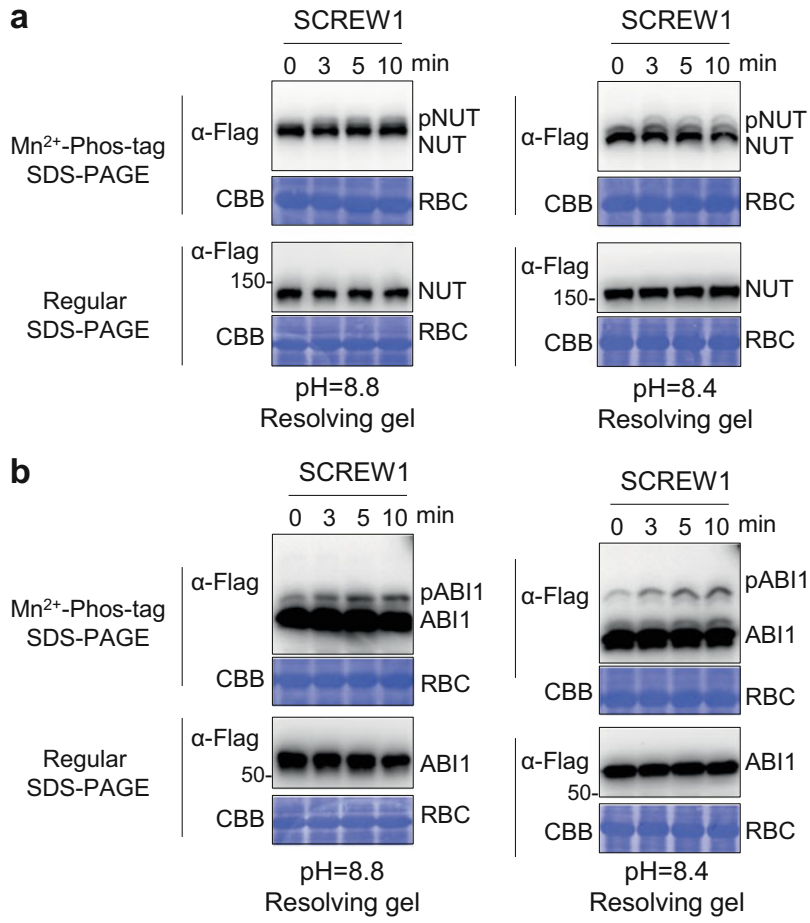


Fig. 1 SCREW-triggered NUT (**a**) and ABI1 (**b**) phosphorylation. Protoplasts were co-transfected with *NUT-FLAG* or *ABI1-FLAG* and incubated for 12 h at room temperature, followed by treatment with 1 μM SCREW1 for the indicated time. Total proteins were separated with Mn^{2+}-Phos-tag (top two) or regular SDS-PAGE (bottom two) with indicated pH in resolving gel and detected with α-FLAG antibodies. The protein loading is shown by Coomassie Brilliant Blue (CBB) staining of the PVDF membrane to visualize RuBisCO (RBC)

7. WI solution: 0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7.

2.3 Immunoblotting Reagents and Equipment

1. 30% (w/v) acrylamide solution.
2. 10 mM Phos-tag™ Acrylamide AAL-107 (Fujifilm Wako Chemicals): add 10 μL methanol to dissolve 10 mg first, and then add 1.6 mL distilled water for a total of 1.6 mL Phos-tag™ Acrylamide with the concentration of 10 mM and store at 4 °C.
3. PVDF (polyvinylidene difluoride) blotting membranes.
4. 10 mM $MnCl_2$ solution: dissolve 0.1 g $MnCl_2{\cdot}4H_2O$ (FW: 198) in 50 mL of distilled water.

5. 10% (w/v) ammonium persulphate solution (APS): dissolve 0.1 g ammonium persulphate in 1 mL distilled water.
6. 2 × SDS loading buffer: 125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 0.05% (w/v) bromophenol blue.
7. 10 × running buffer: 1% SDS, 0.25 M Tris, 1.92 M glycine.
8. 10 × transfer buffer: 250 mM Tris, 1.92 M glycine.
9. Mn^{2+}-Phos-tag SDS-PAGE gel washing buffer: 10 mL 10 × transfer buffer, 10 mL methanol, 2 mL 0.5 M EDTA, add distilled water to 100 mL.
10. 1 × transfer buffer for regular gel: 100 mL 10 × transfer buffer, 200 mL methanol, 0.5 mL 20% (w/v) SDS, add distilled water to 1 L.
11. 1 × transfer buffer for Mn^{2+}-Phos-tag gel: 100 mL 10 × transfer buffer, 100 mL methanol, 2 mL 0.5 M EDTA, 0.5 mL 20% (w/v) SDS, add distilled water to 1 L.
12. Regular resolving gel:

ddH_2O	1.133 mL
30% (w/v) acrylamide solution	1.33 mL
1 M Tris-HCl (pH 8.8 or 8.4)	1.5 mL
10% SDS	20 μL
10% APS	12 μL
TEMED	5 μL

13. Mn^{2+}-Phos-tag SDS-PAGE resolving gel (*see* **Notes 3** and **4**):

ddH_2O	1.098 mL
30% (w/v) acrylamide solution	0.94 mL
1 M Tris-HCl (pH 8.8 or 8.4) (*see* **Note 5, 6**)	1.32 mL
10% SDS	35 μL
10 mM Phos-tag™ acrylamide AAL-107	7 μL
10 mM $MnCl_2$	70 μL
10% APS	25 μL
TEMED	5 μL

14. Stacking gel (*see* **Notes 3** and **4**):

ddH_2O	1.434 mL
30% (w/v) acrylamide solution	0.316 mL

(continued)

1 M Tris-HCl (pH 6.8)	0.253 mL
10% SDS	10 μL
10% APS	10 μL
TEMED	3.3 μL

15. Anti-FLAG-peroxidase conjugated antibodies (e.g., Sigma-Aldrich).
16. Blocking buffer: 5% (w/v) skim milk powder in TBST.
17. Semi-dry western blotting system for electrophoretic transfer of proteins.
18. Wet/Tank Blotting System.
19. Western blot imaging system for chemiluminescence detection.
20. Coomassie Brilliant Blue (CBB) staining buffer: 0.1% (w/v) CBB, 10% (v/v) acetic acid, 50% (v/v) methanol in H_2O.
21. CBB destaining buffer: 10% (v/v) acetic acid, 50% (v/v) methanol in water.
22. Tris-buffered saline with Tween 20 (TBST) buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20.
23. Reagents for chemiluminescent western blot detection (e.g., Clarity™ Western ECL Substrate from Bio-Rad).

3 Methods

3.1 Protoplast Isolation and Transfection [23]

1. Prepare the enzyme solution.
2. Cut approximate 30 Arabidopsis leaves from one-month-old plants into around 1-mm strips with fresh razor blades and digest the leaf strips in 10 mL enzyme solution in a Petri dish for 3 h.
3. Filter the enzyme solution containing protoplasts with a 35- to 75-μm nylon mesh into a 30 mL round-bottom tube.
4. Pellet the protoplasts by spinning for 2 min at 100 × *g* and resuspend the protoplasts in 10 mL W5 solution by gently shaking.
5. Keep the protoplasts in the W5 solution on ice for at least 30 min.
6. The protoplasts should settle to the bottom of the tube in 5–10 min. Before PEG-Ca^{2+} transfection, pipet the W5 solution out and resuspend the protoplasts in MMg solution to a density of 5×10^5/mL.

7. Add 50 μL (around 80 μg) of the mixed vectors into a 15 mL round-bottom tube, then add 1 mL protoplasts in MMg solution into the tube and mix well by gently tapping the tube.
8. Add 1050 μL of 40% PEG into the tube, and immediately mix well by gently reverting the tube.
9. Incubate at room temperature (23 °C) for 5 min, stop the transfection by adding 8 mL W5 solution, and mix well by gently reverting the tube.
10. Spin at 100 × *g* for 2 min, remove the supernatant and then resuspend the protoplasts gently with 200 μL WI. Transfer the protoplasts into one well of a six-well tissue culture plate containing 1 mL of WI (1.2 mL total volume).
11. Incubate the protoplasts under desirable conditions overnight (*see* **Note 7**).
12. Distribute the protoplast suspension as six 200 μL aliquots into the six wells of the plate.
13. Add 1 μM SCREW peptides (diluted 1:1000 from the 1 mM stock solutions) and incubate for the time indicated (Fig. 1).
14. Harvest protoplasts by spinning at 100 × *g* for 5 mins and remove the supernatant. Immediately freeze and store the samples at −80 °C.
15. Take the samples from −80 °C freezer and add 100 μL of 2 × SDS loading buffer. Boil the samples for 5 min and then spin down cell debris at 15,000 × *g* for 2 min.

3.2 Mn^{2+}-Phos-Tag SDS-PAGE and Western Blot

1. To make sure the protein-of-interest is expressed at the predicated molecular weight and the band shift is due to the Phos-tag instead of the protein itself, run the protein samples on regular SDS-PAGE and Mn^{2+}-Phos-tag SDS-PAGE gels in parallel.
2. Prepare regular SDS-PAGE and Mn^{2+}-Phos-tag SDS-PAGE gels preparation with different pH resolving gel (*see* **Notes 3, 4** and **8**).
3. Load the protein samples; do not use the wells at the extreme left and right (*see* **Notes 9** and **10**).
4. Perform the electrophoresis at the voltage of 60 V for 30 min at room temperature.
5. Increase the voltage to 90 V until the bromophenol blue exits from the gel (*see* **Note 11**).
6. After the electrophoresis, wash the Mn^{2+}-Phos-tag SDS-PAGE gel with washing buffer for 30 min at room temperature with gentle agitation.

7. Prepare a PVDF membrane of the same size as the gel. Activate the membrane by soaking it in methanol for 1 min, then equilibrate it in the transfer buffer for 5 min.
8. For Mn^{2+}-Phos-tag SDS-PAGE gel, assemble the transfer sandwich and place the cassette in the transfer tank with an ice bath. Transfer proteins at constant voltage of 55 V for 3 h. For the conventional SDS-PAGE gel, use a semi-dry blotting apparatus at constant current of 1.5 mA for 25 min (*see* **Notes 12** and **13**).
9. After transfer, briefly rinse the gel with TBST and incubate in blocking buffer for 1 h at room temperature with gentle agitation.
10. Incubate the gel with anti-FLAG antibody diluted 1:2000 in blocking buffer at 4 °C overnight, or at room temperature for 3 h.
11. Wash the membrane with TBST, four times for 5 min each.
12. Prepare the chemiluminescence detection reagents according to the manufacturer's instructions, spread them evenly over the membrane, and incubate for 1 min.
13. Capture the chemiluminescent signals using a western blot imaging system.
14. Stain the membrane with CBB staining buffer followed by CBB detaining buffer to visualize RuBisCO as a loading control.

4 Notes

1. Four-week-old plants for protoplast isolation should have grown under conditions without any pathogen and pest infection nor water/drought/light/temperature stresses. Do not use plants with purple petiole or dark green leaves for protoplast isolation.
2. DNA quality is critical for protoplast transfection and protein expression. Therefore, high-quality DNAs without any trace amount of RNAs, proteins, polysaccharides and phenolic compounds are important to obtain a high protein transfection efficiency and expression level.
3. The Mn^{2+}-Phos-tag SDS-PAGE gel cannot be stored for a long time, and it should be freshly prepared prior to use. Also note that the alkaline pH of the resolving gel provides only short-term stability for the separated phosphoproteins. Therefore, gels should not be stored prior to blotting.
4. Cast the gel at a cool temperature and wash the wells with 1× running buffer after pulling out the comb.

5. For the best resolution of the gels, prepare Tris-HCl freshly and make sure the pH is correct.
6. Slightly decreasing the pH of the resolving gel will increase the migration for large proteins (Fig. 1). Don't lower the pH too much, as this will not achieve a better effect.
7. The incubation conditions, such as light and temperature, depend on the purpose of the experiments. For most experiments, protoplasts could be incubated at room temperature under low light (30–50 μmol/m^{-2}/s).
8. The Mn^{2+}-Phos-tag SDS-PAGE gel also has limitations in detecting the phosphorylation of certain proteins. Zn^{2+}-Phos-tag SDS-PAGE gel could be an option if Mn^{2+}-Phos-tag SDS-PAGE gel does not work well for the target protein. Be aware that the buffer for making and running Zn^{2+}-Phos-tag SDS-PAGE gel differs from Mn^{2+}-Phos-tag SDS-PAGE gel [5].
9. Be careful to avoid any precipitates at the bottom of the tube. Insoluble debris will block the gel wells and affect protein migration.
10. Run the protein samples without freeze-storages. Some phosphorylated proteins might not be stable even under the freeze condition.
11. Adjust the running time depending on the molecular weight of the protein.
12. Make sure that no air bubbles are trapped in the sandwich cassette when performing the membrane transfer.
13. Increase the transfer efficiency for proteins with a large molecular weight by extending transfer time.

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Part III

Peptide-Receptor Interaction



Chapter 16

In-vivo Cross-linking of Biotinylated Peptide Ligands to Cell Surface Receptors

Ronja Burggraf and Markus Albert

Abstract

In-vivo cross-linking of biotinylated peptides is a technique to analyze the interaction of small proteins or peptide ligands with their corresponding receptors. Here, we describe an in-vivo method in which leaves of living plants, transiently expressing receptor proteins, are infiltrated with biotinylated peptides. The interaction between ligand and receptor is irreversibly fixed by the infiltration of a cross-linking agent. Subsequently, co-immunoprecipitation is used to pull down the receptor-ligand pair. After western blotting, the biotin tag of the ligand peptide cross-linked to the receptor can be detected by streptavidin-AP conjugate on the membrane.

Key words Protein expression in plants, Peptide-receptor interaction, Biotinylated peptides, In-vivo cross-linking, Co-immunoprecipitation (Co-IP), SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot

1 Introduction

In plant biochemistry, several methods have been established to demonstrate the interaction between receptors and their proteinaceous ligand molecules. The one described here, co-immunoprecipitation (Co-IP) followed by protein detection on western blot, detects protein-protein interactions on a qualitative level, and has often been used to demonstrate receptor–co-receptor interactions [1, 2]. In-vivo cross-linking of synthetic biotinylated peptide ligands to their corresponding receptors facilitates the detection of direct protein-peptide interaction in their natural plant cell environment. The method works irrespective of ligand size and it allows the demonstration of physical interactions between short peptides of only about 20 amino acids to their cognate receptor. The approach, however, is limited by the amino acid sequence, as it indispensably requires residues with free primary amino groups for cross-linking like the one present in the side

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chain of lysines. Additionally, an appropriate amino acid residue for biotinylation is required, and possibilities for potential biotinylation sites should be elaborated carefully. The N-terminal α-amino group of peptides can be utilized for biotinylation [3]. Also the ε-amino group of lysine residues within the peptide sequence or additionally attached to the C-terminus of the peptide can be biotinylated [4]. Importantly, the biotin-tag should be spatially separated from the peptide part that is expected to directly associate with the binding pocket of the receptor. It may be necessary to insert extra amino acid residues functioning as a spacer between peptide and biotin-tag [5]. Random biotinylation of the available free amines should be avoided, as it may affect the binding properties of the ligand protein or peptide to its cognate receptor. Additionally, it may limit the number of free amines that are still available for the cross-linking procedure. Therefore, it is rather recommended to have the biotinylated peptide ligands custom-synthesized, and the biotinylated amino acid residue integrated during peptide synthesis. Irrespective of the production or biotinylation method, the labeled peptides should always be tested for functionality. EC50 values of the biotinylated and the unlabeled peptide should be compared in bioassays, to make sure that the biotin-tag does not hinder the ligand from binding to and activating the receptor.

On the surface of plant cells, a multitude of membrane-bound receptors are present which detect endogenous signals such as hormones or damage-associated molecular patterns, and are also important to sense exogenous signals deriving from pathogenic invaders. Ligands include polypeptide hormones, PAMPs or DAMPs (pathogen−/damage-associated molecular patterns) originating from different pathogenic organisms like bacteria, fungi, oomycetes, or parasitic plants. Leucine-rich repeat receptor-like kinases (LRR-RLKs) and receptor-like proteins (LRR-RLPs) very often serve as pattern-recognition receptors (PRRs) and mostly recognize proteinaceous ligands. The sensing of PAMPs, DAMPs or hormones by PRRs is the essential first step in plant immunity and switches on the cellular signaling cascades leading to plant defense and resistance against invaders [6–8].

As an important tool to study and characterize plant PRR protein properties, subcellular localization and function, *Agrobacterium tumefaciens*-mediated plant transformation and *PRR* overexpression is utilized. A very convenient tool compared to the generation of stable transgenic lines is the transient overexpression of (receptor-) proteins in *Nicotiana benthamiana* [9]. Combining this transient receptor protein overexpression with the infiltration of biotinylated proteins or peptides into leaves allows the ligand-receptor interaction and cross-linking in-vivo. Thereby the receptor and corresponding ligand are covalently linked and thus can easily be isolated and detected. To date, the interaction of small peptide epitopes with their corresponding PRRs has already been

demonstrated in numerous cases with the method described herein. The procedure has been used first in 2015, when the 20 amino acid peptide ligand nlp20, originating from necrosis and ethylene-inducing proteins (NLPs) of oomycetes, fungi, and bacteria, was shown to interact with the membrane-bound LRR-RLP23 from *Arabidopsis thaliana* [10]. In subsequent years, cross-linking of biotinylated peptides has been utilized to demonstrate the interaction of several peptide ligands with LRR-RLPs [11] or LRR-RLKs [8, 12]. Here, we describe this method in detail, from transient expression to protein detection on western blot, highlighting the critical steps to avoid potential pitfalls.

2 Materials

All solutions are prepared with ultrapure Milli-Q water and analytical-grade reagents. They are prepared and stored at room temperature unless otherwise specified. 70% denatured ethanol is used for all cleaning steps. Adhere to current regulations for waste disposal.

2.1 *Transient Expression of* PRRs *in* Nicotiana benthamiana *Leaves*

1. Infiltration medium: 10 mM $MgCl_2$, 150 μM acetosyringone (3,5-dimethoxy-4-hydroxyacetophenon). Weigh 0.238 g $MgCl_2$ in a 250 mL bottle, adjust to 250 mL with water, and add 250 μL of a 150 mM acetosyringone stock solution. Store at −20 °C.
2. 150 mM acetosyringone: Dissolve 0.294 g acetosyringone in 10 ml DMSO, prepare 1 mL aliquots in 1.5 mL reaction tubes, store at −20 °C.
3. *Agrobacterium tumefaciens* (strain GV3101, genotype T-DNA- vir^+ rif^r, pMP90 gen^r), carrying the overexpression construct for the tagged receptor-of-interest. For example, the plasmid pGWB17 [13] containing a 4× Myc-tag for the overexpression of *35S::receptor:myc* [11].
4. *A. tumefaciens* GV3101 carrying the expression plasmid for the p19 suppressor of silencing [14].
5. 4-week-old *N. benthamiana* plants, grown under long day conditions (16 h light/8 h dark, 22 °C, humidity 60%).
6. 1 mL disposable plastic syringes.
7. Photometer.
8. Shaking incubator for bacterial cultures.

2.2 *Infiltration and Cross-linking of Biotinylated Peptides*

1. Custom-synthesized biotin-labeled peptide, unlabeled competitor, and any unrelated peptide as unspecific competitor control [11] (*see* **Note 1**).

2. Ethylene glycol bis(succinimidyl succinate) (EGS).
3. Dimethylsulfoxide (DMSO).
4. Cross-linker buffer: 50 mM HEPES, pH 7.5. Adjust the pH with NaOH. Prepare 50 mL and store at −20 °C (*see* **Note 2**).
5. 1 mL disposable plastic syringes.
6. 2 mL safe-lock reaction tubes.

2.3 Protein Analysis

2.3.1 Co-immunoprecipitation

1. 1 M Tris-(hydroxymethyl)-aminomethan (Tris), pH 8: Dissolve 60.57 g Tris (free base) in 400 mL water, adjust the pH with HCl and the volume to 500 mL with water.
2. 4 M NaCl: Dissolve 23.36 g NaCl in 50 mL water and adjust to 100 mL with water.
3. Wash buffer: 25 mM Tris, pH 8, 150 mM NaCl. Mix 25 mL 1 M Tris (pH 8) and 37.5 mL 4 M NaCl and adjust to 1 L with water. Store at 4 °C.
4. Solubilization buffer: 1% (v/v) Nonident-40 [Igepal] (NP40), 0.5% (w/v) sodium deoxycholate (DOC) in wash buffer. For 20 mL solubilization buffer, mix 20 mL wash buffer (*see* 2.3.1) with 200 µL NP40 and 100 mg DOC (*see* **Note 3**). Keep it on ice.
5. Plant protease inhibitor cocktail prepared according to the manufacturer's instructions.
6. 1 M dithiothreitol (DTT): Dissolve 3.09 g DTT in 20 mL water, dispense into 1 mL aliquots. Store at −20 °C.
7. Beads covered with specific anti- or nanobodies, binding the tagged receptor, e.g., Myc-Trap® Agarose (ChromoTek GmbH) [11].
8. Rotary mixer.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis

1. Acrylamide:bis-acrylamide mix (37.5:1), store at 4 °C.
2. Separating gel buffer: 1.5 M Tris, pH 8.8. Dissolve 90.86 g Tris (free base) in 400 mL water, adjust the pH with HCl and the volume to 500 mL with water.
3. Stacking gel buffer: 1 M Tris, pH 6.8. Dissolve 60.57 g Tris (free base) in 400 mL water, adjust the pH with HCl and the volume to 500 mL with water.
4. 10% SDS: 10% (w/v) sodium dodecyl sulfate in water.
5. 10% APS: 10% (w/v) ammonium persulfate in water (*see* **Note 4**).
6. N,N,N′,N′-Tetramethylethylenediamine (TEMED).
7. 3× SDS loading buffer: 0.5 M Tris/HCl, pH 6.8, 30% (v/v) glycerol, 3% (w/v) SDS, 0.05% (w/v) bromophenol blue, 10 mM DTT (*see* **Note 5**).

8. 1× SDS running buffer: 25 mM Tris, 192 mM glycine, 1 g/L SDS (*see* **Note 6**).
9. Pre-stained marker proteins (e.g., PageRuler™, Thermo Scientific).
10. Polyacrylamide gel electrophoresis system (e.g., the Mini-PROTEAN® Tetra handcast systems with glass plates (7.25 cm × 10 cm × 1 mm) and the Mini-PROTEAN® Tetra cell, Bio-Rad).
11. Electrophoresis power supply.

2.3.3 Wet Blot Protein Transfer

1. 1× transfer buffer: 25 mM Tris, 192 mM Glycine, 20% (v/v) methanol (*see* **Note 7**).
2. Nitrocellulose membrane (pore size 0.45 μM).
3. 3 mm gel blotting paper.
4. Electrophoretic transfer cell (e.g., Mini-Trans-Blot® cell, with foam pads, holder cassette and cooling unit, Bio-Rad).
5. Power supply (constant voltage).

2.3.4 Protein Detection on Western Blot

1. Ponceau S: dissolve 200 mg Ponceau S in 190 mL water and add 10 mL acetic acid (*see* **Note 8**).
2. Blocking buffer: 5% (w/v) skim milk powder in 1× PBS-T. Store at 4 °C.
3. 1× PBS-T: 137 mM NaCl, 22 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% (v/v) TWEEN 20 (*see* **Note 9**).
4. 1× Washing buffer: 50 mM Tris, pH 8, 0.05% TWEEN. Dissolve 6 g Tris (free base) in 900 mL water, adjust the pH with HCl and the volume to 1 L with water, then add 500 μL TWEEN 20.
5. Biotin-free blocking buffer: 50 mM Tris/HCl, pH 8, 0.05% (v/v) TWEEN 20, 5% (w/v) biotin-free BSA. Dissolve 2.25 g BSA in a total of 45 mL washing buffer (*see* 2.3.4). Store at 4 °C (*see* **Note 10**).
6. 1× Assay buffer: 20 mM Tris/NaOH, pH 9.8, 2 mM $MgCl_2$ (*see* **Note 11**).
7. Primary antibody: the antibody has to be chosen to match the tag fused to the receptor protein, e.g., anti-myc (Sigma-Aldrich) in the experiment described here [11].
8. Secondary antibody: alkaline phosphatase (AP) conjugated IgG to detect the primary antibody, e.g., anti-rabbit IgG (Sigma-Aldrich).
9. Streptavidin-alkaline phosphatase conjugate (Strep-AP, Sigma-Aldrich).

10. Tropix® Nitro-Block II™ and Tropix® CDP-Star™ (Sigma-Aldrich).
11. Chemiluminescence detection system (e.g., UVP ChemStudio Analytic Jena GmbH).
12. Horizontal shaker.
13. Foil (e.g., empty disposal bags).

3 Methods

All steps are carried out on the bench at room temperature unless otherwise indicated.

3.1 Transient Expression of Receptors in N. benthamiana

1. Grow *A. tumefaciens* strains containing the p19 construct and the receptor construct of interest each in 5 mL LB containing the appropriate antibiotics for 36 h at 29 °C and 200 rpm.
2. Centrifuge 3–5 mL of the two bacterial cultures (5 min, 5000 rpm, 4 °C) and resuspend the cell sediments in 1 mL infiltration medium. Mix 100 μL of the bacterial suspensions with 900 μL infiltration medium, transfer them to cuvettes and measure the optical density at 600 nm (OD_{600}) with a photometer (*see* **Note 12**). Adjust the bacterial suspensions to OD_{600} = 1 with infiltration medium to a final volume of 5 mL ($\frac{OD_{600}\text{wanted} = 1}{OD_{600}\text{measured}} * \text{volume}_{\text{end} = 5\ \text{ml}} = \text{volume}_{\text{bacteial stock}}$).
3. Incubate the bacteria on the bench in a tube with slightly opened lid for 90 min.
4. Mix 1 volume of each of the bacterial suspensions and add 8 volumes of infiltration medium to reach OD_{600} = 0.1 for each of the constructs. Use p19 alone as negative control, also with an OD_{600} = 0.1. Calculate 2 mL of infiltration mixture per leaf.
5. Infiltrate (*see* **Note 13**) 4-week-old *N. benthamiana* plants, 2–3 leaves per plant and bacterial inoculum. If you intend to test several peptides in one experiment, you will need one plant per peptide treatment. Keep plants on the bench for 48 h (Fig. 1, *see* **Note 14**).

