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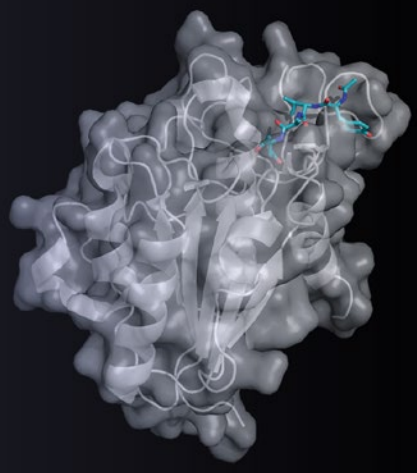

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Plant Proteases and Plant Cell Death

Methods and Protocols

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Plant Proteases and Plant Cell Death

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Preface

Plant cell death and regulatory proteolysis are two fascinating, highly interlinked fields of research. Many plant proteases have been recognized as key regulators of hormonal signaling and stress responses. Likewise, programmed cell death is vitally important for plant developmental processes and responses to microbial challenges. Many proteases have been implicated in programmed cell death in plants, which is one of the most intense areas of research on plant proteases. Indeed, in recent years, both fields have become a vibrant community with regular joint meetings.

This issue of Methods in Molecular Biology on *Plant Proteases and Plant Cell Death* aims to reflect this and presents a wide variety of current protocols for experiments in organisms ranging from unicellular algae to flowering plants. Chapters include protocols for *in vitro* production and characterization of plant proteases, tools for detecting and manipulating