3.2 Infiltration and Cross-linking of Biotinylated Peptides

1. Prepare biotin-labeled peptides to a final concentration of 100 nM alone and in combination with unlabeled competitor (10 μM) or unlabeled competitor control (10 μM), respectively, in ultrapure MilliQ water (Fig. 2a, *see* **Note 1**). 0.5–1 mL per leaf should be enough.
2. Prepare 2 mM EGS cross-linker solution: Dissolve 0.027 g EGS in less than 300 μL DMSO (*see* **Note 15**), mix with 30 mL 50 mM HEPES, pH 7.4.

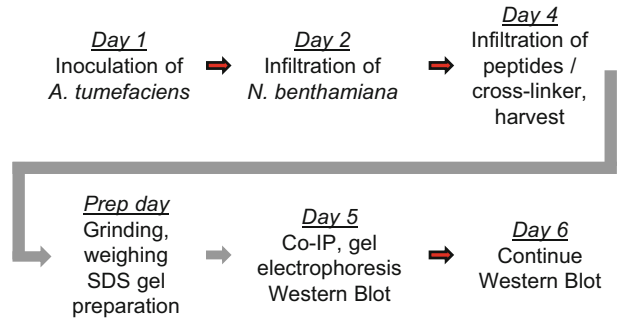


Fig. 1 Workflow for the experimental procedure. For optimal results, follow the time intervals marked by red arrows as indicated here and as described within the text. Steps marked by grey arrows are more flexible and can be performed whenever they fit in the time schedule. Samples and intermediates generated on "preparation (prep) day" can be stored and the procedure continued later

3. Infiltrate (*see* **Note 13**) 1 or 2 leaves (*see* **Note 16)** expressing the receptor constructs per plant with one peptide solution. Incubate for 5 min.
4. Infiltrate the same leaf or leaves with the EGS solution. Incubate for 30 min (*see* **Note 17**).
5. Harvest the leaves, freeze them directly in liquid nitrogen. Grind the deep-frozen leaf material to a fine powder in liquid nitrogen, then weigh out 250–300 mg into 2 mL safe-lock reaction tubes (Fig. 2b, *see* **Note 18**). Store the leaf material at −80 °C, unless you use it directly for further analyses.

3.3 Protein Analysis

Carry out all steps on ice, unless otherwise stated.

3.3.1 Co-immuno-precipitation (Fig. 2b)

1. Mix 250–300 mg plant material as prepared in **step 3.2.5** with 1.7 mL solubilization buffer (*see* **Note 3**), add plant protease inhibitor cocktail and 3.5 µL of 1 M DTT directly to each sample. Incubate for 1 h at 4 °C in a rotary mixer at 5 rpm, avoid air bubbles.
2. During the extraction process, prepare the Co-IP beads according to the supplier's instructions (*see* **Note 19**). Prepare 20 µL bead slurry per sample + 10% (e.g., for 8 samples, 160 µL + 16 µL). Wash the beads by adding 500 µL solubilization buffer and mix gently by snipping the tube. Centrifuge at 2500 × *g*, 4 °C, 2 min and carefully remove the supernatant. Repeat this in total three times. After the last washing step, add 100 µL solubilization buffer per sample + 10% (e.g. for 8 samples, 800 µL + 80 µL).
3. Continue with the plant material. Centrifuge the plant material 60 min, 4 °C, >20,000 × *g* (*see* **Note 20**), if available, use an ultracentrifuge at ~70,000 × *g*. Meanwhile, prepare fresh reaction tubes. For each sample you need two 1.5 mL reaction

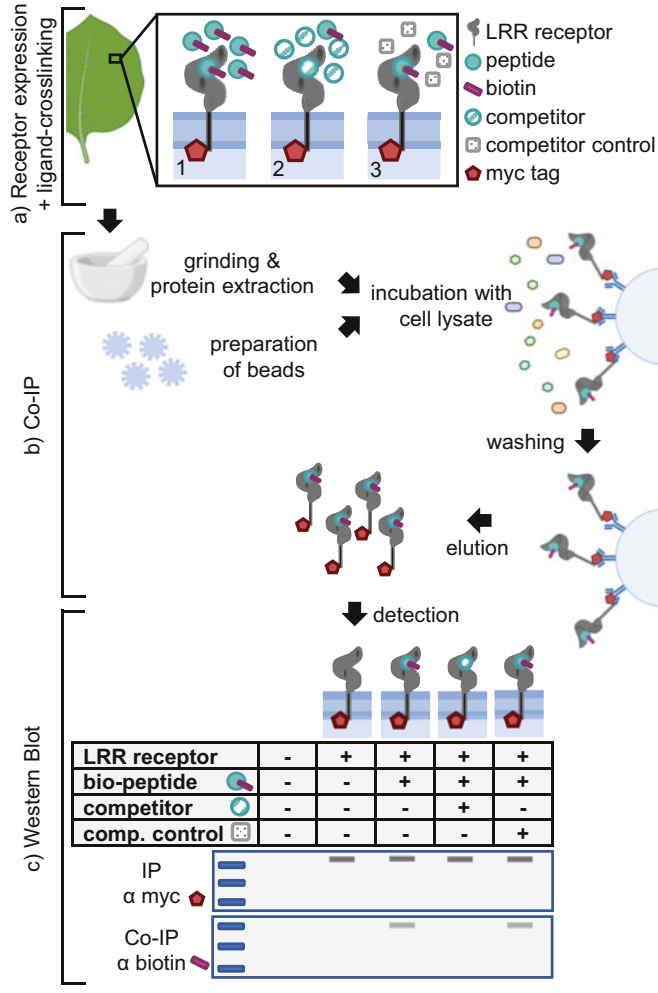


Fig. 2 Scheme of main steps of the in-vivo cross-linking of biotinylated peptides to cell surface receptors. (**a**) Transient receptor expression in *N. benthamiana* leaves. Infiltration of biotinylated peptides, competitor, and competitor control indicated in the black box. Tested combinations are the biotinylated peptide alone (1), the bio-peptide with an excess of unlabeled competitor (2) and the unspecific competitor control (3). (**b**) Illustrates the Co-IP. After harvesting of the plant material, the leaves are ground and proteins are extracted. The cell lysate is incubated with the prepared beads, to bind the tagged receptor. After washing and elution, the tagged receptors and biotinylated peptides are detected on western blot (**c**). The tagged receptor can be detected in all samples except the empty control. The biotinylated peptide can be detected after binding to the receptor in samples (1) and (3), the biotinylated peptide alone and in combination with the competitor control, respectively. No biotinylated peptide can be detected in sample (2) because it is specifically competed out by the unlabeled competitor

tubes, one for the "input" (it will be used later for protein expression control), and one for the following immunoprecipitation (IP). After the centrifugation, transfer the supernatant into the "IP" reaction tube. Then, transfer 40 µL of the supernatant as "input" into the "input" tube. Keep on ice.

4. Mix the prepared bead suspension by carefully pipetting up and down using a cut pipet tip, then add 100 µL bead suspension to each "IP" reaction tube. Distribute any remaining bead suspension drop by drop into the "IP" tubes. Incubate for 1 h at 4 °C in a rotary mixer at 5 rpm.
5. Continue with the "input" samples. Mix each sample with 20 µL 3× SDS loading buffer and boil them for 5 min at 95 °C. Keep at room temperature.
6. Continue with the "IP" samples. Centrifuge at 2500 × *g*, 4 °C, 2 min, discard the supernatant carefully by pipetting. Wash the beads as described in **step 3.3.1.2**, twice with solubilization buffer and twice with wash buffer. Remove as much as possible of the wash buffer from the beads after the last washing step.
7. Add 40 µL 3× SDS loading buffer, mix by snipping and boil the samples for 5 min at 95 °C. Keep at room temperature.
8. Centrifuge all "IP" and "input" samples 3 min at 14,000 × *g* at room temperature. Continue with SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot on the same day.

3.3.2 SDS-Page

You will need to prepare four gels, two for the "IP" samples and two for the "input" samples.

1. To prepare the resolving gel of a 10% SDS polyacrylamide gel (*see* **Note 21**), mix 1.9 mL water, 1.7 mL 30% acrylamide mix, 1.3 mL resolving gel buffer, 50 µL 10% SDS, 50 µL 10% APS and 2 µL TEMED in a 15 mL culture tube and mix gently by overhead turning. Cast the gel and leave space for the stacking gel. Overlay with isopropanol to flatten the gel surface.
2. For the stacking gel, mix 680 µL water, 170 µL 30% acrylamide mix, 130 µL stacking gel buffer, 10 µL 10% SDS, 10 µL 10% APS and 1 µL TEMED in a 15 mL culture tube and mix gently by overhead turning. Remove the isopropanol from the resolving gel by turning the cassette, cast the liquid stacking gel and immediately insert a 10-well comb.
3. Arrange the four gels in the electrophoresis chamber, fill up with 1× SDS running buffer. Load 20 µL of the prepared samples (*see* **step 3.3.1.8**) and the prestained marker proteins. Load two gels with the "input" samples and two gels with the

"IP" samples. Perform electrophoresis at 25 mA per gel until the dye front reaches the bottom of the resolving gel.

3.3.3 Wet Blot Protein Transfer

This procedure is performed for all four gels in parallel.

1. Immediately after electrophoresis, open the glass plates and remove the stacking gel and the dye front with a spatula. Place the gel into a flat container of appropriate size filled with 1× transfer buffer.
2. Soak the nitrocellulose membrane, six pieces of gel blotting paper and two foam pads in 1× transfer buffer. Assemble sandwiches with a foam pad, three pieces of gel blotting paper, gel, nitrocellulose membrane, three pieces of gel blotting paper and a foam pad in the gel holder cassette, the gel facing the minus pole.
3. Assemble two gel holder cassettes in a transfer chamber, fill up the 1× transfer buffer and add one cooling unit (*see* **Note 22**). Run the transfer at 100 V for 60–80 min.

3.3.4 Protein Detection on Western Blot

1. After the protein transfer, disassemble the blotting sandwiches, transfer each membrane in one small plastic container and rinse them once in water.

   From now on, the blots are processed differently for detection of the biotinylated peptide (one "input" and one "IP" blot) as described in procedure 3.3.4.1 and for detection of the tagged receptor (one "input" and one "IP" blot) as described in procedure 3.3.4.2.

Detection of Biotinylated Peptides

1. Block both membranes in 10 mL biotin-free blocking buffer for 1 h on a horizontal shaker.
2. Incubate both membranes with streptavidin-AP diluted 1:1000 in biotin-free blocking buffer overnight at 4 °C.
3. Wash the membranes, twice in washing buffer and twice in 1× assay buffer, each for 5 min.
4. The "IP" is processed first. Place the membrane on foil (e.g., an empty disposal bag), add 500 µL 1× Nitro Block, distribute evenly by covering with a second foil and incubate for 5 min (*see* **Note 23**). Unwrap the blot, wash it for 1–2 min in 1× assay buffer. Place it on foil, add 500 µL 1× CDP-star, distribute evenly by covering with a second foil. To detect chemiluminescence, immediately place the membrane into an imager (e.g., UVP ChemStudio) (Fig. 2c).
5. Develop the "input" blot as described for the "IP" blot in the step above.

Detection of Tagged Receptors

1. Incubate the "input" blot in 10 mL Ponceau A and destain it in water until the bands are clearly visible on a white background (*see* **Note 24**). Wrap the membrane in foil (e.g., an empty disposal bag, or two sheets of cling film) and take a photograph.
2. Unwrap the membrane and wash it in water.
3. Incubate both membranes in 10 mL blocking buffer for 1 h on a horizontal shaker.
4. Incubate both membranes with the primary antibody diluted in 1:5000 in blocking buffer (*see* **Note 25**) overnight at 4 °C.
5. Remove the primary antibody, wash the membranes twice in 1× PBS-T for 5 min.
6. Incubate both membranes with the secondary antibody diluted 1:50000 in blocking buffer (*see* **Note 25**) for 1 h at room temperature on a horizontal shaker.
7. Wash the membranes, twice in 1× PBS-T and twice in 1× assay buffer, each for 5 min.
8. Develop the blots as described in **steps 4** and **5** of Subheading 3.3.4.1.

4 Notes

1. The biotin-labeled peptide is the candidate ligand of the receptor-of-interest. The unlabeled peptide or full-length protein can be used as specific competitor, which is used in 100-fold molar excess to out-compete the biotin-labeled ligand. Any peptide that does not bind to the receptor, ideally a peptide consisting of the same amino acids in scrambled sequence order, can serve as an unspecific competitor control.
2. Different buffers can be used, but avoid buffers with amino groups or any other groups that potentially may influence the cross-linking reaction.
3. You will need 1.7 mL buffer for each 250 mg tissue sample. In case the receptor is only expressed at low levels, you may want to use tissue samples of 500 mg. Keep in mind to increase the buffer volume accordingly. Always prepare fresh extraction buffer directly before usage.
4. Dissolve 1 g in 10 mL water, dispense into 1 mL aliquots. Store at −20 °C.
5. Prepare 40 mL and keep it on the bench; always add DTT freshly to the loading buffer. For more efficient reduction of disulfide bridges replace DTT by β-mercaptoethanol.

6. Prepare 10× buffer by dissolving 30.28 g Tris (free base), 144 g glycine and 10 g SDS in a total of 1 L of water. For the 1× buffer mix 900 mL water with 100 mL 10× buffer. Do not shake it but slightly swivel to avoid bubbles.
7. Instead of methanol, you can use non-denatured ethanol which is less toxic. Prepare 10× buffer by dissolving 30.28 g Tris (free base) and 144 g glycine in a total of 1 L water. For the 1× buffer mix 100 mL 10× buffer with 200 mL methanol or ethanol and 700 mL water.
8. Ponceau S can be reused several times, do not discard after usage.
9. Prepare 10× buffer by dissolving 80 g NaCl, 16.4 g KCl, 14.2 g $Na_2HPO_4 \times H_2O$, and 2.7 g KH_2PO_4 in a total of 1 L of water. For the 1× buffer mix 900 mL water with 100 mL 10× buffer and add 0.5 mL TWEEN 20.
10. It is of high importance to distinguish between biotin-free blocking buffer and standard blocking buffer. The detection of biotin on a membrane with streptavidin-AP requires biotin-free buffers, therefore the membrane should be blocked with biotin-free BSA, otherwise the streptavidin will bind non-specifically to free biotin.
11. pH 9.8 is necessary while working with alkaline phosphatase-conjugated antibody/streptavidin. Prepare 10× buffer by dissolving 24.2 g Tris (free base) and 1.9 g $MgCl_2$ in 900 mL water, adjust the pH with NaOH and fill up with water to 1 L. Dispense into 50 mL aliquots. Store at −20 °C. Add one 50 mL aliquot to 450 mL of water and check and adjust the pH again.
12. 1:10 dilution is necessary to correctly measure the OD since photometers only provide a reliable result up to an OD of 1.
13. Use a plastic syringe without needle for infiltration. Apply gentle pressure to inject the bacterial suspension into the abaxial side of the leaf, supporting the leaf on the opposite side with your index finger.
14. If your lab is particularly cold or dark it is better to keep the plants in the greenhouse under long-day conditions (16 h light/8 h dark, 22 °C).
15. Store EGS at 4 °C in a box with silica gel and place it at room temperature 30 min before use to prevent water draw (hygroscopic effects!) of the powder. The concentration of DMSO may not exceed 1% (v/v) in the cross-linker solution. DMSO is necessary to solve the EGS, but at concentrations too high, it may stress plant cells and membranes. Once dissolved in water, EGS is only stable for a couple of hours! Always prepare freshly.

16. Use one leaf if the area infiltrated with *Agrobacteria* containing the expression construct is rather large, or if the receptor-of-interest is expressed at comparatively high levels. Otherwise use two leaves. Receptor expression levels need to be determined experimentally.
17. Alternatively, it is possible to mix the peptide and EGS solutions and infiltrate them simultaneously. This may help, if the leaves are hard to infiltrate.
18. Make sure that there is no liquid nitrogen left over in the plant powder, otherwise the reaction tube may explode (wear safety glasses)!
19. Always cut the pipet tip when pipetting the beads to minimize shear forces.
20. Centrifuge 45 min 80,000–100,000 × *g* at 4 °C if the sample volume exceeds 2 mL.
21. Prepare the SDS gels whenever it is fitting the time schedule. Either during the Co-IP or the day before and store them in wet paper and in plastic foil at 4 °C. Prepare 10% gels or 8% gels, if your receptor is 140 kDa or more in size. During the assembly of the gel casting cassette, it is important to wear gloves and to clean the glass plates with ethanol prior to use in order to remove any traces of biotin.
22. Depending on the size of the cooling unit you need to exchange it once or twice during the transfer, to prevent heating of the buffer and melting of the gels.
23. We used the Tropix chemiluminescence detection system distributed by Sigma-Aldrich, including Nitro-Block II™ and Tropix® CDP-Star™. For other detection kits, the procedure may have to be modified according to the manufacturer's instructions.
24. This will serve as your loading control, if the same amount of plant material is loaded in every sample. Do not stain the input for the detection of biotin with Ponceau A to prevent biotin contaminations.
25. The 1:5000 dilution applies to the anti-myc antibody that was used in the experiment described here [11]. It may differ for other antibodies. Please follow the manufacturer's instructions.

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Chapter 17

Evaluation of Direct Ligand-Receptor Interactions by Photoaffinity Labeling

Hidefumi Shinohara and Yoshikatsu Matsubayashi

Abstract

Binding assays provide ultimate proof that a particular peptide and receptor kinase (RK) do indeed function as a ligand-receptor pair. Among available binding assays, proximity-induced photoaffinity labeling is superior for confirming direct contact between the peptide ligand and the receptor. Our binding assay employs covalent photoaffinity labeling followed by immunoprecipitation to specifically evaluate the ligand binding activity of the target RKs. Here, we describe a protocol for the synthesis of photoactivatable peptide ligands and the UV-induced formation of covalent bonds between photoaffinity ligands and RKs.

Key words Photoaffinity labeling, Receptor kinase, Peptide ligand, Ligand-receptor interaction

1 Introduction

In general, RKs are functionally divided into two groups. One group consists of ligand-perceiving receptors that are involved in direct ligand binding. The other group consists of co-receptors that do not bind the ligand directly, but heterodimerize with ligand-perceiving receptors to facilitate signal transduction. It is important to define the ligand-perceiving activity of receptors, because the binding of ligands to their receptors is the initial event that triggers ligand-dependent cell-to-cell communication.

Photoaffinity labeling is a classical and reliable biochemical technique for detecting direct ligand-receptor interactions. This method is commonly used for the detection of direct interactions between peptide ligands and their specific receptors, e.g., CLV3-CLV1 [1], CLV3/CLE9-BAM1/2 [2], TDIF-TDR [3], CLE45-SKM1 [4], CEP-CEPR [5], RGF-RGFR [6], AtPep1-PEPR [7], and PSY-PSYR [8]. Our photoaffinity labeling method utilizes photoactivatable ligands derivatized with radiolabeled 4-azidosalicylic acid. Upon UV light irradiation, the azide group of the ligand photolyzes to form a short-lived reactive nitrene,

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which forms a covalent bond with proximal amino acid residues within the receptor proteins. Crosslinked ligand-receptor complexes can be detected by SDS-PAGE and autoradiography.

Here, we describe a protocol for photoaffinity labeling to visualize the direct interaction between peptide ligands and their receptors, using the interaction of PSY6 peptide and its receptor PSYR3 as an example. We include methods on (1) how to prepare the photoactivatable peptide derivatives (Subheading 3.1), (2) how to introduce radioactive iodine into the peptide (Subheading 3.2), and (3) how to detect ligand-receptor interactions using photoaffinity labeling (Subheading 3.3).

2 Materials

2.1 Preparation of Photoactivatable 4-Azidosalicylic Acid (ASA)-Conjugated Peptide

1. [Lys]-substituted and Fmoc-protected peptide derivative (e.g., Fmoc-[Lys^{12}]PSY6) (*see* **Notes 1–3**).
2. 50% (v/v) Acetonitrile.
3. $NaHCO_3$.
4. pH test paper, for alkaline pH.
5. *N*-hydroxysuccinimidyl-4-azido salicylic acid (NHS-ASA).
6. Small orbital shaker for microfuge tubes.
7. Centrifuge evaporator with a vacuum pump.
8. 2× Deprotection solution: 50% (v/v) piperidine, 25% (v/v) acetonitrile in water.
9. Freeze dryer.
10. Centrifuge for 1.5 mL microfuge tubes.
11. HPLC system.
12. Conventional reverse-phase C_{18} HPLC column (4.6 × 150 mm).
13. Solvent A: 0.1% ammonium acetate in HPLC-grade water.
14. Solvent B: 50% (v/v) acetonitrile, 0.1% (v/v) ammonium acetate in HPLC-grade water.

2.2 Radio-Iodination of ASA-Conjugated Peptide

1. 500 mM NaH_2PO_4-NaOH buffer, pH 7.5.
2. 0.4 mM ASA-conjugated peptide solution (from Subheading 3.1).
3. Glove box.
4. 0.5 mM NaI.
5. Na[^{125}I] (PerkinElmer, NEZ033A, 37 MBq) (*see* **Note 4**).
6. 1 mM Chloramine T (*see* **Note 5**).
7. 10% (v/v) Trifluoroacetic acid (TFA).

8. HPLC system.
9. Conventional reverse-phase C_{18} HPLC column (4.6 × 150 mm).
10. Eluent A: 0.1% (v/v) TFA in HPLC-grade water.
11. Eluent B: 0.1% (v/v) TFA in HPLC-grade acetonitrile.
12. Dose calibrator (e.g., AcroBio, CRC-25R).

2.3 Photoaffinity Labeling

1. Membrane preparation (50 μg protein/μL) of plants or cell lines expressing the receptor of interest fused to an affinity tag for immunoprecipitation. Here, we used microsomal fractions prepared from tobacco BY-2 cells expressing HaloTag-fused receptor kinases prepared according to reference [9] (*see* **Note 6**).
2. [^{125}I]ASA-conjugated peptide solution.
3. Binding buffer: 50 mM MES-KOH, pH 5.5, 50 mM sucrose.
4. 0.4 mM Unlabeled peptide solution.
5. Wash buffer: 50 mM MES-KOH, pH 5.5, 500 mM sucrose.
6. Ultracentrifuge.
7. Aspirator.
8. Hand-held UV lamp.
9. Solubilization buffer: 20 mM HEPES-KOH, pH 7.5, 150 mM KCl, 1% (v/v) Triton X-100.
10. Sonicator bath.
11. Microfuge tube rotator.
12. Chamber or refrigerator set at 4 °C.
13. Anti-HaloTag polyclonal antibody (pAB) (e.g., Promega, G9281).
14. ProteinA affinity matrix (e.g., nProteinA-Sepharose, GE Healthcare).
15. 2× SDS sample buffer: 0.1 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 12% (v/v) β-mercaptoethanol, 8% (v/v) glycerol, 0.02% (w/v) bromophenol blue.
16. Heat block set at 95 °C.
17. Precast 7.5% Acrylamide gel for SDS-PAGE.
18. SDS-PAGE running buffer: 25 mM Tris-HCl, pH 8.2, 192 mM glycine, 0.1% SDS.
19. Electrophoresis tank and power supply for SDS-PAGE.
20. Gel dryer.
21. Imaging plate/storage phosphoscreen (e.g., Fujifilm, MS 2025).

22. Imaging plate/storage phosphoscreen cassette.
23. Imaging analyzer/phosphoimager (e.g., Typhoon FLA 9000, GE Healthcare).

3 Methods

3.1 Preparation of Photoactivatable 4-Azidosalicylic Acid (ASA)-Conjugated Peptide

In this section, we describe the introduction of the photoactivatable group to the target peptides using an NHS ester-activated cross-linker. The [Lys]-substituted and Fmoc-protected peptide derivative PSY6 (Fmoc-[Lys^{12}]PSY6) [8] is used as a model for this study (Fig. 1). It takes approximately 2–3 days to complete the entire protocol described in this chapter.

1. Place 4.5 mg of Fmoc-[Lys^{12}]PSY6 peptide (2.4 μmol) into a 1.5-mL microtube.
2. Add 200 μL of 50% acetonitrile and dissolve the peptides.
3. Add 1.0 mg of $NaHCO_3$ and vortex. Check the pH of the solution using a pH test paper. Confirm that the pH is higher than 8 (*see* **Note 7**).

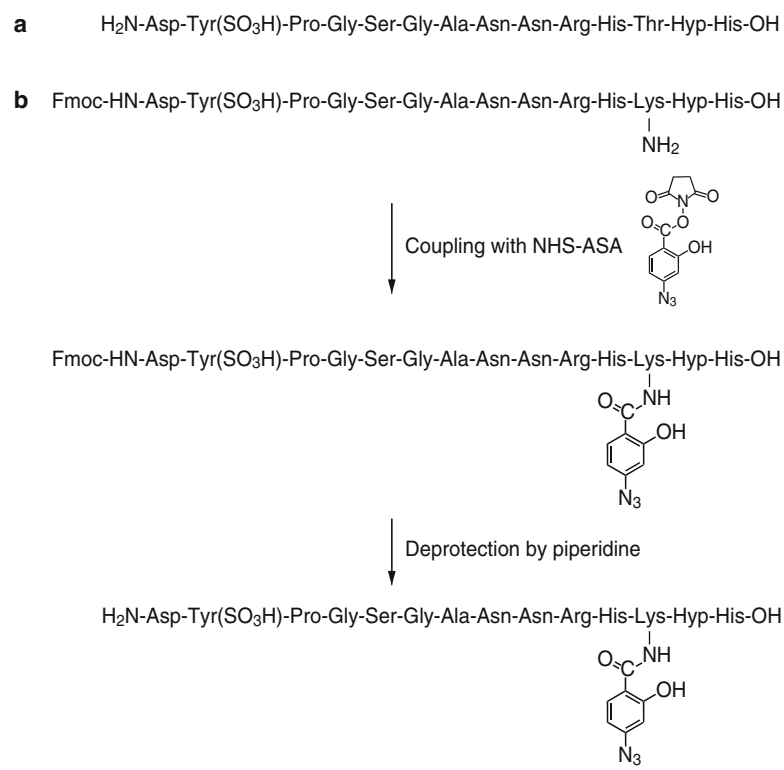


Fig. 1 Schematic outline of the preparation of ASA-PSY6 peptide. (**a**) Structure of PSY6 peptide. (**b**) Stepwise procedure for the synthesis of ASA-PSY6 peptide

4. Add 1.3 mg (4.8 μmol) of *N*-hydroxysuccinimidyl-4-azido salicylic acid (NHS-ASA) into the reaction solution. Vortex and sonicate to dissolve the NHS-ASA powder.
5. Stir for 2 h in dark at room temperature on a small orbital shaker at 800 rpm (*see* **Note 8**).
6. Add 200 μL of 2× deprotection solution and stir for 30 min at room temperature on a small orbital shaker at 800 rpm.
7. Place the reaction tube in a centrifuge evaporator and evaporate to dryness (≈ 1 h).
8. Add 200 μL of water and vortex well to dissolve the ASA-conjugated peptides (*see* **Note 9**).
9. Centrifuge at 15,000 rpm (18,800 × *g*) for 10 min at room temperature. Transfer the supernatant to a new microtube.
10. Set up the HPLC. Equilibrate the reverse-phase C18 HPLC column at 1 mL/min.
11. Load aliquots of the reaction mixture (50 μL) onto the HPLC system.
12. Use a gradient of 20–60% eluant B over 15 min for elution.
13. Collect the peaks containing ASA-conjugated peptides.
14. Repeat **steps 11–13** four times.
15. Pool all collected samples together in a single microtube.
16. Place the sample in a centrifuge evaporator and evaporate the acetonitrile for 30 min.
17. Lyophilize the sample (*see* **Note 10**).
18. Weigh the lyophilized peptide and dissolve in water to a final concentration of 0.4 mM.

3.2 Radio-Iodination of ASA-Conjugated Peptide

In this section, we describe the radio-iodination of ASA-derivatized peptide with ^{125}I by the chloramine T method. The photoactivatable ASA-PSY6 is used as a model for this study (Fig. 2).

1. Mix 5 μL of 500 mM NaH_2PO_4-NaOH buffer, 25 μL of 0.4 mM ASA-peptide, and 5 μL of 0.5 mM NaI solution in a 1.5-ml microtube.
2. Put the reaction mixture in the isolation glove box (*see* **Note 11**).
3. Add 5 μL of Na[^{125}I] (18.5 MBq) and mix.
4. Add 10 μL of 1 mM chloramine T solution.
5. Mix briefly and incubate for 10 min without shaking at room temperature.
6. Add 5 μL of 10% TFA to stop the reaction.

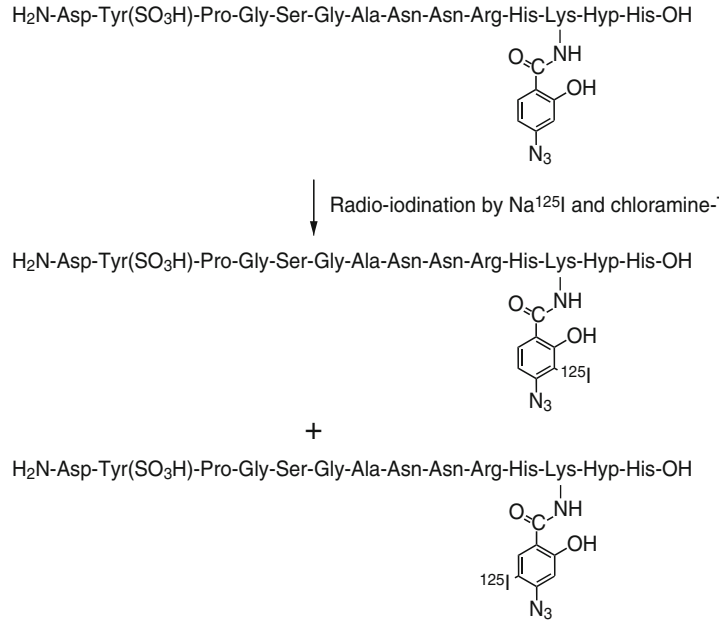


Fig. 2 Schematic outline of the radio-iodination of ASA-PSY6 peptide. Two monoiodinated peptides, *ortho*-[^{125}I]ASA-PSY6 (upper) and *para*-[^{125}I]ASA-PSY6 (lower), are obtained

7. Set up the HPLC. Equilibrate the reverse-phase C18 HPLC column in eluent A at 1 mL/min.
8. Load the reaction mixture onto the reverse-phase C_{18} HPLC column.
9. Use a gradient of 20–50% eluant B over 15 min to elute peptides.
10. Collect the peaks of iodinated peptides (Fig. 3) (*see* **Note 12**).
11. Measure the volume of the collected sample.
12. Measure the radioactivity of the collected sample.
13. Calculate the amounts of iodinated peptides in the collected sample (*see* **Note 13**).
14. Calculate the concentrations and specific radioactivities of the iodinated peptides (*see* **Note 14**).
15. Store at 4 °C (*see* **Note 15**).

3.3 Photoaffinity Labeling

In this section, we describe the protocol for photoaffinity labeling using ^{125}I-labeled photoactivatable peptides. [^{125}I]ASA-PSY6 and its receptor PSYR3 are used as a model for this study.

1. Prepare two microtubes, each with 15 μL (≈ 750 μg) of the microsomal fraction derived from BY-2 cells expressing HaloTag-fused PSYR3 in 250 μL of binding buffer (*see* **Note 6**).

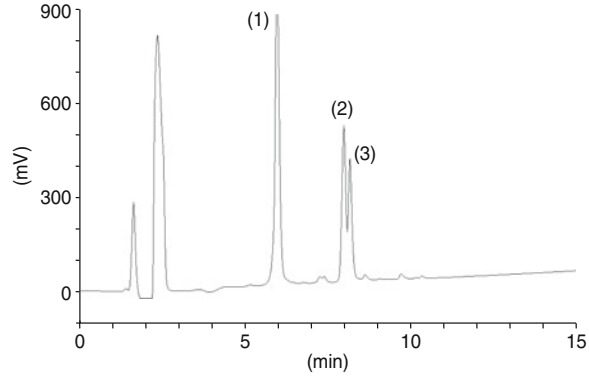


Fig. 3 Purification of iodinated peptides by HPLC on a reverse-phase HPLC column. Two monoiodinated peptides (*ortho* or *para* iodinated) were obtained at a retention time of 8.0 min (peaks 2 and 3). We have not investigated which peak is the *ortho*-substituted product. Peak 1 is chloramine T

2. To the first tube add [^{125}I]ASA-PSY6 solution to a final concentration of 30 nM.
3. To the second tube, for the competition assay, add the [^{125}I] ASA-PSY6 solution and 5.7 μL of 0.4 mM unlabeled peptide solution to a final concentration of 9 μM as the competitor.
4. Mix briefly and incubate on ice for 5 min.
5. Carefully overlay the reaction mixture onto 900 μL of wash buffer (*see* **Note 16**).
6. Centrifuge at 100,000 *g* for 5 min at 4 °C (*see* **Note 17**).
7. Aspirate to remove the supernatant (*see* **Note 18**).
8. Place the tubes sideways on ice.
9. Place the UV lamp at a distance of 1 cm from the tubes and irradiate UV at 365 nm for 10 min.
10. Add 400 μL of solubilization buffer and place in a sonicator bath to dissolve the pellet.
11. Rotate for 20 min at 4 °C.
12. Centrifuge at 100,000 *g* for 20 min at 4 °C.
13. Transfer the supernatant into a new microtube and add 2 μL (≈ 2 μg) of Anti-HaloTag pAb.
14. Rotate for 1 h at 4 °C.
15. Add 30 μL of nProteinA-Sepharose beads.
16. Rotate for 1 h at 4 °C.
17. Centrifuge at 3000 *g* for 5 min at 4 °C.
18. Discard the supernatant.
19. Add 600 μL of solubilization buffer and vortex to wash.

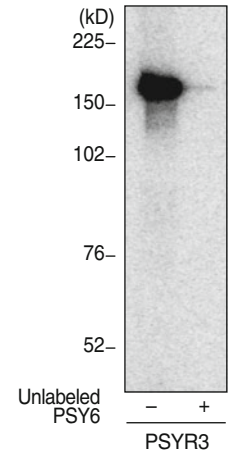


Fig. 4 Photoaffinity labeling of HaloTag-fused PSYR3 by [^{125}I]ASA-PSY6. The binding specificity of [^{125}I]ASA-PSY6 was confirmed by adding a 300-fold excess of unlabeled PSY6 [8]

20. Repeat **steps 17–19** three times.
21. Remove as much supernatant as possible.
22. Add 30 μL of 2× SDS sample buffer.
23. Heat at 95 °C for 5 min.
24. Centrifuge at 3000 *g* for 5 min at 4 °C.
25. Load the supernatant of the sample onto a 7.5% acrylamide SDS-PAGE gel and run under standard conditions.
26. Dry the gel with a gel dryer.
27. Place the dried gel onto an imaging plate (IP).
28. Expose the IP for the desired amount of time (1–3 days) in the cassette.
29. Analyze the IP using the Typhoon FLA 9000 with the storage phosphor-scanning method (Fig. 4).

4 Notes

1. NHS ester-activated labeling reagents react with primary amines (-NH_2) in the peptides. Primary amines exist at the N-terminus of the peptides and in the side chains of lysine (Lys) residues. N-terminal modifications, however, often interfere with the biological functions of the peptides. In such cases, [Lys]-substituted peptide derivatives should be prepared as described below.
2. In order to preserve bioactivity, select a residue that is not conserved among homologs for substitution with Lys.

3. Fmoc protection is required to prevent the reaction of NHS-ASA with the N-terminal α amino group.
4. The half-life of ^{125}I is ≈ 60 days.
5. Fresh chloramine T solution should be prepared for each experiment. Chloramine T powder is unstable and should be stored at −20 °C until use.
6. We usually employ HaloTag as a protein tag [9], but a conventional GFP tag can also be used for immunoprecipitation in combination with appropriate antibodies. Any expression method can be used as long as the protein is functional.
7. Alkaline conditions are important for the reaction of NHS and the primary amine group of Lys in the peptide.
8. ASA is light-sensitive. Avoid direct sunlight when handling solutions containing ASA. Fluorescent light in the room is acceptable.
9. The released Fmoc-protecting group is insoluble in water.
10. Ammonium acetate may not be completely removed in one freeze-drying cycle. In such cases, repeat lyophilization until dry peptide powder is obtained.
11. Perform the following experiments in a glove box. Handle radioactive ^{125}I according to the approved guidelines of your institution.
12. Under this condition, two mono-iodinated (*ortho* or *para* position of the hydroxyl group; Fig. 2) peptides are obtained (peaks (2) and (3) in Fig. 3, respectively). In some cases, these two peptides may have different crosslinking efficiencies for their specific receptors. In photoaffinity labeling experiments, we recommend using both peptides as ligands.
13. The amounts of iodinated peptides are calculated by comparing the peak area of the collected sample with that of a known concentration of non-iodinated ASA-peptides.
14. Under this condition, the specific radioactivity of radio-iodinated peptides is 50 Ci/mmol to 200 Ci/mmol.
15. Do not freeze the sample, because freezing triggers the volatilization of radio-iodine.
16. Since the wash buffer has a higher sucrose concentration, the reaction mixture can be layered on top of the wash buffer. Be careful not to mix the upper and lower layers.
17. The receptor-bound and free [^{125}I]ASA-peptides are separated in this step.
18. Be careful not to leave any droplets on the wall of the tube, as the upper layer contains a high concentration of unbound ligand. This operation can be done with a pipette, but it is easier to do with an aspirator.

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Chapter 18

Rapid Identification of Peptide-Receptor-Coreceptor Complexes in Protoplasts

Xiaoyang Wang and Xiangzong Meng

Abstract

Secreted signaling peptides, also called peptide hormones, play crucial roles in regulating plant growth, development, and immunity. Plant peptide hormones are perceived by plasma membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) that harbor specific extracellular domains to bind and recognize the corresponding peptide ligands. Binding of a peptide ligand to its receptor usually induces the hetero-dimerization of the cognate receptor and a coreceptor, followed by the phosphorylation and activation of the receptor complex to transduce downstream signaling. Therefore, matching peptide ligands with their respective receptors/coreceptors is crucial for elucidating peptide hormone signaling pathways. In this chapter, using the RGF7 peptide-RGI4/RGI5 receptor-BAK1 coreceptor complex as an example, we describe a rapid method to identify the peptide ligand-receptor-coreceptor complexes via co-immunoprecipitation assays using recombinant proteins transiently expressed in Arabidopsis protoplasts.

Key words Signaling peptide, Peptide receptor, Coreceptor, Co-immunoprecipitation, Protoplast transfection, Immuno-blotting

1 Introduction

Communications between cells and between a cell and its environment are critical to plant growth, development, and adaptation to the environment. Secreted peptides (also called peptide hormones) act as short- and long-distance signaling molecules and play crucial roles in plant cell communication [1–4]. The functions of peptide hormones span from the regulation of growth and developmental processes to defense against pathogens and abiotic sensing [1–4]. Plants utilize distinct plasma membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) to perceive various peptide hormones [5–8]. A typical RLK contains a unique extracellular domain, a single transmembrane domain, and a cytoplasmic kinase domain, whereas RLPs lack the kinase domain and possess a short cytoplasmic domain. Specific extracellular domains of RLK-

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and RLP-type receptors recognize and bind the corresponding peptide ligands. Binding of peptide ligand to a RLK-type receptor often induces hetero-dimerization of the receptor and a regulatory RLK (also called coreceptor), usually followed by the phosphorylation and activation of both RLKs to transduce downstream signaling. In contrast, a RLP-type receptor without the kinase domain often associates with a RLK for signal transduction. Similar to RLK-type receptors, the peptide ligand-induced activation of a RLP-RLK receptor complex also usually requires its interaction with another regulatory RLK-type coreceptor [5–8].

Most of the known peptide hormone receptors in plants are RLKs or RLPs harboring extracellular leucine-rich repeat (LRR) domains (i.e., LRR-RLKs or LRR-RLPs) [5–8]. In Arabidopsis, there are more than 200 LRR-RLK and LRR-RLP members [9], many of which function as bona fide receptors for peptide hormones to regulate plant growth, development, and defense responses [5–8]. In contrast to the cognate peptide receptors, a subgroup of LRR-RLKs, known as the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERKs), including SERK1, SERK2, BAK1/SERK3, and BKK1/SERK4, function as shared coreceptors in multiple peptide signaling pathways through hetero-dimerization with distinct LRR-RLK-type receptors or RLP-RLK receptor complexes [10]. Structural studies revealed that the SERK coreceptors are also engaged in peptide ligand binding during their dimerization with cognate receptors upon ligand perception [11]. In addition to SERKs, another subgroup of LRR-RLKs, designated as CLAVATA3 INSENSITIVE RECEPTOR KINASES (CIKs), including CIK1 to CIK6, were recently shown to also function as coreceptors for several LRR-RLK-type receptors to mediate the perception of CLAVATA3/EMBRYO SURROUNDING REGION (CLE) family peptides [12–14].

Given the large number of peptide ligands, RLKs, and RLPs in plant genomes [15, 16], an enormous number of peptide-receptor/coreceptor complexes are expected to exist. Matching peptide ligands with their respective receptors/coreceptors is crucial for elucidating peptide hormone signaling pathways. Several genetic, physiological, biochemical, or structural biological approaches have been used to match peptide ligands with their receptor/coreceptors [3, 17]. However, most of these approaches are time-consuming. Additionally, since most peptide hormones are processed and modified from nonfunctional prepropeptide precursors to produce mature functional peptides [2, 3, 18, 19], some approaches used to match peptide-receptor pairs rely on the identification and chemical synthesis of mature peptides [3, 17]. In this chapter, using the RGF7 peptide-RGI4/RGI5 receptor-BAK1 coreceptor complex as an example [20], we describe a rapid method without the use of synthetic mature peptides to identify the peptide

ligand-receptor-coreceptor complexes via co-immunoprecipitation (Co-IP) assays using recombinant proteins transiently expressed in Arabidopsis protoplasts.

2 Materials

2.1 Protoplast Expression Plasmids for Peptide Precursor, Receptor, and Coreceptor

The expression constructs to be used depend on the specific research question. In the experiment described, we used expression constructs for the RGF7 precursor, the RGI4 and RGI5 receptors, and the BAK1 coreceptor [20]. To generate these constructs, the coding sequences of *preRGF7*, *RGI4*, *RGI5,* and *BAK1* were PCR-amplified from Arabidopsis cDNA, then were cloned into the protoplast expression vectors *pHBT-35S:GFP*, *pHBT-35S:FLAG,* or *pHBT-35S:2HA* to generate the protoplast expression plasmids *35S:preRGF7-GFP, 35S:RGI4-FLAG, 35S:RGI4-2HA, 35S:RGI5-FLAG, 35S:RGI5-2HA,* and *35S:BAK1-FLAG* used for protoplast transfection (*see* **Note 1**).

2.2 Protoplast Isolation and Transfection

1. Plant material: *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) seeds were sown in soil and grown at 22 °C in a growth chamber with 12 h-light (60 μE m^{-2} s^{-1})/12 h-dark cycles and 65% humidity for 4 weeks (*see* **Note 2**).
2. Enzyme solution: 1.5% (w/v) cellulase R10, 0.4% (w/v) macerozyme R10, 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 10 mM $CaCl_2$ (*see* **Note 3**).
3. 90 × 15 mm Petri dish (for 10 mL enzyme solution).
4. Razor blade.
5. Vacuum desiccator.
6. Nylon mesh (with a mesh pore size of 75 μm).
7. 15-mL and 30-mL round-bottom centrifuge tubes.
8. Hemacytometer.
9. Light microscope.
10. Swinging-bucket centrifuge.
11. W5 solution: 154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$, 2 mM MES, pH 5.7.
12. WI solution: 0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7.
13. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7.
14. PEG transfection solution: 40% (w/v) polyethylene glycol (PEG), 0.2 M mannitol, 100 mM $CaCl_2$ (*see* **Note 4**).
15. 6-well cell culture dishes.

2.3 Co-IP

1. Protein extraction buffer: 10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_3, protease inhibitor cocktail (Thermo Scientific, A32965, 1 tablet for 50 mL) (*see* **Note 5**).
2. Anti-FLAG M2 Affinity Gel (Sigma, A2220).
3. GFP-Trap Agarose (ChromoTek, gta-20).
4. Co-IP washing buffer: 10 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100.
5. 50 mM Tris-HCl, pH 7.5.
6. Rotating wheel shaker for microfuge tubes.

2.4 Immunoblotting

1. 4× protein loading buffer: 0.25 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 0.1% (w/v) bromophenol blue, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol (added just before use).
2. Prestained protein standard.
3. Bis-Tris Precast Protein Gels (4–12% gradient, e.g. Tanon, 180-9215H) with matching electrophoresis system (e.g., Tanon VE180).
4. SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.0.
5. Immun-Blot PVDF Membrane.
6. Transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
7. Semi-Dry Electrophoretic Transfer Cell.
8. TBST buffer: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20.
9. Blocking and antibody incubation buffer: 5% (w/v) skim milk in TBST buffer.
10. Antibodies: anti-HA-horseradish peroxidase (HRP) (Roche, 12013819001), anti-FLAG-HRP (Sigma-Aldrich, A8592), anti-GFP (Roche, 11814460001), goat anti-mouse IgG-HRP (Abmart, M21001).
11. Rocking shaker.
12. Enhanced chemiluminescence (ECL) Western blotting substrate.
13. Chemiluminescence Imaging System.

3 Methods

3.1 Protoplast Isolation [21, 22]

1. Prepare fresh enzyme solution and put it into a 90 × 15 mm Petri dish.
2. Cut the middle part of well-expanded leaves (usually the 5–7th true leaves) into 0.5–1 mm strips using a razor blade (*see* **Note 6**).
3. Transfer leaf strips immediately and gently into the enzyme solution using a blunt-ended forceps (*see* **Note 7**).
4. Cover the Petri dish with aluminum foil and vacuum-infiltrate the enzyme solution at 200 mbar for 30 min using a vacuum desiccator.
5. Slowly release the vacuum and continue the enzyme digestion for another 2.5 h in the dark at room temperature (*see* **Note 8**).
6. Gently shake the Petri dish by hand to release protoplasts into the solution (*see* **Note 9**), add an equal volume of W5 solution to dilute the protoplast solution, then filter through a 75-μm nylon mesh into a 30-mL round-bottom tube.
7. Centrifuge the protoplast solution at 1000 g for 2 min to pellet the protoplasts with a swinging-bucket centrifuge (*see* **Note 10**), then remove as much of the supernatant as possible.
8. Add 10 mL W5 solution to the tube and gently resuspend the protoplast pellet.
9. Pellet the protoplasts again by centrifugation at 1000 g for 1 min, then remove as much of the supernatant as possible.
10. Resuspend the protoplasts with MMG solution and count them using a hemocytometer and an optical microscope, then adjust the protoplasts in MMg solution to a density of 2×10^5 cells/mL.

3.2 Protoplast Transfection [21, 22]

1. Prepare fresh PEG transfection solution and high-quality plasmid DNA at 1.5 μg/μL (*see* **Note 11**).
2. To test the interaction between the RGF7 peptide ligand and the RGI4 or RGI5 receptor, mix 50 μL of *35S:preRGF7-GFP* plasmids with 50 μL of *35S:RGI4-FLAG* or *35S:RGI5-FLAG* plasmids in a 15-mL round-bottom centrifuge tube (*see* **Note 12**). To test the RGF7 peptide-induced interaction between the RGI4/RGI5 receptors and the BAK1 coreceptor, mix 30 μL of *35S:preRGF7-GFP*, 30 μL of *35S:BAK1-FLAG*, and 40 μL of *35S:RGI4-2HA* or *35S:RGI5-2HA* plasmids in 15-mL tubes. Use *pHBT-35S* empty vector plasmids as controls.
3. Add 1 mL of protoplasts in MMg solution into each tube, and gently mix the protoplasts with plasmid DNA.

4. Immediately add an equal volume (1.1 mL) of PEG transfection solution, then mix gently and thoroughly.
5. Incubate the transfection mixtures at room temperature for 6–8 min.
6. Add 7 mL of W5 solution and mix well by gently inverting to stop the transfection.
7. Pellet the protoplasts by centrifugation at 1000 g for 1 min, then remove the supernatant.
8. Resuspend the protoplasts gently with 1 mL of WI solution, then transfer the protoplasts into a 6-well cell culture dish.
9. Incubate the protoplasts for 12 h at room temperature under low light (~30 μE m^{-2} s^{-1}).
10. Harvest the protoplasts in 1.5 mL tubes by centrifugation at 1000 g for 1 min and remove the supernatant.
11. Immediately freeze the transfected protoplast samples with liquid nitrogen and store them at −80 °C until further analysis.

3.3 Co-IP

1. Put the frozen protoplast samples on ice, resuspend each sample with 500 μL protein extraction buffer by vigorous vortexing (*see* **Note 13**), then incubate the samples on ice for 5 min to lyse the protoplasts.
2. Centrifuge the extracts at 13,000 g for 10 min at 4 °C, then transfer the supernatant to new 1.5-mL tubes.
3. Transfer 30 μL of each extract into a new tube as input sample, add 10 μL of 4× protein loading buffer, then heat the input samples for 5 min at 95 °C and store them at −20 °C.
4. For IP samples, wash the Anti-FLAG M2 Affinity Gel and GFP-Trap Agarose twice with 1 mL of protein extraction buffer for each washing.
5. Aliquot equal amounts of pre-washed Anti-FLAG M2 Affinity Gel or GFP-Trap Agarose (5 μL gel/agarose per 500 μL extract) into the corresponding protoplast extracts as indicated in Fig. 1, then incubate the samples at 4 °C for 2 h on a rotating wheel (*see* **Note 14**).
6. Pellet the gels/agaroses by centrifugation at 5000 g for 1 min, then remove the supernatants.
7. Wash the gels/agaroses four times with 1 mL of protein extraction buffer for each washing.
8. Wash the gels/agaroses one time with 1 mL of 50 mM Tris-HCl, pH 7.5.
9. Pellet the gels/agaroses by centrifugation at 5000 g for 1 min, then remove the supernatants.

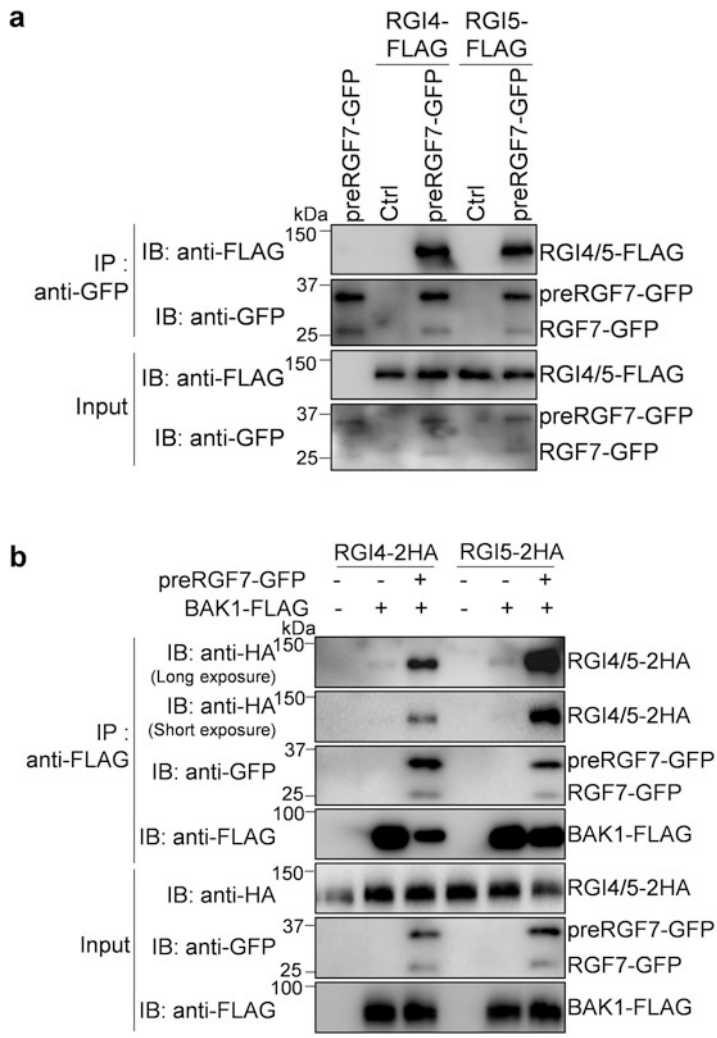


Fig. 1 Identification of the RGF7-RGI4/RGI5-BAK1 complex via Co-IP assays in Arabidopsis protoplasts. (**a**) The RGF7 peptide interacts with RGI4 and RGI5 receptors in Arabidopsis protoplasts. The RGF7 precursor (preRGF7)-GFP fusion protein was co-expressed with the fusion protein RGI4-FLAG or RGI5-FLAG in Arabidopsis protoplasts. The proteins immunoprecipitated from protoplast extracts using GFP-Trap Agarose (IP: anti-GFP) were analyzed by immunoblotting with anti-GFP (IB: anti-GFP) or anti-FLAG antibody (IB: anti-FLAG) (top two panels). The protein inputs were shown by immunoblotting with the indicated antibodies (bottom two panels). Ctrl, vector control. (**b**) The RGF7 peptide induces the interaction between the RGI4/RGI5 receptors and the BAK1 coreceptor in Arabidopsis protoplasts. Co-IP results also indicate that the RGF7 peptide interacts with the BAK1 coreceptor. Different combinations of preRGF7-GFP, RGI4/RGI5-2HA, and BAK1-FLAG fusion proteins as indicated were co-expressed in Arabidopsis protoplasts. The proteins immunoprecipitated from protoplast extracts using Anti-FLAG M2 Affinity Gel (IP: anti-FLAG) were analyzed by immunoblotting with anti-HA (IB: anti-HA), anti-GFP (IB: anti-GFP) or anti-FLAG antibody (IB: anti-FLAG) (top four panels; the top two panels show different exposure times of the same blot). The protein inputs were analyzed by

10. Add 30 μL of 1× protein loading buffer to each IP sample, then heat the IP samples for 5 min at 95 °C and store them at −20 °C for immunoblotting analyses.

3.4 Immunoblotting

1. Dissolve and mix the input and IP samples, spin down the samples briefly.
2. Load 10 μL of each sample to Bis-Tris Precast Protein Gels, and separate the total proteins by SDS-PAGE.
3. Electrotransfer the proteins from the gels to PVDF membranes using a semi-dry electrophoretic transfer cell.
4. Incubate the membranes in the blocking buffer with gentle shaking for 1 h at room temperature.
5. Incubate the membranes in the blocking buffer containing the corresponding primary antibodies (diluted 1:2000) as indicated in Fig. 1 for 2 h at room temperature under gentle shaking.
6. Wash the membranes three times for 10 min each with TBST buffer at room temperature.
7. Incubate the membranes with anti-GFP as primary antibody in the blocking buffer containing the second antibody (goat anti-mouse IgG-HRP, diluted 1:8000) for 1 h at 4 °C under gentle shaking.
8. Wash the membranes three times for 10 min each with TBST buffer at room temperature.
9. Proceed detection of immunoblotting signals with ECL reagent and a chemiluminescence imaging system. The Co-IP result indicating the interaction between the RGF7 peptide and the RGI4/RGI5 receptors is shown in Fig. 1a. Figure 1b shows Co-IP results indicating the RGF7 peptide-induced interaction between the RGI4/RGI5 receptors and the BAK1 coreceptor. Results in Fig. 1b also indicate the interaction between the RGF7 peptide and the BAK1 coreceptor.

4 Notes

1. The *pHBT-35S:GFP*, *pHBT-35S:FLAG,* and *pHBT-35S:2HA* vectors are protoplast transient expression vectors with the backbone of pUC18, which carry GFP, FLAG, or 2HA tag under the control of CaMV 35S promoter and NOS terminator.

Fig. 1 (continued) immunoblotting with the indicated antibodies (bottom three panels).. (This figure was originally published in the New Phytologist. Wang et al. [20]. © 1999–2022 John Wiley & Sons, Inc)

2. Healthy Arabidopsis plants grown at the proper conditions are critical for protoplast isolation. Protoplasts isolated from stressed leaves usually show lower transfection efficiency.
3. Firstly, the enzyme solution without $CaCl_2$ should be made and heated at 55 °C for 10 min to inactivate proteases and enhance enzyme solubility. Then, 10 mM $CaCl_2$ is added into the enzyme solution after it is cooled to room temperature. The enzyme solution should be freshly prepared.
4. The PEG transfection solution should be prepared freshly. Freshly prepared PEG solution shows better protoplast transfection efficiency.
5. The DTT, NaF, Na_3VO_3, and protease inhibitors should be added into the protein extraction buffer just before use.
6. Use a sharp razor blade to quickly cut leaves on a piece of clean white paper. Avoid crushing the tissue at the cutting site.
7. Submerge leaf strips completely in the enzyme solution to increase the enzyme digestion efficiency. It doesn't matter whether leaf strips face up or down.
8. The enzyme digestion time will be different for different plant species. Therefore, digestion time should be tested and optimized for plant species other than Arabidopsis.
9. After the release of protoplasts, most of the leaf strips will be digested away, and the enzyme solution becomes green.
10. Centrifugation at a higher speed may break the protoplasts due to excessive pressure.
11. Purity and quality of the plasmid DNA are critical for successful protoplast transfection. Low-quality plasmid DNA may decrease the viability of protoplasts and lead to transfection failure. We usually isolate plasmids using the large-scale alkaline lysis method, then purify plasmid DNA from contaminating RNA and protein via CsCl/ethidium bromide density gradient centrifugation to prepare high-quality plasmid DNA as previously described [23].
12. Usually, a maximum of 200 μg plasmid DNA can be used for the transfection of 1 mL protoplasts (2×10^5 protoplasts/mL). When a mixture of more than two plasmids is used, the ratio of the added plasmids can be adjusted based on the protein expression levels derived from different plasmids.
13. Usually, 500 μL protein extraction buffer is used for protein extraction from 2×10^5 protoplasts.
14. Make sure the gel/agarose slurries are thoroughly resuspended before adding to different samples using a tip with its end cut off. 5 μL gels/agaroses for each sample is appropriate. Adding excessive gels/agaroses may cause non-specific protein binding

and may therefore increase the non-specific background in the Co-IP analyses.

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Chapter 19

Acridinium-Based Chemiluminescent Receptor-Ligand Binding Assay for Protein/Peptide Hormones

André Guilherme Daubermann, Keini Dressano, Paulo Henrique de Oliveira Ceciliato, and Daniel S. Moura

Abstract

Chemiluminescent acridinium esters (AE) have been extensively used for oligonucleotide probing and peptide-binding assays in molecular research due to labeling efficiency, lack of radioactivity, and ease of application. In addition to being a powerful and reliable alternative to radiolabeling, AE can be directly bound to the target molecule, with high specificity. Here, we describe an AE-based protein/peptide labeling method and the use of the labeled protein/peptide in a ligand-binding assay.

Key words Chemiluminescence, Peptide labeling, Peptide purification, Binding assay, Ca^{2+} mobilization assay, Aequorin, Receptor-ligand interaction, HPLC purification

1 Introduction

Many biological processes are controlled by protein or peptide hormones. These proteins/peptides trigger signaling cascades usually mediated by cell membrane receptors. Unveiling the interaction of specific molecules with their receptors through receptor-ligand binding assays conventionally required the use of radioligands containing radioisotopes such as iodine-125 (^{125}I) [1–3]. However, current methods use bioluminescence, chemiluminescence, or fluorescence as alternative detection strategies and molecules that are safer, more stable, and highly sensitive [4–7]. Chemiluminescence relies on light emission during chemical reactions without the need for a catalyst, unlike bioluminescence. Molecules that undergo chemiluminescent reactions are of great interest, as they may be used as receptor-ligand interaction detectors. In ligand-binding assays, for instance, such molecules are used instead of the conventional radiolabeled molecules, which have been extensively used in ligand-binding assays [1–3, 8–11]. Of these chemiluminescent molecules, AEs, which undergo

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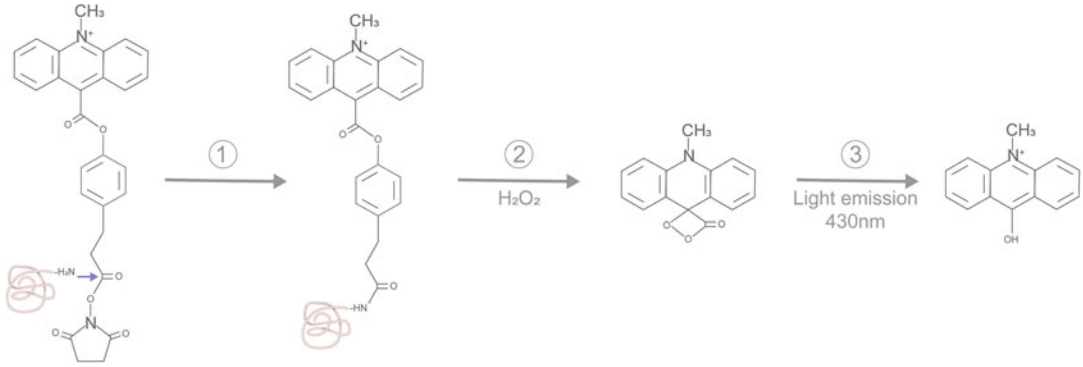


Fig. 1 The addition of hydrogen peroxide induces light emission at 430 nm from acridinium-labeled proteins/peptides. The acridinium NHS ester is amine-reactive and thus will bind to any primary amino group on proteins/peptides (1). Addition of the trigger solution (2) will result in the formation of a highly strained dioxetane intermediate, that upon elimination of CO_2 and emission of light (430 nm) converts to the acridinone (3)

chemiluminescent reactions when exposed to hydrogen peroxide (Fig. 1), have been used in receptor-ligand binding assays since 1994 [1, 3, 12]. In plants, chemiluminescent acridinium NHS ester has been successfully applied in peptide binding assays [13–18].

In this chapter, we present a step-by-step protocol for the preparation and application of AEs in a peptide-ligand binding assay (Fig. 2). The first step includes the peptide labeling reactions, purification of the peptide, separation of the labeled fraction, and quantification (Subheading 3, methods 3.1–3.3). The second step comprises an initial activity assay, which is employed to verify whether the AE-labeled peptide is as active as the unlabeled control (3.4), and the ligand-binding assay itself (3.5). The AtRALF1 and AtRALF34 peptides have been AE-labeled in previous research for binding assays [18, 19]. The AtRALF1 peptide is used in this chapter as successful example.

2 Material

All the procedures described in the acridinium labeling steps (Subheading 3.1) follow the Acridinium Labelling kit (Cayman Chemical, Michigan, USA, 200201) supplier's instructions, with optimization for peptide labeling and purification as detailed below. Other acridinium-based labeling kits, such as the Chemiluminescent Labelling kit (Enzo Chemical, ADI-907-001), may have different procedures and may or may not contain all the labeling reagents.

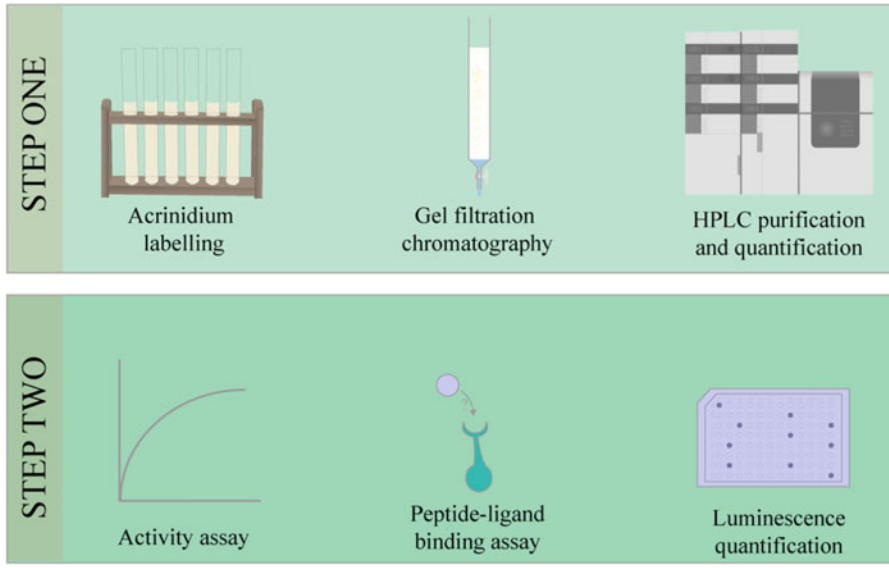


Fig. 2 Scheme representing the overall methodology for acridinium-based peptide-ligand binding assay. In this scheme we divided the whole assay into two major steps. The first step includes the peptide labeling with acridinium NHS ester, desalting through gel filtration chromatography, and further purification and quantification in HPLC. The second step (step two) includes an activity assay, which aims to confirm whether the labeled peptide kept its activity, as well as the peptide-ligand binding assay and the luminescence quantification

2.1 Acridinium Labeling

2.1.1 Equipment

1. Room temperature shaker.
2. pH meter.
3. Desiccator.

2.1.2 Reagents and Solutions

1. 5 mM acridinium NHS ester (Solution I): weigh 1 mg of the acridinium NHS ester, dilute it in 316 μL anhydrous dimethylformamide, and store the stock solution at −20 °C in a desiccator.
2. Acridinium working solution (Solution II): dilute Solution I in a 1:9 ratio. Add 9 μL of Solution I to 81 μL of anhydrous dimethylformamide in a small test tube.
3. Acridinium labeling solution (Solution III): 0.1 M phosphate buffer, pH 6.3, 0.15 M NaCl (*see* **Note 1**).
4. Quenching solution (Solution IV): 0.1 M phosphate buffer, pH 6.3, 0.15 M NaCl, 1% glycine. The stock solution should be stored at 4 °C.

2.2 Desalting Column and Peptide Detection

2.2.1 Equipment

1. Nanodrop Spectrophotometer.

2.2.2 Consumables

1. Glass or plastic column (*see* **Note 2**).
2. 2 mL test tubes.

2.2.3 Reagents and Solutions

1. Desalting column: syringe packed with Sephadex G25-80. (*see* **Note 3**).
2. Column equilibration solution (Solution V): 0.1% (v/v) formic acid in ultrapure water.
3. Purification buffer (Solution VI): 1 M phosphate buffer, pH 6.3, 1% BSA, 1.5 M NaCl, 0.1% sodium azide. Dilute the purification buffer 1:10 (purification buffer: UltraPure water). Store the solution stock at 4 °C (*see* **Note 4**).

2.3 HPLC Purification

2.3.1 Equipment

1. High-performance liquid chromatography (HPLC) system equipped with two pumps and a photodiode array detector (PDA).
2. C18 Column: reversed-phase C18 column, 250 mm long, 4.6-mm diameter, 5 μm particle size, 100 Å-spherical silica (e.g., Kromasil®, Nouryon, Sweden).
3. C18 reversed-phase Narrow-bore column, 150 mm long, 2 mm diameter, 5 μm particle size, 100 Å-spherical silica (e.g., Nucleosil, Machery-Nagel, Duren, Germany).
4. Vacuum pump.
5. Ultrasonic bath sonicator.
6. All-Glass Filter Holder – Kit (Merck Millipore).
7. Lyophilizer.
8. Glass material (e.g., beakers, graduated cylinders, solvent bottles).

2.3.2 Consumables

1. Cellulose nitrate membrane filter (0.45 μm).
2. Regenerated cellulose membrane filter (0.45 μm).
3. 1 mL syringe.
4. Hypodermic needle, blunt tip, 22 ga, 51 mm length.
5. 10 mL test tubes.

2.3.3 Reagent and Solutions

1. Solvent A: 1 mL formic acid in 1 L ultrapure water. Formic acid must be HPLC grade. The solvent must be vacuum-filtered (All-Glass apparatus) over a 0.45 μm regenerated cellulose membrane filter and degassed for 15 min in a sonicator bath with occasional shaking.
2. Solvent B: 1 mL formic acid in 1 L acetonitrile. All reagents must be HPLC grade, vacuum-filtered (All-Glass apparatus) over a 0.45 μm cellulose nitrate filter and degassed for 15 min in a sonicator bath with occasional shaking.

2.4 Ca^{2+} Mobilization Activity Assay

1. Arabidopsis seeds, genotype p35S:aeq [20].

2.4.1 Plant Material

2.4.2 Equipment

1. Microplate reader (Biotek ELx800).
2. Laminar flow hood.
3. Growth cabinet set to 24 °C.

2.4.3 Consumables

1. 96-well plates.
2. White filter paper.
3. Sterile Petri dishes, 90 mm diameter, 15.9 mm height.

2.4.4 Reagents and Solutions

1. Sterilization solution: 50% (v/v) sodium hypochlorite, 0.01% Tween X-80 in sterile distilled water.
2. Liquid culture medium: Half-strength Murashige and Skoog medium [21] (0.5 × MS) without sucrose or vitamins. Adjust pH to 5.6 with KOH and autoclave.
3. Semi-solid culture medium: Half-strength Murashige and Skoog medium (0.5 × MS), without sucrose or vitamins, and 0.4% (w/v) agar. Adjust pH to 5.6 with KOH and autoclave.
4. 2.5 mM coelenterazine.

2.5 Peptide-Ligand Binding Assay

1. Five-day-old Arabidopsis seedlings (mutants and/or transgenic lines can be used) grown in semi-solid culture medium.

2.5.1 Plant Material

2.5.2 Equipment

1. Microplate reader (Biotek ELx800).
2. pH meter.
3. Temperature-controlled shaker (4 °C).

2.5.3 Consumables

1. 24-well plates.

2.5.4 Reagents and Solutions

1. Acridinium-labeled peptide of interest.
2. Unlabeled peptide of interest.
3. Trigger solution: 20 mM hydrogen peroxide (H_2O_2), 0.1 M NaOH. Stock solutions of both hydrogen peroxide and sodium hydroxide should be stored at 4 °C (*see* **Note 5**).

3 Methods

The procedure described below was used to label AtRALF peptides. Other peptides/proteins with distinct size, isoelectric point, stability, etc., might require optimization.

3.1 Labeling of AtRALF1 with Acridinium

1. Peptide dilution: in a small test tube, dilute 150 μg of AtRALF1 in 300 μL of the labeling buffer (Solution III) (*see* **Note 6**).
2. Peptide labeling reaction: add 9 μL of the acridinium working solution (Solution II) to the peptide solution (*see* **Note 7**).
3. Incubate with gentle agitation at room temperature for 30 min.
4. Reaction quenching: add 100 μL of the quenching solution (Solution IV) (*see* **Note 8**).
5. Incubate with gentle agitation for another 30 min at room temperature.

3.2 Desalting and Detection

According to the supplier's instruction, after proceeding with the labeling of the peptide/protein, the labeled peptide/protein should be desalted and the eluant collected in test tubes. Alternatively, we directly purified the labeling reaction by HPLC, eliminating the desalting step.

1. Desalting: apply the labeled protein to the top of the Sephadex G25–80 column washed and equilibrated in Solution V. Collect 1 mL of the eluant in a test tube.
2. Column wash: repeatedly add 1 mL of the purification buffer (Solution VI) and collect the eluant in individual test tubes (1 mL in each tube). Repeat this procedure 8–10 times to ensure that all protein/peptide has eluted from the column.
3. Protein detection: measure the absorbance at 220 nm of all fractions collected in a Nanodrop spectrophotometer. A single peak is observed and pooled fractions, typically three, are further HPLC purified (*see* **Note 9**).

3.3 Peptide Purification and Quantification

If the labeled peptide is desalted using the Sephadex G25-80 column, subsequent HPLC purification will enable the separation of the labeled and the unlabeled protein/peptide of interest. If the desalting step is omitted, HPLC purification will desalt and separate labeled from unlabeled peptides.

1. Equilibrate the C18 column with Solvent A at a flow rate of 1 mL/min.
2. Load the fractions collected from the desalting column onto the C18 column (*see* **Note 10**).

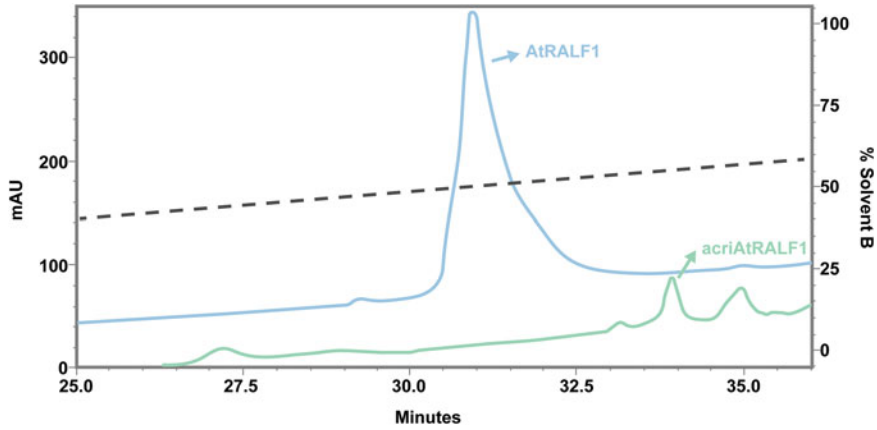


Fig. 3 HPLC profile of the unlabeled (AtRALF1) and the acridinium-labeled (acriRALF1) peptides. %acetonirile: percentage of acetonitrile. mAU: milli-absorbance unit at 220 nm. Arrows indicate the unlabeled (AtRALF1) or acridinium-labeled (acriRALF1) peptides. (Modified and adapted from Campos et al. [18])

3. Elute the labeled peptide fraction in a 60-min gradient ranging from 0 to 40% Solvent B. Labeled and unlabeled peptides were distinguished based on their retention time. Both were monitored using absorbance at 220 nm (Fig. 3). Alternatively, the spectral absorbance profile generated by the photodiode array may be used to differentiate both labeled and unlabeled peptides.
4. Select the fraction containing the labeled peptide (Fig. 3) and concentrate it by lyophilizing (*see* **Note 11**).
5. The working solution is prepared by dissolving the lyophilized peptide in water. Quantification is performed in a C18 narrow-bore column. The column is equilibrated with Solvent A at a flow rate of 0.2 mL/min. Load an aliquot onto the C18 narrow-bore column and elute the labeled AtRALF1 using a 60-min gradient ranging from 0% to 100% Solvent B. Elution is monitored at 220 nm (*see* **Note 12**).

3.4 Activity Assay

As the peptide AtRALF1 induces changes in cytoplasmic Ca^{2+} levels, a Ca^{2+} mobilization assay was performed to test whether the labeling process affected AtRALF1 activity. The test for AtRALF1-induced increase in cytoplasmic Ca^{2+} was performed in Arabidopsis seedlings expressing the bioluminescent calcium sensor aequorin (p35S:aeq). The overall activity of the acridinium-labeled AtRALF1 was like the unlabeled control peptides (Fig. 4).

1. Surface-sterilize p35S:aeq Arabidopsis seeds with the sterilization solution for 20 min and wash the seeds with sterilized ultrapure water in a laminar flow cabinet, as all the subsequent steps are done under sterile conditions.

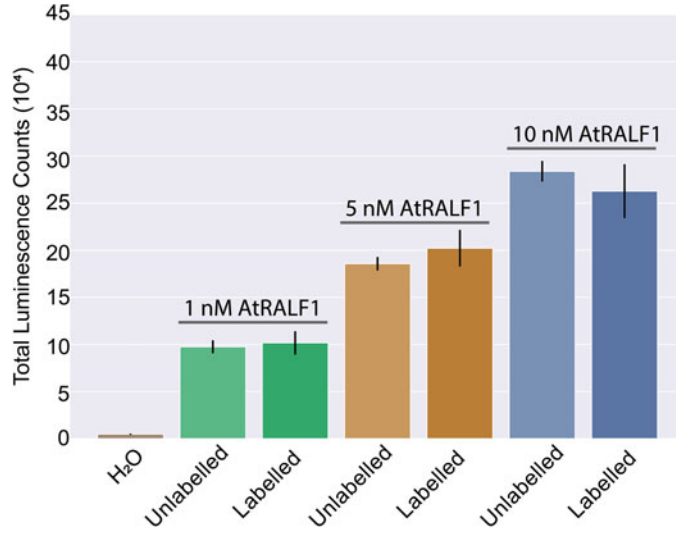


Fig. 4 Evaluation of Ca^{2+} mobilization induced by labeled or unlabeled AtRALF1. Concentrations of the unlabeled AtRALF1 ranging from 1 nM to 10 nM were used as control. The same concentrations were used for the labeled AtRALF1 (acriRALF1). (Modified and adapted from Campos et al. [18])

2. Stratify p35S:aeq Arabidopsis seeds for 72 h at 4 °C and then allow it to germinate in plates containing liquid culture medium.
3. Seedling incubation: transfer four-day-old p35S:aeq Arabidopsis seedlings to 96-well microplates (one seedling per well) containing liquid culture medium with 2.5 mM coelenterazine.
4. Incubate the 96-well microplate with the seedlings in the dark at 24 °C for 16 h.
5. Add 1, 5, or 10 nM of the labeled or unlabeled peptide to each well of the microplate. Use an equal volume of water as control.
6. Measure the resulting luminescence in a microplate reader for 160 s in a total of 20 measures (Fig. 4).

3.5 Peptide-Ligand Binding Assay

Receptor binding of peptide-ligands can be assayed in whole seedlings, specific organs, or microsomal fractions. We successfully applied acridinium-labeled AtRALF1 (acriRALF1) in an assay using whole seedlings. Wild-type and control seedlings (Col-0 and *bak7*, respectively) and four different mutants lacking the AtRALF1 receptors BAK1 and FER [19, 22] (Col-0, *bak1–1*, *bak1–3*, *bak1–4*, and *fer4*) were incubated with acriRALF1. All RALF-receptor mutants showed lower acriRALF1 binding when compared to controls (Col-0 and *bak7*) (Fig. 5).

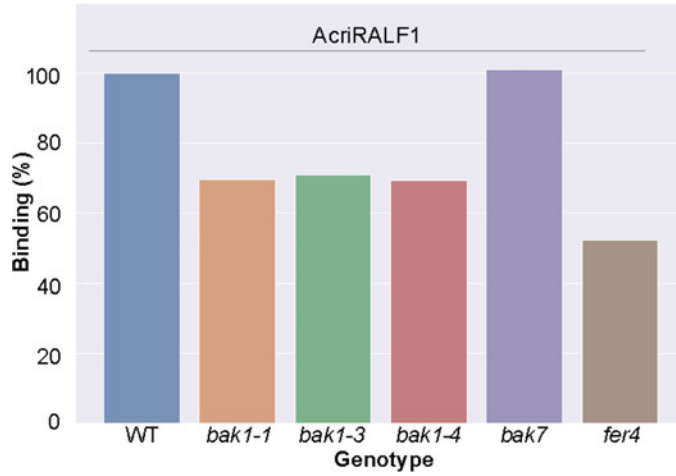


Fig. 5 Binding of acridinium-labeled AtRALF1 to whole Arabidopsis seedlings. Values represent the luminescence of each genotype relative to the binding with wild-type (WT) plants. (Modified and adapted from Dressano et al. [19])

1. Surface-sterilize p35S:aeq Arabidopsis seeds with the sterilization solution for 20 min and wash the seeds with sterilized ultrapure water in a laminar flow cabinet, as all the subsequent steps are done under sterile conditions.
2. Stratify Arabidopsis seeds for 72 h at 4 °C and then allow them to germinate at 22 °C and constant light (150 μmol photons m^{-2} s^{-1}) in Petri dishes containing semi-solid culture medium.
3. Seedling incubation: transfer four- to five-day-old Arabidopsis seedlings to 96-well microplates (one seedling per well) containing 1 mL of liquid culture medium.
4. Incubate the seedlings with 7 nM acridinium-labeled protein/peptide for 15 min at 4 °C on a shaker. To demonstrate binding specificity, protein/peptide is completed out using an excess (e.g., 10, 100, and 500 times) of unlabeled protein/peptide.
5. Carefully wash the seedlings at least three times.
6. Measure the resulting luminescence emission in the whole seedlings or roots using a microplate reader 2 s after injecting 50 μL of the trigger solution. The assay should be independently repeated at least three times, using a minimum of three replicates each.

4 Notes

1. Supplier's instructions recommend pH 8.0. Nonetheless, we decreased the pH of this solution to 6.3 to prevent protein precipitation. This optimization should be done to each protein. The stock solution should be stored at 4 °C.

2. We used a 5-mL plastic syringe.
3. The desalting column may be provided in the kit by the supplier; however, the Sephadex G25-80 column was suitable in our case.
4. Alternatively, 0.1% formic acid (v/v) (HPLC grade) can be used instead of the purification buffer.
5. The trigger solution is prepared fresh, immediately prior to use, by making a 1:1 mixture of sodium hydroxide and hydrogen peroxide solutions that may be supplied in the kit.
6. The optimum amount of peptide required in this step may vary depending on the peptide of interest. We optimized it by trial and error.
7. Working volume depends on the molecular weight of the protein. Molar ratio for optimum labeling varies and should be tested previously.
8. Use of 1% lysine instead of 1% glycine was equally effective.
9. This step is not mandatory; however, it may save you time when purifying these aliquots by HPLC. Alternatively, you may choose to purify all the aliquots by HPLC.
10. Before running the labeled peptide in the HPLC, we recommend loading a sample containing only the unlabeled peptide to confirm the elution time.
11. The fraction containing the labeled peptide will elute later than the unlabeled fraction.
12. A standard curve using known concentrations of AtRALF1 must be previously made to determine the concentration of your labeled sample [19].

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Chapter 20

LuBiA (Luciferase-Based Binding Assay): Glowing Peptides as Sensitive Probes to Study Ligand-Receptor Interactions

Louis-Philippe Maier, Georg Felix, and Judith Fliegmann

Abstract

The quantitative and qualitative biochemical description of molecular interactions is fundamental to the study of ligand/receptor pairs and their structure/function relationships. Bioactive peptides often are active at (sub-)nanomolar concentrations, indicating they have a high affinity for their sites of action, notably binding sites on receptors. Since such receptor proteins are commonly of low abundance, highly sensitive detection methods are required to study these ligand/receptor interactions. We present a protocol for an inexpensive luminescence-based detection setup in which the peptide ligand of interest is extended with the 11-amino acid HiBiT tag. This tag can be quantified easily down to fmol amounts by its ability to reconstitute the enzymatic activity of LgBiT, a truncated version of the *Oplophorus gracilirostris* luciferase.

Key words Competition binding assay, Peptide ligand, Immunoprecipitation, GFP-trap, Nano-Glo, Luciferase, HiBiT

1 Introduction

Quantitative assessments of high-affinity binding of ligands to their cognate receptor proteins require sensitive methods to accurately measure minute amounts of the bound ligand. Classically, this was achieved by labeling ligands with radioactive isotopes with relatively short half-lives (e.g., ^{125}I and ^{35}S), which allowed sensitive and specific detection of the labeled ligand by radioactive emission. Alternative methods, avoiding the instability and biohazard of radiolabels, have since been developed for competitive binding assays (see e.g., [1]). Luminescent labels offer a convenient alternative because light can be detected with high sensitivity and over a wide range of signal intensities. Covalent modification of peptides with chemiluminescent acridinium esters, for example, has been used to assign ligand peptides like IDA, systemin, and inceptin elicitor peptides [2–4] to their corresponding receptors in plants. Labeling of peptides with acridinium esters can be achieved by

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derivatization of primary amines and subsequent purification of the labeled ligand. Oxidation of the acridinium ester in the presence of a base leads to an activated intermediate state that rapidly decays with the concomitant emission of a photon [5]. Measurement of the light flash produced by this reaction allows sensitive quantification of ligands labeled with acridinium esters.

More persistent and further amplified luminescence can be generated by luciferase enzymes. Recently, a new split-luciferase-based system was developed to study protein/protein interactions (Nano-Glo®, [6]). It is based on a small luciferase (Nluc) from the deep-sea shrimp *Oplophorus gracilirostris*, which was split and modified into two enzymatically inactive parts, LgBiT and HiBiT. Importantly, LgBiT (18 kDa) and HiBiT (11 aa residues) rapidly interact with a very high affinity ($K_D = 0.7$ nM), thus reconstituting a stable enzyme with an exceedingly high luciferase activity [6, 7]. The relative amount of reconstituted luciferase is directly proportional to the HiBiT concentration if LgBiT and luciferase substrate are provided in excess, thus allowing direct quantification of HiBiT labels.

We have developed a versatile, quantitative binding assay for peptide ligand/receptor pairs based on the HiBiT label (*see* **Note 1**); a method that we refer to as Luciferase-based Binding Assay (LuBiA): In short, peptide ligands extended by the HiBiT peptide (at either terminus) are allowed to interact with their immobilized receptor binding sites. Immobilization, e.g., by affinity adsorption to solid beads, is required to remove unbound ligands by rapid, extensive washing. After washing, ligands bound to the immobilized receptors are released by heat treatment and quantified by the HiBiT-mediated luciferase reconstitution.

LuBiA has been developed with the intent to facilitate the evaluation of putative peptide/receptor pairs with regard to binding specificity. It is a versatile and adaptable method that will be useful also for the quantification of binding affinities, or for the screen of immobilized protein libraries in search of candidate receptors.

Advantages of this method are

- Its exquisite sensitivity.
- Low background due to minimal non-specific binding (holds for the peptides tested in our lab so far).
- The overall low cost (standard laboratory procedures and affordable labeling).
- The stability of the HiBiT label and absence of biohazards.
- No requirement for special training.
- No sophisticated equipment other than a luminometer needed.

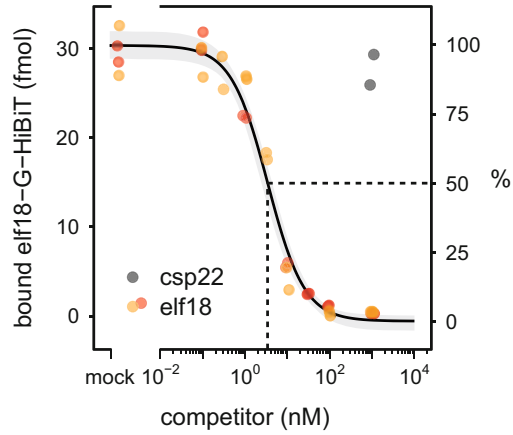


Fig. 1 Characterization of the binding specificity of EFR for different peptides. Shown is a binding competition experiment with 300 pM elf18-G-HiBiT and various concentrations of unlabeled competitors. The authentic ligand of the receptor (elf18) is able to displace the labeled peptide with an IC_{50} of 3 nM, while an unrelated peptide not recognized by EFR (csp22) fails to do so when added at 3000-fold excess. Data of two independent experiments are shown for elf18 (indicated by color hues); the competition curve was obtained with a non-linear mixed effects model (shaded area indicates 99% confidence interval; 1000 bootstraps)

In the detailed protocol below, application of LuBiA is demonstrated with the well-studied Elongation Factor-Tu Receptor (EFR) and its ligand elf18, whose binding properties have previously been published [8, 9] (Fig. 1). For this, we transiently expressed EFR with a GFP-tag in *Nicotiana benthamiana* leaves and immobilized the solubilized protein via its tag on magnetic GFP-Trap® affinity beads.

2 Materials

2.1 Buffers and Solutions

1. LB medium: 1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract; sterilized by autoclaving.
2. Antibiotic stocks: 100 mg/mL kanamycin in water; 100 mg/mL spectinomycin in water, 50 mg/mL rifampicin in DMSO; final concentrations in medium are 0.1% (v/v) of stocks.
3. Resuspension buffer (*Agrobacterium*): 10 mM MES, pH 5.7, 10 mM $CaCl_2$.
4. Induction buffer (*Agrobacterium*): 10 mM MES, pH 5.7, 10 mM $CaCl_2$, 150 μM acetosyringone.
5. Extraction buffer: 50 mM MES, pH 5.7, 100 mM NaCl (*see* **Note 2**).

6. Solubilization buffer: 25 mM Tris/HCl, pH 8.0 at 4 °C, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate (*see* **Note 2**).
7. IP wash buffer: 25 mM Tris/HCl, pH 8 at 4 °C, 150 mM NaCl.
8. Binding buffer: 50 mM MES, pH 5.7, 100 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$ (*see* **Note 3**).
9. Binding wash buffer: 50 mM MES, pH 5.7, 150 mM NaCl, 50 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1% (v/v) Tween-20 (*see* **Note 3**).
10. Elution buffer: 100 mM Tris/HCl, pH 8 at RT, 100 mM NaCl.
11. Blocking reagent (BSA/NaCl): 1% (w/v) Bovine Serum Albumin (BSA), 100 mM NaCl.
12. Detection solution: 1× Nano-Glo® blotting buffer, 0.5% (v/v) LgBiT protein solution, 0.2% (v/v) substrate solution. Prepare shortly before use and keep dark, on ice.

2.2 Reagents, Equipment

1. GFP-Trap® magnetic agarose beads (affinity resin; anti-GFP antibody conjugate, agarose-matrix with magnetic core, by *Chromotek*™; *see* **Note 4**).
2. Nano-Glo® HiBiT Blotting System kit (*Promega*™); contains a proprietary buffer optimized for Nano-Glo® luciferase activity and concentrated solutions of the LgBiT protein and the furimazine substrate [6].
3. Magnetic rack for 1.5 mL reaction tubes (e.g., *SureBeads*™ from *Bio-Rad*).
4. Micropipettes (1–10 µL, 10–100 µL, 0.1–1 mL, 1–5 mL), with disposable polypropylene pipette tips, the latter sterilized by autoclaving.
5. Multi-dispenser pipettes (0.1 and 1 mL steps) with disposable tips.
6. Mortar and pestle, ceramic, with abrasive surfaces.
7. White 96-well plates, polystyrene, flat bottom (e.g., Lumitrac® by *Greiner Bio-One*™).
8. 1.5 mL reaction tubes, polypropylene.
9. 15 mL and 50 mL reaction tubes, polypropylene.
10. 1 mL disposable plastic syringe, blunt-ended.
11. Cuvettes for photometric estimation of the optical densities of bacterial suspensions.
12. Photometer to be used with the above-mentioned cuvettes.

13. Microplate luminometer (e.g., Centro LB 960 by *Berthold Technologies*™).
14. Cooled centrifuges, able to accommodate 15 or 50 mL reaction tubes at up to 5000 × *g* (preferably with swing-out rotor), and 15 mL reaction tubes at >16,000 × *g*.
15. Shaking incubator for microbial cultures.
16. Greenhouse, or plant growth cabinet.
17. Freezers (−20 °C and −80 °C) and fridge (4 °C).
18. Heat block or water bath, to boil samples in 1.5 mL reaction tubes.
19. Laboratory scale.
20. Ice and liquid nitrogen.
21. Over-head shaker for 1.5 mL and 15 mL reaction tubes inside a fridge or cold-room (4 °C).

2.3 Receptors, Plants, and Bacteria

Receptors are tagged with GFP for immobilization on GFP-Trap® beads. Expression in transiently transformed *Nicotiana benthamiana* plants according to standard procedures should be feasible for most receptors of interest. In the example detailed here, we used a construct encoding EFR with a C-terminal GFP tag, and co-expressed it with the silencing suppressor protein P19 under control of the CaMV 35S promoter.

1. *N. benthamiana* plants: Grow for 4–6 weeks under long-day conditions in a greenhouse at about 22 °C and 50% relative air humidity.
2. *Agrobacterium tumefaciens* GV3101 (*rifampicin*^{R}) harboring a binary expression vector encoding the recombinant receptor (*p35S::EFR:GFP*; *spectinomycin*^{R}).
3. *Agrobacterium tumefaciens* GV3101 (*rifampicin*^{R}) harboring the silencing suppressor (*p35S::P19*; *kanamycin*^{R}).

2.4 Synthetic Peptide Ligands (see Notes 5 and 6)

Have peptides custom-synthesized (e.g., at 95% purity). Prepare 10 mM stock solutions in water or DMSO, depending on the solubility of the peptide in the respective solvent. Dilute to working concentrations in the blocking reagent (BSA/NaCl) to decrease potential loss of peptides by adherence to plastic surfaces. Store peptide stocks and dilutions at −20 °C.

1. elf18: ac-SKEKFERTKPHVNVGTIG (N-terminally acetylated).

2. elf18-G-HiBiT: ac-SKEKFERTKPHVNVG-TIGG**VSGWRLFKKIS** (N-terminally acetylated, and C-terminally extended by a glycine residue as a flexible linker [underlined] and the 11 aa residues of the HiBiT tag [bold]).
3. csp22: AVGTVKWFNAEKGFGFITPDDG.

3 Methods

3.1 Transient Transformation and Expression in N. benthamiana

1. Grow the two *Agrobacterium* strains (carrying P19 and recombinant receptor constructs) overnight at 200 rpm, 28 °C in 5 mL LB medium (i.e., enough for the infiltration of at least 15 plants) containing the appropriate antibiotics.
2. Harvest cultures by centrifugation at 3500 × *g* for 10 min, discard the supernatant, and wash the pellet with an excess of resuspension buffer. Centrifuge again and resuspend in induction buffer. Adjust OD_{600} to 1 and incubate for 2 h at RT.
3. Prepare the infiltration solution (ca. 2 mL per plant): Combine resuspensions of both strains in a 1:1 ratio, and dilute with induction buffer to a final OD_{600} of 0.4.
4. Infiltrate fully expanded middle-aged leaves of *N. benthamiana* from the abaxial side with the infiltration solution using a 1 mL syringe without needle.
5. Wait for the infiltrated solution to transpire completely (i.e., until leaf color returns to normal) before moving the plants back to the greenhouse. Grow the infiltrated plants under long-day conditions for 3 days.

3.2 Preparation of Solubilized Receptor Proteins

1. Freeze plant leaves expressing the receptor in liquid nitrogen. Using a pre-cooled mortar and pestle, grind the frozen material to a fine powder. You will need 200 mg of powdered plant material per sample and replicate (*see* **Notes 7** and **8**).
2. Transfer the required amount of frozen plant powder to a pre-cooled 15 or 50 mL reaction tube, depending on the total amount. Resuspend the powder in ice-cold extraction buffer (0.5–1 mL of buffer per 200 mg) by vigorous manual shaking until completely thawed.
3. Harvest cell debris by centrifugation at 5000 × *g* for 5 min and 4 °C. Discard the supernatant.
4. Wash the cell debris twice with extraction buffer (at least 1 mL of buffer per 200 mg): Each time, add ca. 10% of final buffer volume and resuspend by pipetting, then top up to the final volume, briefly vortex, and centrifuge as above.

5. Resuspend cell debris in ice-cold solubilization buffer (1 mL per 200 mg plant powder), transfer to 15 mL reaction tubes, and incubate at 4 °C for 1 h by over-head shaking (15 rpm).
6. Centrifuge at >16,000 × *g* for 25 min at 4 °C, then transfer the supernatant (solubilizate) to a new tube.

3.3 Immobilization on Affinity Beads

1. For each sample, transfer 7.5 μL of the GFP-Trap® magnetic agarose beads slurry to one 1.5 mL tube.
2. Add 300 μL of solubilization buffer to each tube and invert them four times.
3. Insert the tubes into the magnetic rack and incubate for a couple of seconds to immobilize the beads. Remove the buffer with a pipette and subsequently release the beads from the tube walls by removing either the magnet, or the tubes from the rack, depending on the rack's design.
4. Repeat the equilibration procedure starting from **step 2**, and keep the tubes on ice.
5. Add 900 μL of the solubilizate (from 200 mg plant powder, **step 3.2.6**) to each tube and incubate by over-head shaking (15 rpm) for 1 h at 4 °C.
6. Fix the beads to the tube walls with the help of the magnetic rack, as above. Remove the supernatant with a pipette.
7. Wash twice with solubilization buffer: Each time, add 1 mL of buffer per tube, release the beads from the tube walls by removing either the magnet, or the tubes from the rack, invert four times, fix the beads with the magnet to the tube walls, and remove the supernatant by aspiration.
8. Wash twice with IP wash buffer (*see* **Note 9**), as above.
9. Wash twice with binding buffer, as above.

3.4 Binding and Removal of Unbound Ligand

1. After removal of the last wash solution, release the tubes from the magnet and start the binding reaction by adding 200 μL of the binding buffer containing the HiBiT-tagged ligand and the unlabeled competitor to be tested. For the binding competition experiment shown in Fig. 1 we used 0.3 nM elf18-G-HiBiT-peptide, either alone or together with different concentrations of elf18 or csp22.
2. Incubate for 20 min by over-head shaking at 15 rpm (*see* **Note 10**).
3. Fix the beads with the help of the magnetic rack to the tube walls and remove the supernatant by aspiration.
4. Wash the beads four times with 1 mL of binding wash buffer (duration of the wash steps should be short and uniform for all

samples; use a multi-dispenser pipette and *see* **Note 11**): same handling as done before in **3.3.7**.

5. Resuspend beads in 200 µL of binding wash buffer and transfer the slurry to new 1.5 mL tubes (safe-lock, preferentially; *see* **Note 12**).
6. Immobilize the beads with the aid of the magnet and replace the supernatant with 200 µL of elution buffer.
7. Elute the bound peptides from their binding sites by boiling for 10 min at 95 °C (*see* **Note 13**). The samples can be stored at −20 °C until quantification (pause point).

3.5 Quantification of the HiBiT Label

1. Incubate the 96-well plate with blocking reagent (BSA/NaCl, 100 µL per well) for 20 min at RT.
2. Remove the blocking reagent (*see* **Note 14**) and place the plate on ice to prevent the drying out of the surface film.
3. Add 10 µL of eluted peptides (boiled slurry of **3.4.7**) to each well (if you had stored the samples at −20 °C, re-heat for 3 min at 95 °C and cool on ice before use).
4. With the aid of a multi-dispenser pipette, quickly add 90 µL of freshly prepared detection solution to each well.
5. Monitor the luminescence with a plate reader (*see* **Note 15**).

3.6 Evaluation of Binding Data

1. Extract the measured luminescence values (RLU) from a time point where the light emission has reached a plateau and is but slowly declining (e.g., 10 min in Fig. 2a).
2. Use a dilution series of a known concentration of HiBiT-labeled ligand to generate a calibration curve and calculate the amounts of HiBiT present in the samples of the binding assays (Fig. 2b).
3. To evaluate your ligand/receptor pair of interest with regard to binding specificity, perform competition binding experiments comparing the unlabeled ligand with structural analogs that exhibit a biological activity distinct from the authentic ligand (e.g., truncated ligands, variation in peptide aa motif, unrelated peptides). Determine the amount of total and unspecific binding, which is the amount of labeled ligand, bound in the absence and in the presence of an excess of unlabeled competitor, respectively. Calculate the amount of specific binding, that is the difference between the total and unspecific binding (Fig. 3). Express the concentrations of competing unlabeled peptides that displace 50% of specific binding sites as IC_{50} values (Fig. 1; *see* **Note 16**).

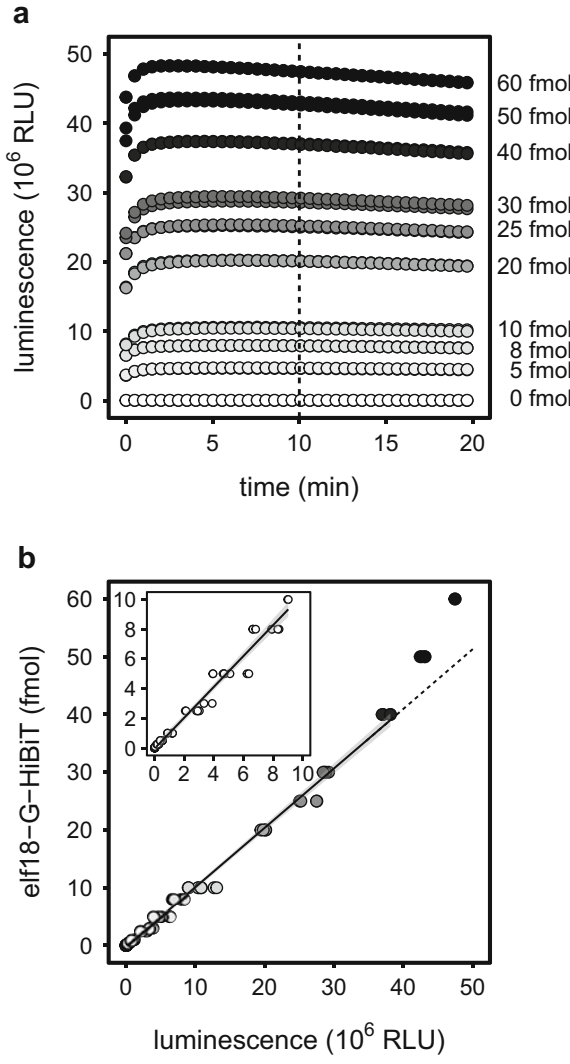


Fig. 2 Luminescence kinetics of a dilution series of HiBiT-labeled ligand, which was used to generate a calibration curve. Dilutions of elf18-G-HiBiT peptide were assayed in place of binding samples according to the described protocol. Luminescence values obtained at 10 min after luciferase reconstitution – corresponding to a stable phase, marked by a vertical line in (**a**) – were plotted against the known amount of peptide standard in (**b**). The calibration curve was obtained from data of four independent experiments, one of which (**a**) was part of. The inset zooms in on the lower end of the concentration range. The correlation between luminescence and ligand amount is near-linear between 0.01 and 40 fmol, a range that covers the usual amounts of bound ligand, and can be approximated by a linear regression model ($R^2 \sim 0.99$; shaded area indicates 99% confidence interval): $n\ (L) = (1.03 \pm 0.01) \times L - 0.19$, with n being the molar amount of HiBiT-labeled ligand (fmol) added to the assay, and L being the measured luminescence (10^6 RLU). Quantification outside of this range requires a more complicated, logistic regression model

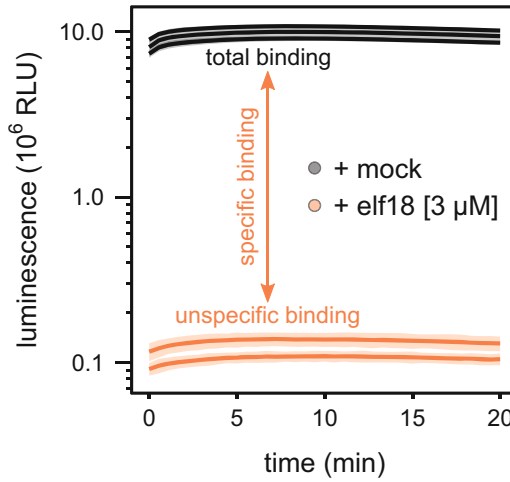


Fig. 3 The LuBiA assay displays an excellent signal-to-noise ratio and low amounts of unspecific ligand binding. Only less than 2% of total binding was unspecific; this was achieved by efficient washing and HiBiT label-specific detection. Binding of elf18-G-HiBiT [3 nM] to EFR was assayed in the absence (gray, total binding) or presence (orange, unspecific binding) of an excess of the unlabeled competitor elf18 [3 µM], in three and two replicates, respectively. Shown are luminescence kinetics following luciferase reconstitution by the HiBiT-labeled ligand retained in the binding samples; lines indicate mean values and ribbons the residuals of two technical replicates

4 Notes

1. The Nano-Glo® HiBiT Blotting System has been developed for detection of HiBiT-labeled proteins on Western blots. In our hands, with proteins from plant or bacterial extracts, this rapid developing system shows excellent sensitivity, high specificity, and low background on nitrocellulose membranes (data not shown). It is worth noting that the HiBiT label could also be used in experiments to detect crosslinked ligand/receptor complexes on blots. It can thus provide a valuable alternative for crosslinking of biotinylated ligands to receptors [10] and subsequent detection via streptavidin-coupled enzymes.
2. Supplementation of extraction and solubilization buffers with a cocktail of protease inhibitors may be needed to reduce protease action. They were not required in the presented example.
3. Composition of the binding and wash buffers may need to be adjusted for the ligand/receptor pair of interest. The current protocol assumes elf18/EFR interaction to occur at apoplastic pH of 5.7. A neutral pH should be considered for interactions with cytosolic receptor proteins. High salt and detergent concentrations are proposed to be added to the buffers to

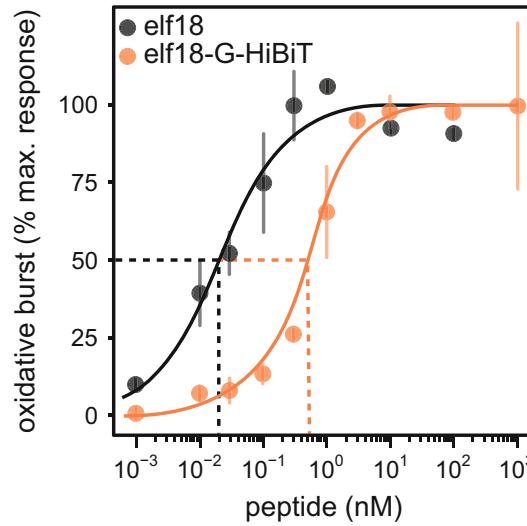


Fig. 4 The HiBiT-labeled elf18 peptide retained good biological activity. elf18-G-HiBiT showed a lower activity (EC_{50} ~ 0.5 nM) in comparison to unlabeled elf18 (EC_{50} ~ 20 pM). However, the labeled ligand was still active at sub-nanomolar concentrations in the dose-dependent induction of the oxidative burst in *Arabidopsis thaliana* (Col-0). The assay was performed as described [2, 15]. Results represent % of maximal ROS response reached with saturating concentrations of elf18. Values are means of two (elf18) or four (elf18-G-HiBiT) independent repetitions, with four technical replicates each

minimize nonspecific interactions and background problems. These components may have to be reduced or omitted if they disturb the specific interaction tested.

4. Magnetic affinity beads allow for very rapid and extensive washing. Prolonged duration of the washing process (>10 min), such as with washing by sedimentation of beads, may work well for ligands binding with high affinity but could provide problems for ligands with lower affinity and a higher dissociation rate, leading to considerable loss of the labeled ligand. Rapid washing with non-magnetic beads can be achieved with self-made columns (pipette tips with filter discs for bead retention) powered by a vacuum manifold.
5. HiBiT-labeled peptide ligands can be generated easily by standard custom peptide synthesis. Alternatively, the HiBiT label may also be encoded genetically as a tag. This can be useful for the labeling of larger proteinaceous ligands that are too difficult or expensive for in vitro synthesis, or that need expression in a specific host organism for proper structural folding and modifications; the recombinant ligand protein may be expressed and purified with systems and methods of choice.
6. HiBiT labels can be added to either terminus of the peptide ligand. Labeled ligands need to be tested in suitable bioassays

for their specific biological activity and compared to unlabeled versions of the ligand (*see* Fig. 4). If ligand function is affected, then consider placement of the HiBiT tag at the opposite terminus of the peptide.

7. This protocol assumes a membrane-localized receptor that mainly remains associated with the cell wall debris after plant homogenization (such as EFR). If the receptor of interest is mainly present in the soluble or microsomal membrane fraction, then skip **steps 2–5** and resuspend the frozen plant powder [20% (w/v)] directly in solubilization buffer.
8. If you want to interrupt the protocol at this stage, you can store the frozen leaves or powdered material at −80 °C for several months.
9. Thorough washing is required to remove sodium deoxycholate, which could precipitate at pH lower than 6.6 in the next step.
10. Incubation for 20 min at 4 °C is proposed for the interaction step. Some ligand/receptor interactions may require significantly longer incubation or a higher temperature (RT), depending on their kinetics of interaction.
11. Rapid washing is critical for ligand/receptor complexes that show considerable rates of dissociation. Dissociation can be slowed down by performing these steps at 4 °C.
12. Transfer to fresh tubes aims at reducing potential background originating from HiBiT-labeled ligands adhering to the plastic surface of the tube.
13. We propose to heat-denature the samples to release the labeled ligand from the binding sites. Direct quantification of the HiBiT label might also be possible but the ligand/receptor complex could affect the reconstitution of the luciferase due to steric hindrance.
14. The blocking reagent can be reused a couple of times and should be stored at −20 °C in between uses.
15. The HiBiT label is quantified by measuring the amount of luciferase activity which is reconstituted in the presence (of an excess of) LgBiT protein and substrate. The required exposure time depends on the sensitivity of the luminometer, 1 s was used in the presented example.
16. The IC_{50} enables the relative quantification of the receptor's binding affinity for different competitors only within a specific experimental setup. LuBiA can also be used for the estimation of general binding affinities, provided the system reaches equilibrium during the binding step. The affinity of the receptor for the HiBiT-labeled ligand (K_D) can be determined in saturation binding experiments. Its affinity to unlabeled versions (K_i) is

subsequently estimated according to their IC_{50} values. A potential pitfall in the evaluation of the binding data is the choice of the correct model. The classical model implicitly assumes that the total concentration of the receptor is much lower than the dissociation constant. This assumption is violated for high-affinity interactions with low values for K_D and K_i, in particular if the receptor protein was enriched, leading to inflated estimates for apparent affinities. In order to estimate adequate K_D values, a more complex model for an intermediate binding regime has to be applied [11]. Similar considerations apply to the estimation of K_i values. The K_i is calculated not only from the experimentally determined IC_{50} and K_D values, but also requires correction by additional terms. A variety of equations have been proposed for this purpose [12–14]. Application of these equations requires, at the least, knowledge of the total receptor concentration present during the binding step (**3.4.1**). This concentration can be derived from the left-over sample (boiled slurry of **3.4.7**), for example, by comparing the signal intensity for the GFP-tagged receptor protein present in the sample, to a dilution series of a GFP standard of known concentration, in quantitative Western blotting with anti-GFP antibody.

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Chapter 21

Microscale Thermophoresis (MST) to Study Rapid Alkalinization Factor (RALF)-Receptor Interactions

Martine Gonneau, Sébastjen Schoenaers, Caroline Broyart, Kris Vissenberg, Julia Santiago, and Herman Höfte

Abstract

Microscale thermophoresis (MST) is a simple but powerful tool to study the in vitro interaction among biomolecules, and to quantify binding affinities. MST curves describe the change in the fluorescence level of a fluorescent target as a result of an IR-laser-induced temperature change. The degree and nature of the change in fluorescence signal depends on the size, charge, and solvation shell of the molecules, properties that change in function of the binding of a ligand to the fluorescent target.

We used MST to describe the interaction between components of a regulatory module involved in plant cell wall integrity control. This module comprises the secreted peptide Rapid Alkalinization Factor 23 (RALF23) and its receptor complex consisting of the GPI-anchored receptor Lorelei-Like Glycoprotein 1 (LLG1) and a receptor kinase of the CrRLK1L family, FERONIA. Here we show how MST can also be used to study three-partner interactions.

Key words Thermophoresis, Soret effect, Molecular interaction, Binding affinity, Receptor complex formation, Lorelei Like Glycoprotein (LLG), Feronia (FER), Rapid Alkalinization Factor (RALF)

1 Introduction

1.1 Microscale Thermophoresis (MST)

Thermophoresis, thermodiffusion, or the Soret effect describes the movement of a molecule in a thermal gradient, in most cases from hot to cold. This biophysical phenomenon has been known for a long time but its theoretical basis remained a subject of debate until the foundational studies of Duhr and Braun in 2006 [1, 2]. They established the major parameters of the Soret effect using fluorescently tagged DNA or polystyrene beads and their movement in salt and temperature gradients. The result is the following S_T equation,

$$S_T = \frac{A}{kT}\left(-S_{\text{hyd}} + \frac{\beta\sigma_{\text{eff}}^2}{4\varepsilon\varepsilon_0 T} \times \lambda_{\text{DH}}\right)$$

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Fig. 1 The Monolith® NT.115

where A is the surface area of the molecule, k is Boltzmann constant, σ_{eff} is the effective charge, S_{hyd} the hydration shell effect, λ_{DH} the Debye length (a measure of a particle's net electrostatic effect in solution and how far its electrostatic effect persists), ε the dielectric constant of the particle and ε_0 is a physical constant, also called electric permittivity of the free space, β is the temperature derivative of ε, and T is the temperature of the system. For a more detailed theoretical background see [1, 3].

In the late 2000s two young German scientists, Stefan Duhr and Philipp Baaske working on a dsDNA unfolding project, used a laser to very rapidly heat fluorescently tagged DNA. They wondered how they could use their experimental process to visualize the Soret effect of thermodiffusion, founded the NanoTemper Technologies GmbH company, and developed the Monolith NT115 (Fig. 1) to make known and apply the microscale thermophoresis principle to measure the strength of interactions between biomolecules in solution.

Microscale thermophoresis (MST) has emerged in the last 10 years as an effective analytical method to study biomolecular interactions. The principle is simple: as long as the buffer and environmental parameters are kept constant, alterations in the thermophoretic behavior of a compound arise when one or more parameters (size, charge, or hydration shell) of the Soret equation change as a result of the interaction with another molecule. In this way, thermophoretic properties of fluorescently labeled macromolecules are altered upon interaction with a large variety of compounds. Consequently, MST can be used to study a wide range of bio-molecular binding events, from the binding of small ions, molecules, or peptides, to the interaction of large proteins in plant and medical science [4, 5], ribosomal complexes [6], liposomes [7], and polysaccharides [8].

In a typical MST experiment, the target protein is loaded into glass capillaries, along with the ligand at different concentrations. Intrinsic fluorescence of the target protein, or an attached fluorescent chemical or protein tag, is used to detect movement of the target protein in a transient temperature gradient. Such a microscale thermal gradient (from +2 to 6 °C) is rapidly and locally established by applying an infrared (IR) laser spot on the microcapillaries holding the samples (Fig. 2a). Before IR-laser activation, the fluorescent target is homogeneously distributed. When the laser is switched on, a sudden drop in fluorescence is observed, which corresponds to the combination of two effects, (1) a Temperature Related Intensity Change (TRIC) seen for the vast majority of fluorophores for which the fluorescence decreases at increasing temperatures, and (2) thermophoretic movement of the fluorescent molecules out of the locally heated region toward the colder edges. The concentration of the molecules in the heated spot is monitored by the fluorescence signal, which decreases until it reaches a steady state on a time scale of about 10–20 s (Fig. 2b). After deactivation of the laser, the fluorescence increases again as a result of passive diffusion and reestablishment of the concentration equilibrium (Fig. 2b). In a typical experiment, the change in MST signal is monitored in the presence of a dilution series of the non-fluorescent ligand (16 concentrations), and quantified as the ratio of fluorescence at a given time after switching on the laser (F_1) to the initial fluorescence (F_0) (Fig. 2c). For the analysis, ΔF_{norm} is expressed as a function of the non-fluorescent ligand concentration. Consequently, a saturation curve is acquired which allows the Kd of the target-ligand interaction to be calculated (Fig. 2d).

Other common methods to study biomolecule interactions are surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). Limitations of these methods are the requirement for sample immobilization (SPR) and relatively large amounts of material (ITC), respectively [9].

MST instead can be carried out in solution in any buffer or even in complex biofluids, and requires only very low amounts of labeled sample. Informative results appear in real time, and a Kd can be determined in less than 15 min. As a result, assay optimization is very easy and MST can even be used as a rapid interaction screening method. It is therefore no surprise that MST was well received and widely adopted by protein biochemists.

1.2 MST to Study RALF-Receptor Interactions Involved in Plant Cell Wall Integrity Signaling

The primary cell wall is a dynamic structure with a critical role in plant growth, development, and immunity. It is a complex network of proteins and polysaccharides such as cellulose, hemicelluloses, and pectins. Plant cell expansion is turgor-driven and requires careful relaxation of the load-bearing crosslinks of the cell wall network. How this process is coordinated to allow cell expansion, without losing the integrity of the cell wall remains a major unsolved question in plant biology [10, 11].

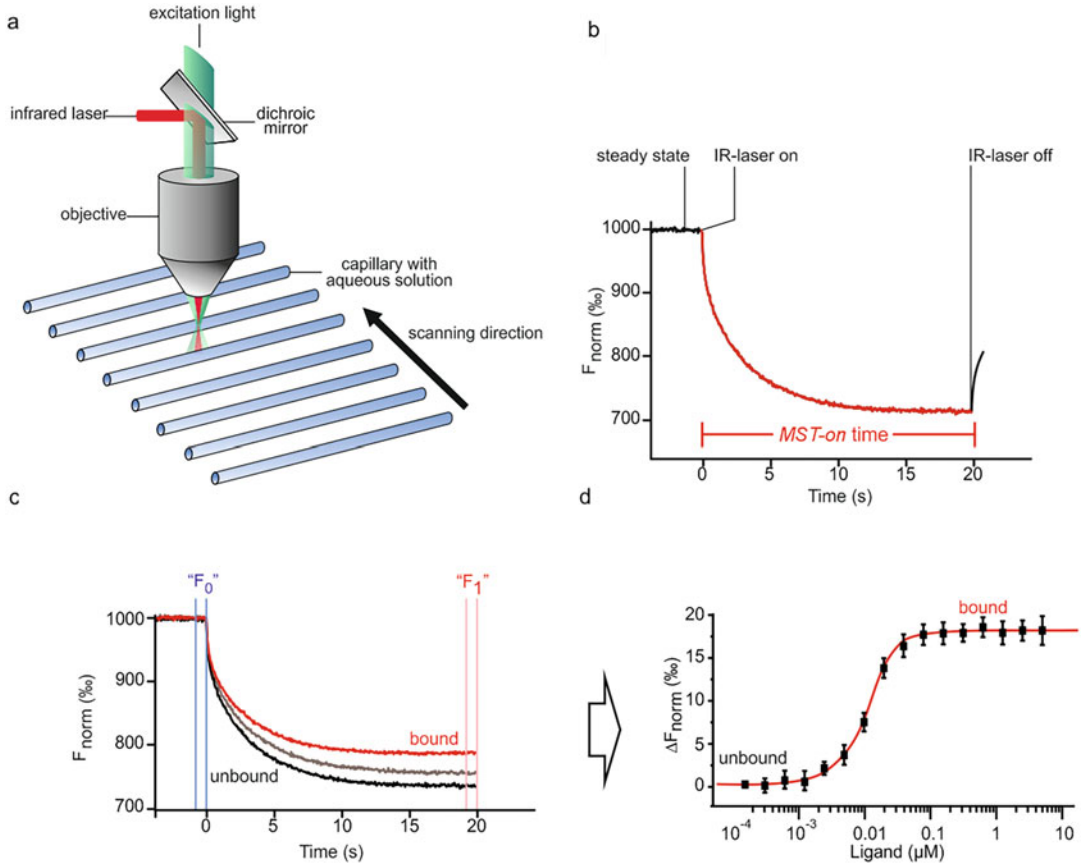


Fig. 2 MST setup and experiments. (**a**) Schematic representation of the Monolith® NT.115 optics. A focused IR laser is used to locally heat a defined sample volume. Laser excitation and sample emission light paths are split using a dichroic mirror. The emission signal is subsequently amplified by a photomultiplier and projected onto a point detector (not shown). (**b**) Before the IR-laser activation, the fluorescent molecule is homogeneously distributed inside the capillary. When the laser is switched on, fluorescence drops because of a Temperature-Related Intensity Change (TRIC) and thermophoretic movement (MST) of the fluorescent molecule out of the locally heated region. (**c**) In a typical experiment, the change in MST signal is monitored in the presence of a dilution series of the non-fluorescent ligand (16 concentrations). The black trace corresponds to the MST signal of the fluorescent unbound form of the target and the red one to the signal observed upon saturated ligand binding. Intermediate thermophoresis curves obtained with increasing amount of ligand bound to the target are represented in a simplified way by the grey curve. (**d**) Titration of the non-fluorescent ligand results in a gradual change in MST, which is plotted as ΔF_{norm} against the ligand concentration to yield a dose-response curve. This curve can be fitted to derive binding constants

Over the last 10 years, a regulatory module involved in the control of cell wall integrity has been intensively studied. This module consists of secreted peptides (Rapid Alkalinization Factors, or RALFs) which bind to a GPI-anchored protein named Lorelei Like Glycoprotein (LLG), thus facilitating the recruitment of a receptor kinase of the *Catharanthus roseus* Receptor-Like Kinase-Like 1 (CrRLKL1) family, such as FERONIA (FER), into a

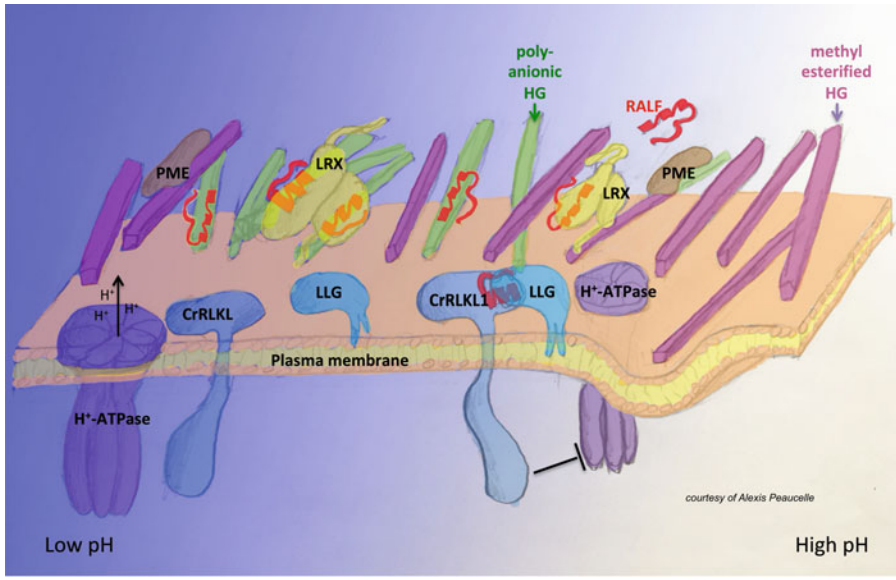


Fig. 3 Schematic model for the regulatory module of plant cell wall integrity control.
Here we describe the analysis of RALF23/LLG1/CrRLKL1-FER^{ECD} complex formation in vitro. In the in vivo context of the plant cell wall, other elements are involved to regulate the assembly and pH-dependent relaxation of the polysaccharide and protein network including homogalacturonan pectin (HG), leucine-rich repeat extensin (LRX) proteins, and pectin methyl esterase (PME) enzymes.

receptor complex [12]. RALF peptides also bind cell wall proteins of the Leucine-Rich repeat Extensin (LRX) family [13] (Fig. 3).

MST has been used successfully to study the binding of RALF peptides to their CrRLKL1 receptors in two-protein complexes [14–16]. However, the ternary LLG2/RALF23/FER complex has only been analyzed by sedimentation-velocity analytical centrifugation, co-immunoprecipitation, and ITC [12]. Here, we first detail the method for MST analysis of binary complex formation between LLG1 and RALF23 (method 3.2), and continue to show how MST can be used to analyze formation of the ternary LLG1/RALF23/FER complex (method 3.3).

2 Material

1. Monolith® NT.115 (NanoTemper Technologies GmbH): The Monolith® NT.115 (Fig. 1) is a full suite hardware to study and quantify molecular interactions between a fluorescence-labeled target and a ligand using microscale thermophoresis (*see* **Note 1**). It is equipped with two excitation lasers (blue/green, blue/red, or green/red) and the optical system to measure fluorescence emission at corresponding wavelengths. Alternatively, NT. label-free models are available that rely on

the intrinsic fluorescence of the protein of interest for detection, but this equipment has a much narrower range of application. In addition to the excitation lasers, the machine is equipped with an IR laser that is used to establish a local microscale thermal gradient (from +2 to 6 °C) in the microcapillaries holding the samples (Fig. 2a).

2. Software: The Monolith® NT.115 comes with proprietary software for experimental design and real-time visualization (MO.Control), and for analysis and export of data (MO.Affinity analysis).
3. Fluorescent target protein (LLG1): ~40 nM of the target protein in 200 µL assay buffer is sufficient for one MST run. Target proteins vary depending on the biological question under investigation. They can either be expressed as a fusion protein linked to a fluorescent protein tag (e.g., GFP), or as an untagged protein that is purified and subsequently labeled using a labeling kit provided by NanoTemper Technologies GmbH. In the experiment described here, we used recombinant LLG1 (lacking the GPI anchor) and the extracellular domain of FERONIA (FER^{ECD}) expressed in insect cells. The proteins were purified using a Strep-9xHis tandem affinity tag, subsequently cleaved by TEV (tobacco etch virus protease), followed by size-exclusion chromatography. Labeling of LLG1 with an amine-reactive fluorophore is described in Subheading 3.1. FER^{ECD} was used as one of the unlabeled binding partners in the ternary complex (Subheading 3.2).
4. Protein labeling kit: RED-NHS second Generation labeling kit (NanoTemper Technologies GmbH).
5. Desalting spin columns (e.g., Thermo Scientific, 89849).
6. Ligand: 1 mM custom-synthesized peptide. Here we used the mature RALF23 peptide custom-synthesized by ProteoGenix at >95% purity. Dissolve the peptide at 1 mM in MilliQ-water. RALFs are cysteine-rich peptides sensitive to oxidation, so after solubilization, flash freeze them into small aliquots and store them under argon (*see* **Note 2**).
7. 1 µM FER^{ECD}: adjust the concentration of purified FER^{ECD} to 1 µM in 10 mM citrate buffer, pH 5.0, 10 mM NaCl.
8. Glass capillaries: Standard and Premium Microcapillaries (NanoTemper Technologies GmbH) (*see* **Note 3**).
9. Assay buffer: NanoTemper Technologies GmbH provides optimized buffers but there are no restrictions with regard to the choice of buffers. In this experiment we used 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% (v/v) Tween-20 (*see* **Note 4**).
10. Single-channel electronic pipets, ranging from 0.2 to 2 µL, 1 to 10 µL, 2 to 20 µL and from 20 to 200 µL.

3 Methods

Prior to the MST experiment, the target protein (here LLG1) needs to be labeled. We used the amine-reactive RED-NHS second generation labeling kit (*see* **Note 5**) and followed the manufacture's (NanoTemper Technologies GmbH) protocol.

3.1 Labeling of the Target Protein

1. In a microfuge tube, mix 7 μL of Dye RED-NHS second Generation freshly prepared in DMSO with 7 μL of Labeling Buffer of the Nano Temper labeling kit.
2. Add 10 μL of this dye solution to 90 μL of the purified protein (10 μM LLG1) in a microcentrifuge tube, mix by pipetting up and down, incubate for 30 min in the dark.
3. Remove the excess of dye on a protein desalting spin column equilibrated in the buffer selected for the MST assay (*see* **Note 4**).
4. Before you initiate the MST experiment, it is recommended to check for proper folding, stability, and integrity of protein samples, e.g. in a thermal unfolding experiment (*see* **Note 6**). Always keep the samples at 4 °C and in the dark, to maintain protein integrity and to prevent destruction of the light-sensitive fluorescent dyes.

3.2 MST Analysis of Binary LLG1-RALF23 Complex Formation

1. Prepare two strips of 8 PCR tubes labeled 1 (tubes 1 to 8) and 2 (tubes 9 to 16).
2. Load 10 μL of assay buffer (taking into account the solvent of the ligand; *see* **Note 7**) in tubes 2 to 16 with a single-channel electronic pipette (*see* **Notes 8** and **9)**.
3. Load 20 μL of the highest ligand concentration (100 μM RALF23; *see* **Note 10**) in tube 1 of strip 1, then transfer 10 μL into the next tube and mix carefully by pipetting up and down, without making bubbles. Concentrations of RALF23 peptide ranges from 100 μM to 3 nM.
4. Repeat until the last tube (tube 16 in strip 2) and do not forget to discard 10 μL of the final dilution (*see* **Notes 8** and **9)**.
5. To each tube of the PCR strips, add 10 μL of labeled LLG1 using a single-channel electronic pipette (*see* **Notes 8** and **9**).
6. Pulse centrifuge the strips to spin down all liquids, and incubate at room temperature for 10 min.
7. Load each sample into a glass capillary (Premium capillaries for labeled LLG1) by dipping the capillary into the liquid, and fill the capillary tray of the Monolith NT115 from position 1 to 16 (*see* **Note 11**).

8. Start the measurement (*see* **Notes 12** and **13**). During an MST experiment, the sample fluorescence in each capillary is recorded, first during a 3-s period at ambient temperature to monitor the steady-state fluorescence, and then after IR-laser activation for a defined MST time of 20 s. The thermophoresis curves appear in real time, and the experimenter can at any time evaluate the quality of the analysis (overall fluorescence intensity, sample-to-sample fluorescence homogeneity, aggregation, etc.). During the run, the change in sample fluorescence (F_{norm}) is monitored by the ratio between the fluorescence at a given time before (F_0) and after IR laser activation (F_1).

$$F_{\text{norm}} = {}^{F_1}\!/_{F_0}$$

$$F_{\text{norm}} = (1 - x)F_{\text{norm}[A]} + x\,F_{\text{norm}[AL]}$$

$F_{norm[A]}$ is the contribution of the unbound fluorescent molecule A, $F_{norm[AL]}$ is the contribution of the fluorescent molecule A and its interacting ligand L, and x is the fraction of fluorescent molecules that formed a target-ligand complex. The dissociation constant, Kd, is obtained by fitting a dose-response curve to the F_{norm} vs ligand concentration plot (*see* **Note 14**). The binding affinity constant is automatically determined at the end of each run without the need for additional data analysis (Fig. 4). All the parameters of the run are archived (left part of Fig. 4), along with the capillary scan to judge homogeneity of the fluorescence signal, signal-to-noise ratio, response amplitude, and other data required for quality control (right part of Fig. 4) (see **Notes 12** and **13**).

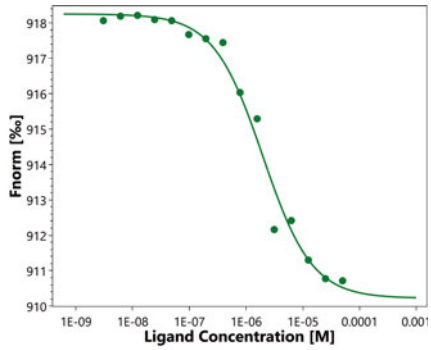
9. If the data quality is satisfactory (see **Notes 15** and **16**), repeat the measurement (**steps 3.2.1** to **3.2.8**) three times in the same conditions, ideally using three different protein preparations, or at least three different dilutions of all the compounds.

10. Present the data in either one of three ways: (1) Plot the fraction of ligand that is bound to the target against the ligand concentration. (2) Plot the normalized fluorescence (F_{norm} in [‰]) against the concentration of the ligand without further processing (Fig. 5a). (3) Plot the baseline-corrected normalized fluorescence ΔF_{norm} [‰] against the concentration of the ligand (Fig. 5b). To obtain ΔF_{norm}, the baseline F_{norm} value (mean F_{norm} value of the unbound target) is subtracted from all data points of the same curve. Presenting the MST data as such allows for a comparison of the Kd of multiple experiments in a single graph (Fig. 5b) (*see* **Note 17**).

a

Experiment type:	Binding Affinity
Filename	C:\Users\Martine\Data Files\LLG1-RALF23-Fer.moc
Date measured	Wed, 12 Feb 2020 14:59:24 GMT
Target:	20 nM LLG1
Ligand:	100 µM RALF23
Buffer:	MST buffer including 0.05 % Tween
Capillary:	Monolith NT.115 MST Premium Coated Capillary (K005)
Excitation Color:	Red
Excitation Power:	100% (Auto detect)
MST Power	Medium
Device	Monolith NT.115 (201503-BR-N031

b



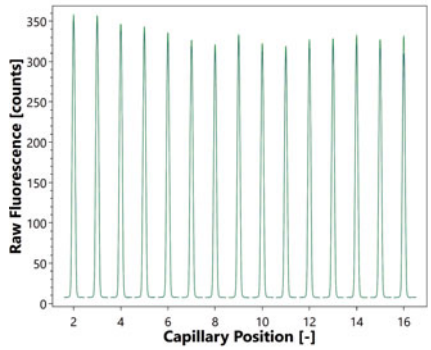
Response Evaluation: On Time 1.5 s

Kd model

Unbound	918.3
Bound	910.2
Kd	1.96 µM
TargetConc	20 nM

Response Amplitude: 8.0
Noise: 0.4
Signal to Noise Ratio: 20.4

c

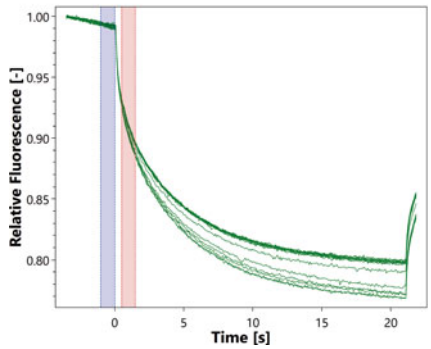


Initial Fluorescence:
Average: 334 counts
Variation: ±7.3 %

No adsorbtion

No Ligand Induced Fluorescence Change

d



Cursor positions:
Cold Region: -1s -0s
Hot Region: 0.5 s - 1.5s

No Aggregation

No Ligand Induced Photobleaching Rate Change

Fig. 4 Example of MST report for the interaction of fluorescently labeled LLG1 and RALF23 as provided by the MO-control software. (**a**) run parameters, including date, ligand and target concentration, buffer, type of capillaries, etc., (**b**) dose-response curve with signal-to-noise ratio and proposed Kd, (**c**) capillary scans with quality control parameters (adsorption to capillary walls, ligand-induced fluorescence change), and (**d**) MST traces with quality control parameters (formation of aggregates, photobleaching)

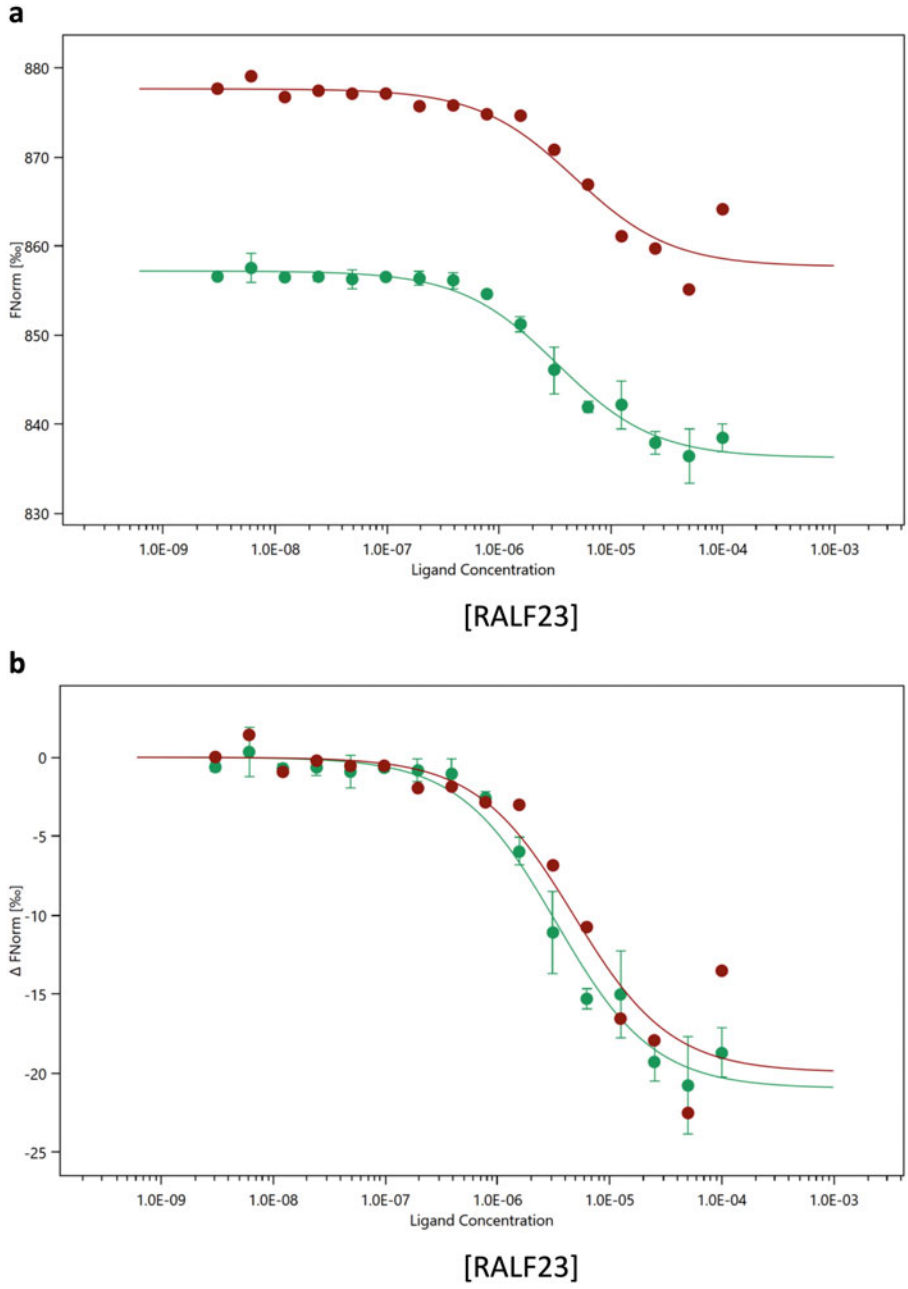


Fig. 5 Two representations of LLG1-RALF23 interaction as proposed by the MO.Affinity Analysis software. (**a**) Normalized Fluorescence (F_{norm} [‰]) of the target (LLG1) is shown for different ligand (RALF23) concentration. In this representation, the curves for individual runs are difficult to compare, as they start at different F_{norm} levels and/or show different amplitudes. (**b**) ΔF_{norm} (in [‰]) is shown for different ligand concentrations. ΔF_{norm} represents the change in normalized fluorescence as obtained by subtracting the mean F_{norm} value of the unbound target from all data points. This is the recommended way to present MST data which allows to compare the amplitude of the binding curves and the Kd of multiple experiments in one graph. The green curve represents the mean of two experiments; the red one an interaction obtained at a different fluorophore excitation intensity. RALF23 interacts with LLG1 with a Kd of 3.61 $\pm$ 1.47 μM, which is consistent with the previously reported binding affinity of 4.95 μM in ITC [12]

3.3 MST Analysis of Ternary LLG1-RALF23-FER^{ECD} Complex Formation

In this experiment, we titrate the pre-formed binary LLG1-RALF23 complex with a dilution series of FER^{ECD} from 1 μM to 0.03 nM. Resulting changes in thermophoretic mobility are recorded by MST as described in Subheading 3.2.

1. To establish the binary complex, add 1 μL of 1 mM RALF23 to 200 μL labeled LLG1 in a microfuge tube. Mix by gently flicking the tube. Incubate at room temperature for 10 min.
2. During the incubation time, prepare two strips of 8 PCR tubes labeled 1 (tubes 1 to 8) and 2 (tubes 9 to 16).
3. Load 10 μL of assay buffer in tubes 2 to 16 with a single-channel electronic pipette (*see* **Notes 8** and **9**).
4. Load 20 μL of the highest ligand concentration (1 μM FER^{ECD}; *see* **Note 10**) in tube 1 of strip 1, then transfer 10 μL into the next tube and mix carefully by pipetting up and down. Be careful not to introduce any bubbles.
5. Add 10 μL of the labeled LLG1-RALF23 complex from **step 3.3.1** to each of the 16 PCR tubes using a single-channel electronic pipette (*see* **Notes 8** and **9**).
6. Proceed with the MST measurements as described in **steps 3.2.6** to **3.2.9**. Plot the results as the baseline-corrected normalized fluorescence ΔF_{norm} [‰] against ligand (FER^{ECD}) concentration as described in **step 3.2.10**. Results are shown in Fig. 6.

4 Notes

1. The MST interaction studies are performed in solution, without the need for immobilization, which may result in the loss or modification of the interaction binding site. The dynamic range of the affinity measurement is broad with a pM/nM to mM Kd-range.
2. Correctly folded RALF peptides that are insensitive to oxidation can be produced in insect cells as a part of a protein complex with the Leucine-Rich Repeat domain of the LRX protein, from which they can be dissociated and eluted at pH 2 [13].
3. Standard and Premium types of capillaries should be tested to select the one that gives better results. Premium capillaries are coated to prevent adhesion of sticky proteins. To test the capillaries, NanoTemper Technologies GmbH recommends to prepare 120 μL of the labeled molecule at the concentration you want to use in the assay. Fill four capillaries of each type. Load the capillaries into the instrument and start a capillary scan. If the labeled protein sticks to capillary walls, irregular

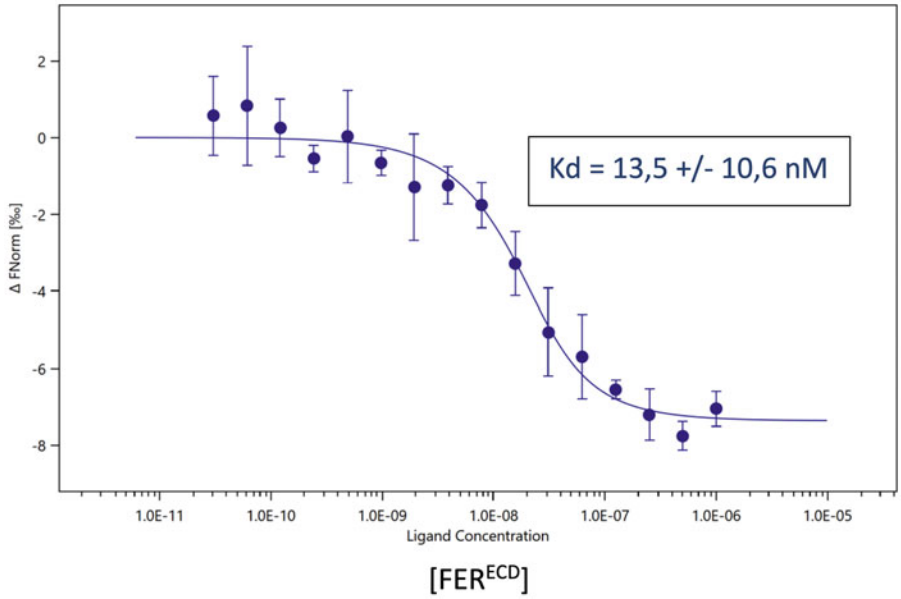


Fig. 6 Binding of FER^{ECD} to the pre-formed LLG1-RALF23 complex. The binary complex of LLG1 and RALF23 has been established three times independently. Binding of FER^{ECD} was then analyzed in three technical MST repeats, as recommended. No interaction could be detected between LLG1 and FER^{ECD} (not shown) which is consistent with previous ITC experiment [12]. Instead, labeled LLG1, preincubated with RALF23 formed a complex with FER^{ECD}, with K_d of 13.5 ± 10.6 nM, which is >25-fold lower than the Kd for the LLG1-RALF23 interaction. The results show that LLG1 binds RALF23 peptide and that the LLG1-RALF23 heterodimer is able to recruit the FER^{ECD} to form a functional ternary heterocomplex.

peaks with higher fluorescence intensity at the capillary wall will be observed. Choose the type of capillary that gives the same symmetrical peak in all 4 test capillaries. If the proteins persist to stick, we recommend to add detergent to the incubation medium and/or to use low-binding capillaries and low-binding tubes for the preparation of all solutions.

4. An unsuitable buffer can promote protein aggregation. As a result, fluorescence intensity curves will appear irregular. To cope with this, the buffer can be optimized by adding a small amount of detergent or by increasing the salt concentration. Different buffers and additives should be tested for optimal performance. NanoTemper Technologies GmbH recommends to test buffers by comparing the time traces obtained in >4 capillaries filled with exactly the same sample.
5. A purified protein can be labeled on lysine or cysteine residues depending on their requirement for protein functionality and folding. This is a critical step and it may be necessary to check for the functionality or the stability of the labeled protein after labeling. A large variety of alternative labeling methods exists and should be carefully considered for each individual protein.

6. Prior to MST, the quality and stability of the labeled protein may be assessed in a thermal unfolding experiment using the Tycho NT.6 (NanoTemper Technologies GmbH). The Tycho NT.6 is a benchtop device based on the principle of nanoDSF (Differential Scanning Fluorimetry). It follows the thermal denaturation of a purified protein submitted to a temperature gradient (30–90 °C, 30°/min) by measuring intrinsic Trp and Tyr fluorescence. The fluorescence increases with temperature and reaches its maximum when the protein is completely unfolded and all the Trp and Tyr residues are solvent exposed. Based on the temperature-dependent denaturation profile and the inflection temperature of protein denaturation (Ti), one can evaluate the effect of the fluorescent labeling on the stability or solubility of the protein.
7. In an MST run of 16 capillaries, all the compounds have to be at the same concentration except the ligand. The software of the Monolith® NT.115 helps in calculating the amounts of all compounds (target, ligand, buffer assay). The concentration of the ligand solubilization buffer needs to be carefully adjusted according to the successive dilutions of the ligand. For example, if a ligand is solubilized in an organic solvent, the concentration of this solvent has to be identical across all capillaries.
8. Take note that precise pipetting is absolutely essential. For the sake of reproducibility, it is advisable to use the same set of precision pipettes and always pipet up and down in the same way.
9. To obtain a homogeneous distribution of the fluorescent compound, we recommend to use a single-channel electronic pipette (the maximum accepted divergence of fluorescence for a correct run of 16 capillaries is ± 10%).
10. The highest concentration required depends on the expected affinity of the ligand for the target, where lower concentrations of ligand are required for higher affinities.
11. The capillaries should be handled with gloves to prevent the deposition of any residue, which could interfere with fluorescence acquisition.
12. MST experiments can be carried out at different intensities defined by the power of the IR-laser. Higher laser power results in a faster and larger temperature increase. To compare runs, the settings of the Monolith® NT.115 parameters have to be the same (temperature of the analysis, IR-laser intensity, and the LED power for excitation intensity of the fluorophore).
13. To optimize the MST parameters (medium versus high MST power for example) the same set of loaded capillaries may be used. Simply displace the capillaries a few millimeters inside the holder to create a hot spot at a different position along the capillary.

14. Details on the equations that express the overall changes in fluorescence, including constants, temperature variation, and Soret coefficient contribution, as well as the calculation of the affinity constant are depicted in detail in the Monolith® NT.115 user manual (V23_2017-01-24) and [17].
15. The signal-to-noise ratio is the recommended parameter to evaluate the robustness of an interaction. The noise is defined as the variations in the MST signal which are not caused by the addition of a ligand. The signal/noise is calculated by dividing the response amplitude by the background fluorescence intensity. A value of more than 5 is desirable while a value of more than 12 corresponds to an excellent interaction.
16. An expert mode for analysis of the MST data is available in MO-affinity analysis software which provides access to all data and parameters. Outliers, e.g., due to protein aggregation or incorrect pipetting, can also be removed.
17. The Monolith® NT.115 is easy to use thanks to user-friendly interfaces. Several tutorials and online tools are available to assist in the interpretation of the results and the presentation of data for publication.

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Chapter 22

Isothermal Titration Calorimetry to Study Plant Peptide Ligand-Receptor Interactions

Judith Lanooij and Elwira Smakowska-Luzan

Abstract

The field of plant receptor biology has rapidly expanded in the past three decades. However, the demonstration of direct interaction between receptor-ligand pairs remains a challenge. Identifying and quantifying protein-ligand interactions is crucial for understanding how they regulate certain physiological processes. An important aspect is the quantification of different parameters of the interaction, like binding affinity, kinetics, and ligand specificity that drive the formation of signaling complexes. In this chapter, we discuss Isothermal Titration Calorimetry (ITC) as a label-free technique to measure thermodynamic parameters of ligand binding with high accuracy and reproducibility. We provide a detailed guideline how to design, perform, analyze, and interpret ITC measurements using as an example the interaction between the SCHENGEN3/GASSHO1 (SGN3/GSO1) leucine-rich repeat receptor-like kinase and its sulfated peptide ligand CASPARIAN STRIP INTEGRITY FACTOR 2 (CIF2).

Key words Isothermal Titration Calorimetry (ITC), Peptide binding, Protein/peptide interactions, Thermodynamic parameters, Dissociation constant, Binding affinity

1 Introduction

Small, secreted peptides are key components of cell-to-cell communication, allowing plants to defend themselves and regulate their development [1–3]. In general, peptide ligands are perceived via plasma membrane–localized receptor kinases (RKs), which transmit extracellular signals across membranes to induce downstream signaling. RKs perceive a wide range of "self" and "non-self" derived signals, like peptides, hormones, or other bioactive molecules, in the extracellular space [4, 5]. RKs are modular proteins, consisting of a structurally conserved intracellular kinase domain (KD), a single transmembrane domain (TD), and a variable Extracellular Domain (ECD), which facilitates receptor-receptor and receptor-ligand interactions. An enormous number of peptide–receptor pairs are expected to exist given the large number of peptide ligands and RKs in plant genomes, and the possibility that one ligand may

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interact with multiple receptors, and one receptor may recognize multiple ligands. However, to date, only a small portion of those possible pairs have been identified and characterized. Therefore, receptor–ligand pair identification and quantification of their interaction are critical steps toward a system-wide understanding of how peptide ligands, as short- and long-distance signaling molecules, orchestrate plant development and integrate internal cues with external environmental stimuli [3].

A crucial aspect of unraveling the biochemical mechanisms behind cell communication is the assessment of the different parameters characterizing the formation of signaling complexes. There are different techniques available to quantify biomolecular interactions that provide binding affinity (how strong the interaction is between two molecules), kinetics (how fast the interaction happens), and ligand specificity (how specific the interaction is between two molecules). In this book chapter we discuss Isothermal Titration Calorimetry (ITC), a label-free technique for the characterization of macromolecular interactions [6].

ITC directly determines the heat exchange that occurs during complex formation, providing information on the thermodynamics of biomolecular binding processes [7]. ITC measures the heat released (exothermic reaction) or absorbed (endothermic reaction) during the binding reaction and allows the distinction between enthalpic and entropic contributions to the binding mode. It is important to mention that ITC is more reliable for measuring entropy-driven interactions as compared to other methods, like NMR relaxation measurements, that is more appropriate for enthalpy-driven reactions [8, 9].

ITC experiment is performed in a calorimeter that uses a power compensation system to maintain the same temperature between the sample cell (containing the receptor protein) and the reference cell (filled with water or buffer) at each ligand titration (Fig. 1a). During the experiment, a titration system injects precise amounts of ligand to the sample cell causing heat to be released or absorbed (depending on the nature of the reaction), and consequently, a temperature imbalance between the sample and the reference cell will occur. Such imbalance is then rapidly compensated by modulating the feedback power applied to the cell heater [10]. The thermogram generated shows a series of peaks that return to baseline, with the area of each peak corresponding to the heat released or absorbed at each ligand injection (Fig. 1b) [11, 12]. As the receptor-binding site becomes saturated with ligand, the peak area decreases gradually until only dilution heat is observed. The binding curve (Fig. 1c) represents the heat of the reaction per titration/injection as a function of the molar mass ratio between the ligand and the receptor protein. Fitting the binding curve to a specific binding model provides the binding stoichiometry (n); the thermodynamic parameters of the binding reaction (enthalpy, ΔH;

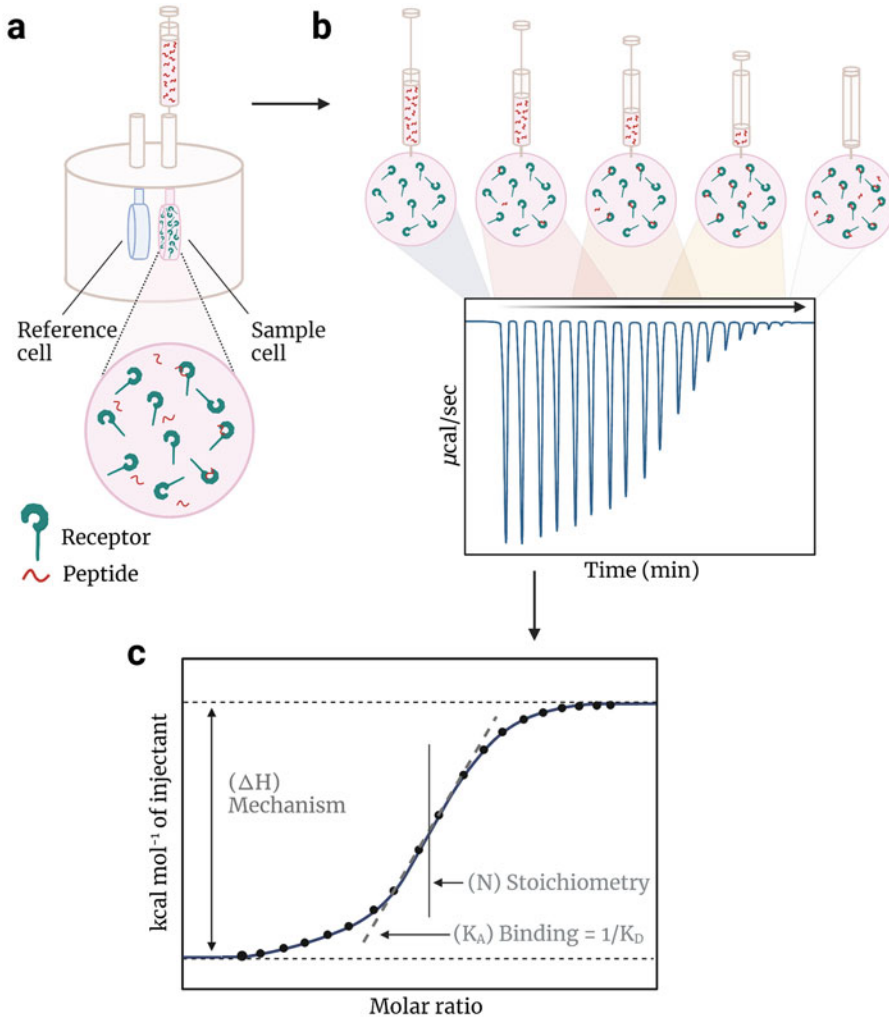


Fig. 1 Basis of Isothermal Titration Calorimetry (ITC). (**a**) The ITC instrument consists of a reference cell (blue) filled with MilliQ water, a sample cell (pink) containing a protein (SGN3 ECD), and an automated injection syringe containing ligand (CIF2 peptide variants) used to titrate the ligand into the sample cell. (**b**) During the ITC measurement, a small volume of the ligand solution, at the defined time intervals, is injected into the cell triggering the binding reaction and producing the characteristic peak sequence in the recorded signal. (**c**) After the measurement is finished, an appropriate software integrates the area under each peak (meaning subtraction of the dilution heat effects and normalization per mol of injected ligand) and the individual heat events are plotted against the molar ratio. Based on this information, using nonlinear regression, it is possible to estimate the thermodynamic parameters N, K_A, and ΔH

entropy, ΔS; and Gibbs free energy, ΔG); the strength of the interaction (the equilibrium association constant K_A, from which the more commonly used equilibrium dissociation constant K_D can be derived) [6, 12].

ITC has two important advantages in comparison to other methods used to assess protein-ligand interactions: (i) the tested

molecules (protein and peptide) are free to move in solution and are not labeled, which ensures a direct characterization of the binding event, unbiased by labeling and/or by limited mobility of the molecules due to their immobilization on a surface; (ii) ITC is the only method that allows a detailed characterization of the binding event by providing not only the binding affinity, but also other critical information including the binding stoichiometry and the thermodynamic parameters [8, 13]. Knowing them can help significantly in the understanding of the molecular mechanism of the binding reaction, even when no structural data are yet available. Furthermore, ITC measurements can be used as complementary data to validate structural results.

The aim of this chapter is to provide detailed know-how on how to set up an ITC experiment, describe the crucial steps and related complications, and provide advice on how to overcome such problems. Moreover, the presented protocol describes the analysis of an ITC experiment measuring the single binding event between the ECD of SGN3 and its peptide ligand CIF2 (sulfated and non-sulfated) [14]. Detailed protocols for the expression and purification of the receptor protein are not included.

2 Materials

2.1 Materials and Reagents

1. Hamilton® syringe, 700 series, fixed needle – different volumes.
2. Disposable borosilicate glass tubes, 0.7 mL.
3. Disposable 5 mL syringe with plastic tubing.
4. 0.22 μm membrane filter.
5. ITC buffer: 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.5, 200 mM NaCl, 5% (v/v) glycerol (*see* **Notes 1** and **2**).
6. MilliQ Water (*see* **Note 3**).
7. Purified receptor protein (*see* **Notes 4–6**): In the experiment described here we used the C-terminally StrepII-9xHis-fused ECD of SGN3. It was produced by secreted expression in baculovirus-infected insect cells and purified by chromatography [14]. Briefly, the ECD of SGN3 was inserted into the baculovirus transfer vectors pMeIBac B1 (Invitrogen) by ligation-independent cloning between the existing Honeybee melittin signal sequence and the C-terminal Strep II-9x histidine tag. The C-terminally tagged SGN3 ECD was harvested from baculovirus-infected High Five insect cells 72 h post-infection. SGN3 ECD was purified by Ni-NTA affinity chromatography (Qiagen) followed by two consecutive runs of gel filtration using a Superdex 200 16/60 column (GE Healthcare) pre-equilibrated with interaction buffer.

Protein purity and identity was checked by SDS-PAGE and anti-His immunoblot (*see* **Note 7**). The protein was subsequently concentrated to 3.5 mg/mL (*see* **Notes 8–10**) via centrifugal filtration with a 20 kDa filter.

8. Peptide ligand: CIF2 (DY(SO_3) GHSSPKPKLVRPPFKLIPN), custom-synthesized in the tyrosine-sulfated and non-sulfated state (*see* **Note 11**). Prepare 10 mM stock solutions of the two peptides in filter-sterilized ITC buffer (*see* **Notes 3, 8, 9,** and **10**).
9. Protein assay: commercial kit for the determination of protein concentration (e.g., the Bradford or Qubit assay systems).

2.2 Equipment

1. Microcalorimeter (we used Malvern Panalytical, model: MicroCal VP-ITC).
2. Vacuum pump (provided together with MicroCal VP-ITC, Malvern Panalytical).
3. Filling syringe (provided together with MicroCal VP-ITC, Malvern Panalytical).
4. 3 mL plastic tubes (provided together with MicroCal VP-ITC, Malvern Panalytical).
5. Benchtop refrigerated centrifuge with rotor.
6. Magnetic stirrer and 7 mm stir bars.
7. UV spectrometer.
8. pH meter.

2.3 Software

1. Software controlling the operation of the VP-ITC calorimeter: VPViewer 2000. Within this software, all ITC experimental parameters are entered, runs are controlled, and data is saved to the hard disk of the computer. When VPViewer 2000 is started it opens a linked copy of a project window in Origin, named VPITCPlot.opj. This project window of Origin is strictly for real-time display of data from VPViewer and is not intended for data analysis or saving data; VPViewer automatically saves all relevant experimental data.
2. Software for data analysis; we used Origin version7 (MicroCal, Malvern Instruments Ltd., RRID: SCR_014212). There is other software like NITPIC for the integration of the ITC data, SEDPHAT for data analysis, and GUSSI which is a program that can illustrate the output of SEDPHAT.

3 Methods

In this section, we first give more general recommendations on how to set up of an ITC experiment (Subheading 3.1). Then we provide the protocol for the characterization of the SGN3-CIF2 interaction by ITC (Subheading 3.2), instructions for the setup of the ITC instrument (Subheading 3.3), and a protocol for data analysis and interpretation (Subheading 3.4).

3.1 General Considerations

1. Decide on how to load sample cell compartment and syringe.

   Typically, the protein is loaded into the sample cell compartment of the calorimeter while the syringe contains the peptide solution (Fig. 1a). This is because it is usually easier to reach high concentrations of the peptide than the protein (*see* **Note 12**).

2. Estimate starting concentrations required for the receptor and the peptide ligand.

   The concentration of the injected ligand should be high enough to reach saturation within the first third to half of the experiment. For a one-site model (when one or several identical sites bind to the same analyte with the same enthalpy and binding affinity), the shape of the titration curve changes according to the product of the K_A, n, and the sample cell concentration (Fig. 2) [12, 15]. The product of these parameters, called the Wiseman "c" parameter, suggests an optimal experimental window where c is between 10 and 500 (for instance for $c = 1$, fitting is not feasible). Figure 2 shows the effect of c on the binding isotherm.

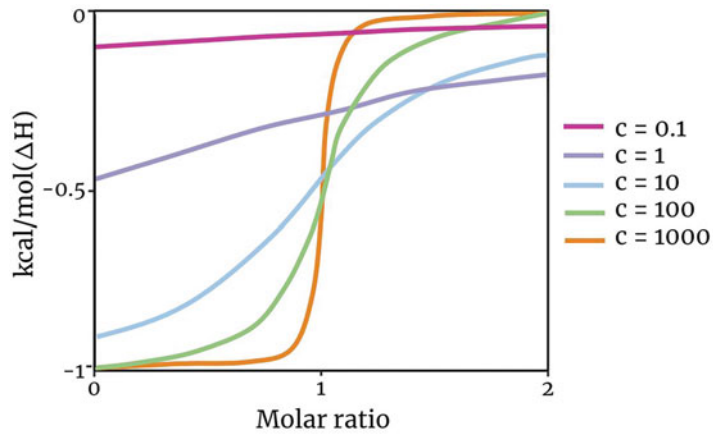


Fig. 2 Simulated ITC titration curves with various c parameters. Each simulated curve has the same ΔH, N, and K_A. Higher c-values result in titration curves that are too steep to resolve K accurately (although n and ΔH are well resolved) because the cell protein concentration is too high relative to K ($c < 100$), whereas lower c-values result in shallow titration curves from which all three parameters (n, K, and ΔH) are poorly resolved ($c = < 10$). Most papers recommend a c-value between 10 and 100

Table 1
Controls and an actual experiment that should be performed to study peptide/protein interactions by ITC

	Run	Syringe	Cell
1	Peptide/Protein	Peptide	Protein
2	Peptide/Buffer	Peptide	Buffer
3	Buffer/Protein	Buffer	Protein
4	Buffer/Buffer	Buffer	Buffer

If the expected stoichiometry of the interaction is 1:1, and assuming that the availability of protein and peptide will not be limiting, a ratio of 10: 1 ([Syringe]: [Cell]) is typically a good starting point (e.g., 100 μM in the sample cell versus 1 mM of the ligand in the syringe) [6, 7]. After obtaining preliminary fit parameters from the first ITC titration, experimental design software can be used to improve the experimental concentrations [6].

3. Choose the proper experimental design.

   Four ITC runs, the experimental run and three controls are required to test how the chosen protein, peptide, and buffers will behave during the ITC assay (Table 1).

   Runs 2 and 3 are important to detect the presence of oligomeric states in the peptide or the protein samples, respectively. For instance, peptide precipitation that may occur at the high concentration used in the syringe will lead to an endothermic response upon dilution into the sample cell filled with the same buffer. Run 4 should not give any signal, unless the two buffers do not match perfectly despite the rigorous preparation of both the syringe and cell solutions (*see* **Note 3**).

3.2 ITC Assay of the SGN3-CIF2 Interaction

To determine the thermodynamic parameters of the SGN3-CIF2 and SGN3-CIF2ns (non-sulfated) interactions, perform the ITC experiment at 25 °C in a microcalorimeter (e.g., VP-ITC Microcalorimeter, Malvern Instruments Ltd).

1. Switch on the computer and the ITC device. Start the VPViewer 2000, software that controls the instrument.
2. Prepare the sulfated (CIF2) and unsulfated (CIF2ns) peptide samples at the final concertation of 12.5 μM in the ITC buffer. The intended volume should be at least 500 μL.
3. Prepare SGN3 ECD at the final concentration of 5 μM in the ITC buffer. The intended volume should be at least 2 mL.
4. Equilibrate all the solutions at room temperature if the experiment is conducted at 20 °C or higher for at least 1 h before

starting the measurement (25 °C for the experiment described here) (*see* **Note 13**).

5. Load the protein and peptide solutions as well as deionized water into the 3 mL plastic tubes together with a 7 mm stir bar and degas them for 5 min using a vacuum pump (*see* **Note 14**).
6. Rinse the reference cell several times with deionized water and the sample cell with the ITC buffer as described by the manufacturer.
7. In the VPViewer 2000 adjust the temperature to 25 °C under the "Thermostat/Calib" tab.
8. Immediately before the experiment, centrifuge the protein and peptide solutions at 2000 × *g* for 2 min to eliminate bubbles.
9. Remove the deionized water from the reference cell and then slowly load the cell with 1600 μL of degassed deionized water (*see* **Note 15**) to allow air bubbles to evacuate through the opening of the cell (*see* **Note 16**).
10. Remove the ITC buffer from the sample cell and then load the cell with 1600 μL of the protein solution (SGN3 ECD) (*see* **Note 16**).
11. Load the injection syringe with 300 μL of the desired peptide solution (CIF2 or CIF2ns). Special attention must be paid to remove any air bubbles from the syringe. The presence of bubbles will lead to unstable baseline and, in consequence, to irreproducible data (*see* **Notes 17** and **18**).
12. Gently wipe the needle with a tissue, and then insert the syringe into the sample cell.
13. Turn on the stirrer at the appropriate speed, typically around 300 rpm, and allow the system to thermally equilibrate until the heat value shown on the calorimeter controller is stable (*see* **Note 19**).

3.3 Data Acquisition

Choosing right ITC run parameters is crucial. In addition to the parameters previously discussed (concentration, stirring speed, cell temperature), pay special attention to the parameters listed below. Use the following settings for:

1. Total Number of Injections: 38. This parameter will be determined by your experimental design and sample concentrations. You will need a minimum of 10–15 injections to define a binding isotherm (*see* **Note 20**).
2. Reference Power: 10 μcal/s. This setting determines the approximate value that the baseline will settle at when the system is equilibrated. Measuring highly exothermic reactions will require a high reference power of about 30 μcal/s, while highly endothermic reactions will require a low reference

power setting of about 2 μcal/s. If you have little information about the expected heats, it will be best to use a reference power setting of 10–15 μcal/s.

3. Volume of injection: 2 μL for the first (*see* **Note 21**), 8 μL for all following injections (*see* **Note 22**). Injection volume is generally between 3 and 15 μL. This range ensures high volumetric accuracy while allowing enough time for the injectant (peptide) to equilibrate to the temperature of the cell before injection. The injectant equilibrates to the cell temperature in the stem prior to reaching the cell. Injections ≥15 μL may result in reduced reproducibility of the injection blank heat. Injection blank heat is the thermal energy associated with the force of the injection and any temperature differences between the cell volume and the injection volume. A water/water and buffer/buffer titration will show the injection blank heat.
4. Duration of the injection: 0.5 μL/s. This is the default value and the slowest rate at which you can inject, which usually does not need to be adjusted. Changes in control peak shape and size that can be obtained by varying the injection duration are very subtle and usually not beneficial.
5. Spacing between injections: 280 s. This parameter is very important. It defines the time interval between two consecutive injections. The spacing needs to be long enough for the baseline to re-establish before the next injection (Fig. 3). Spacing between injections is usually set to 240–360 s (*see* **Note 23**).

Table 2 summarizes the experimental parameters used to estimate affinity of interaction between SGN3 and two versions of its ligand, sulfated CIF2 and non-sulfated CIF2ns (*see* **Note 24**).

3.4 Data Analysis and Interpretation

The most common software for the analysis of calorimetric data is Origin for MicroCal (version7, MicroCal, RRID:SCR_014212). In this section, we provide the protocol for how to process the ITC raw data and for the subsequent analysis. A single-binding model is needed to obtain the binding curve for the SGN3-CIF2 interaction and demonstrate lack of interaction with CIF2ns. Figure 4a shows negative heat changes (μcal/s) during consecutive injections of 8 μL of CIF2 (12.5 μM) into the sample cell containing SGN3 (5 μM). This graph indicates that the SGN3-CIF2 interaction releases heat in the exothermic binding reaction. On the contrary, injections of the non-sulfated CIF2 do not cause any significant heat changes, meaning that there is no interaction. Sulfation is thus required for CIF2 binding to SGN3 ECD (Fig. 4b).

To perform routine ITC Data Analysis and Fitting:

1. Start Origin 7.0 for ITC. The program opens and automatically displays the RawITC plot window.

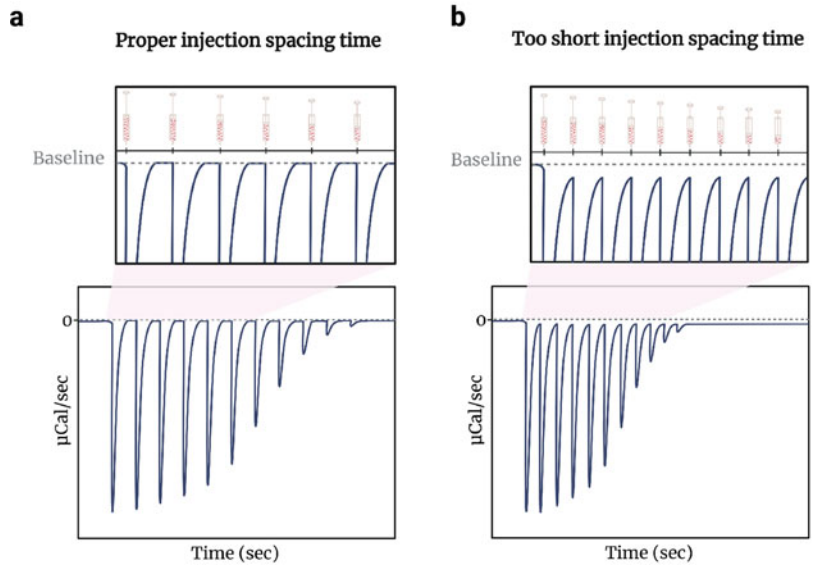


Fig. 3 Spacing between injections: (**a**) an appropriate injection spacing time and (**b**) too short injection spacing time. The heat signal should return to the basal level before proceeding to the next injection. The flat region between two injections will be used to define the baseline, subsequently subtracted from raw data before power integration over time

Table 2
The experimental parameters used to estimate affinity of interaction between SGN3-CIF2 (sulfated) and SGN3-CIF2ns (non-sulfated)

Experimental parameters	CIF2	CIF2ns
Syringe concentration, peptide (µM)	12.5	12.5
Cell concentration, protein (µM)	5	5
Cell temperature (°C)	25	25
Stirring rate (rpm)	300	300
Total number of injections	38	38
Volume, first injection (µL)	2	2
Volume, following injections (µL)	8	8
Injection spacing (s)	280	280
Reference power µcal/s	10	10

2. Click on the *Read Data* button and the dialog box should be open with the *ITC Data* (*.it).
3. Navigate to the *C:\Origin70\Samples* folder, then select *Your-Experiment.itc* from the Files list (*see* **Note 25**). After opening

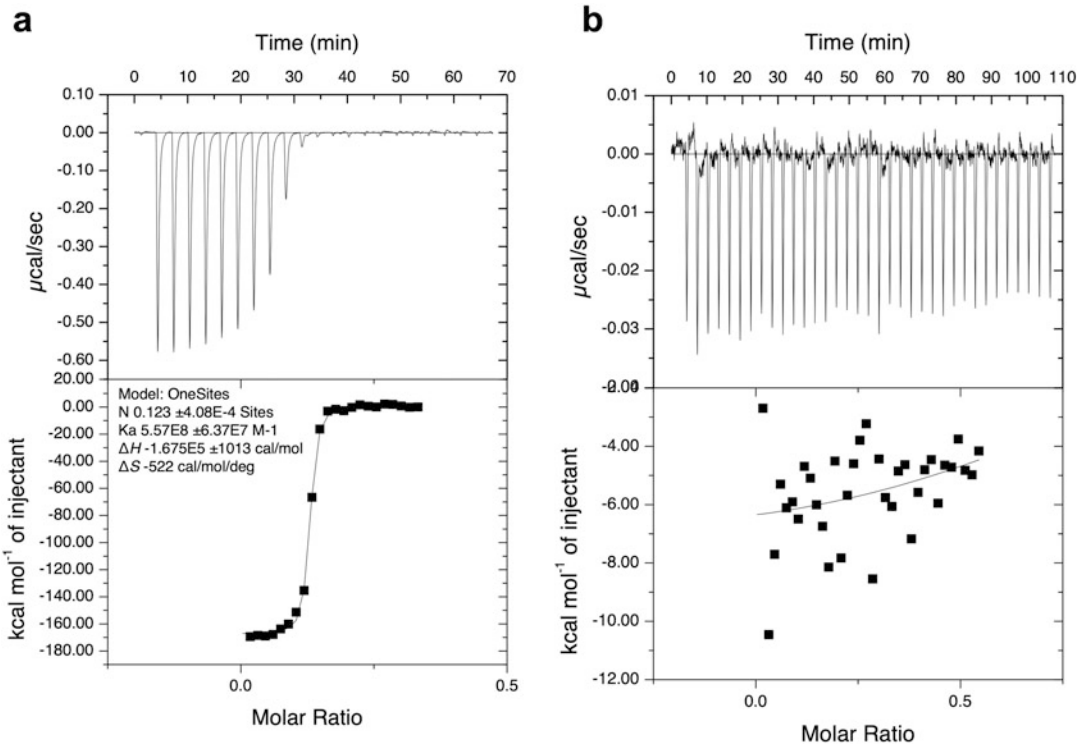


Fig. 4 Determination of CIF2 (**a**) and CIF2ns (**b**) binding affinity with SGN3 extracellular domain by ITC. The association constant (K_A), stoichiometries (N), ΔH, and ΔS are indicated for SGN3-CIF2. No detectable binding dissociation constant was determined for CIF2ns (unsulfated). Similar results were obtained in at least three independent experiments

YourExperiment.itc, this file is read and plotted as a line graph in the *RawITC* window, in units of µcal/second vs. minutes.

4. Manually adjust baseline and integration range for peak integration. Origin performs peak integration automatically. However, two critical integration details, the baseline and the integration range may not be accurate in cases where the signal-to-noise ratio is low. In these cases, both the baseline and the integration range need to be manually adjusted.
5. Origin also automatically opens the DeltaH window and plots the normalized area data rnahhh_ndh, in kcal per mole of injectant versus the molar ratio ligand/macromolecule. The analysis software calculates the area of each peak by integrating the power (µcal/s) over time. Knowing the peptide and protein concentrations, this step results in a plot of heat normalized by the concentration of injectant (leading to the unit of kcal/mol) versus the [peptide]/[protein] molar ratio. The first injection point is systematically discarded to remove the effect of solution diffusion across the syringe tip during the equilibration process (*see* **Note 20**).

6. Select Window: DeltaH to make DeltaH the active window. Before fitting a curve to the data, it is advised to re-check the current concentration values for this experiment.
7. To fit the area data to the "One Set of Sites" model click on the "One Set of Sites" button. A new command menu display bar appears – The Fitting Function Parameters dialog box. Click "1 Iter." to perform a single iteration or "100 Iter." to perform up to 100 iterations. Once you have a good fit, click on the "Done" button and the fitting parameters will be automatically pasted into a text window named "Results Log" and to the DeltaH window in a text label.
8. The fitting procedure yields the association constant (K_A), the stoichiometry ratio (N), and the binding enthalpy (ΔH) and entropy (ΔS).
9. Compare "N" to the expected value of 1.0. "N" is the average number of binding sites per mole of protein in your solution. It is expected to be 1.0 for the one-site non-cooperative binding model of the SGN3 ECD-CIF2 interaction. This expectation is based on the assumptions that all binding sites are identical and independent, you have pure protein (and ligand), you have given the correct protein and ligand concentrations, all your protein is correctly folded and active. This is rarely true in practice! Protein (and ligand) concentration determinations depend on the accuracy of the methods used. Protein extinction coefficients, for example, are rarely known better than ±5%, and are usually worse. Poor measurement techniques, incorrect UV baseline corrections, and attempts to conserve material using "micro" cuvettes, for example, can lead to serious errors. Even if protein concentration is correct, not all the protein may be correctly folded (a common experience with recombinant proteins). Table 3 summarizes potential reasons for N being smaller or higher than 1.

Table 3
Summary of potential reasons for N being smaller or higher than 1

N < 1	N > 1
Protein concentration is lower than estimated	Protein has multiple binding sites
Protein is impure	Ligand concentration is lower than estimated
Protein is pure but not all correctly folded	Non-cooperative binding model isn't correct
Ligand concentration is higher than estimated	
Non-cooperative binding model isn't correct	

4 Notes

1. Tris-HCl and reducing agent DTT (dithiothreitol) are not recommended in the buffer as they are causing intense heat changes, and this can severely affect the measurements. If reducing agent is required, it is recommended to use β-mercaptoethanol or TCEP (tris(2-carboxyethyl) phosphine) solutions at neutral pH.
2. The buffer used in this protocol was optimized to test the interaction between SGN3 ECD and its ligands. Buffer requirements should be optimized for each individual protein-peptide ligand pair. ITC is compatible with aqueous buffers in the range pH 2–12. However, amine buffers such as Tris should be avoided due to large enthalpy of ionization and substituted with HEPES or phosphate buffer. If there is a need to use glycerol, its final concentration should be kept below 20% (v/v) to avoid bubbles and heat distortions. The presence of organic solvents can cause signal artifacts, so if dimethyl sulfoxide is needed, keep it below 10% (v/v) in all your solutions.
3. Use ultrapure MilliQ water for the preparation of all buffers and solutions.
4. We highly recommend using ITC buffer for storage and dilution of both the ligand and the protein, to avoid buffer mismatch that can greatly contribute to measurement artifacts. Therefore, the last purification step of the protein preparation should also be performed in ITC buffer. All buffers used for protein purification and ITC experiments should be filter-sterilized using a 0.22 μm membrane filter, stored at 4 °C and use within 48 h. Prior to the ITC experiment, degas all buffers and solutions for 5–30 min under vacuum with a stirring rate of about 250 rpm.
5. The expression system and purification procedure will have to be adapted to the protein that is being studied.
6. Try to collect as much information as possible about the protein you want to analyze, particularly with respect to protein synthesis, purification, and characterization. This information will be important for troubleshooting and may be helpful in case you encounter problems with interpreting ITC measurements.
7. Protein identity, purity, and homogeneity can also be assessed by mass spectrometry.
8. The accurate determination of protein and peptide concentrations is essential for determination of high-quality thermodynamic parameters. Ideally, concentration of the protein and

peptide should be determined with an error not larger than 5%. For the determination of protein concentration, several reliable assay systems are commercially available. In these assays, the protein samples are added to the assay reagent, producing a color change (Bradford Assay) or increased fluorescence (Qubit Protein Assay) in proportion to the amount added. Protein concentration is determined by reference to a standard curve generated with known concentrations of a reference protein (e.g., bovine serum albumin). Alternatively, concentration can be determined by absorbance at 280 nm, if the protein or peptide sequence contains at least one aromatic residue, using the molar extinction coefficient that can be accurately calculated from the primary sequence. However, determining the concentration of a polypeptide that does not contain any aromatic residue is more challenging. In that case the absorption of the peptide bond at 205 nm, or 1D 1H quantitative NMR can be used.

9. We advised to perform the ITC experiment immediately after determining protein and peptide concentration to limit the effects of possible changes in sample properties over time.
10. Unless specified otherwise, it is not recommended to freeze the protein and the peptide sample as the physical environment of solution changes dramatically and may impact the protein stability, solubility, and activity. In addition, especially during slow-freezing process, the concentration of the polypeptide remaining in the unfrozen phase is increasing, potentially inducing denaturation and/or precipitation.
11. The choice of peptides depends on the receptor to be studied. CIF2 was chosen here as one of the known ligands of SGN3. Usually, in the ITC experiments synthetic peptides are used. They are obtained by solid-phase peptide synthesis and purified by reverse-phase HPLC. As the ITC is a sensitive and quantitative approach, the synthetic peptide should have the highest quality. A purity grade of >95% is recommended, as contaminants may cause unwanted heat effects.
12. Typically protein concentration ranges from the tens to hundreds in the micromolar range, while the peptide concentration is in the millimolar range. In case that protein and peptide availability are not a limiting factor, preliminary tests should be performed to verify that the protein and the peptide do not precipitate at chosen concentrations and conditions used for ITC. This can be done by increment titration of the peptide into protein solution in a glass vial.
13. Typically, the binding constant and heat of binding will be temperature dependent, but not the stoichiometry of binding.

14. The formation of gas bubbles in the ITC cells during the run needs to be avoided, since the resulting data may become noisy due to bubble-driven liquid displacement effects.
15. It is not necessary to refill the reference cell at every run. However, it is good practice to replace the deionized water at least once a week.
16. To obtain reliable measurements, both reference and sample cells must be precisely and sufficiently filled. Therefore, we recommend using a larger volume (2 mL) compared to the maximum capacity of the cells (1.6 mL). Use a 2.5 mL glass Hamilton® syringe, 700 series and insert it into the cell until you gently touch the bottom with the tip of the syringe. Inject the solution slowly and constantly, while retracting the needle from the cell. In this way, the cell is filled from the bottom to the top, and the operation avoids the formation of bubbles. Position the tip of the syringe on the ledge just below the visible portion of the cell port and draw the liquid to remove any excess of liquid from the cell.
17. Connect the plastic tubing of a filling syringe to the filling port of the injection syringe. Place the peptide ligand in borosilicate glass tubes and insert it into the pipette stand of the machine. Slowly withdraw the plunger of the filling syringe to draw up the solution containing the titrant. Purge the bottom "close fill port" as soon as the titrant solution begins to exit the filling port. Perform a refill step to remove any air bubbles from the injection syringe.
18. To avoid any contaminations, two different syringes should be used to transfer the protein and the peptide solutions, respectively.
19. Faster stirring speeds will increase the baseline noise level but may be necessary if solutions are more viscous than water. Also, when binding is extremely tight, a significant error is observed close to the equivalence point, if the injected ligand solution does not mix completely throughout the entire volume of the sample cell. In those cases, you may obtain better values using a stir speed of 500–600 rpm. Also, in case you are studying particulate suspensions, which tend to settle from gravity, more stirring will be needed to keep a uniform suspension.
20. Do not stop the experiment until your binding is saturated, otherwise the data are harder to fit.
21. The first injection is discarded from the data set to avoid the effect of protein diffusion across the syringe tip during the equilibration process. The volume of this first injection is usually smaller than the following ones to maximize the integrated heat observed within the second injection, which is used to evaluate the binding enthalpy of the reaction.

22. The limiting VP-ITC sensitivity is ~0.1 μcal. For precise measurements, each injection should have an average of at least 3–5 μcal of heat absorbed or evolved. If the heats are too small, then you need to increase either the concentration of the reactants or the injection volume.
23. Sometimes it takes longer for the ligand to find unoccupied binding sites after some (or most) have been filled, so the equilibration time is longer in the middle of an experiment than at the start. 600-s intervals between injections are unusual, but not unheard of.
24. The ITC measurements should be repeated at least three times to have enough data for statistics.
25. Data file names should not begin with a number, nor should they contain any hyphens, periods, or spaces. Also, Origin truncates the filenames to the first 15 characters, therefore when reading in multiple files, the first 15 characters of the filename must be a unique combination to prevent overwriting the data.

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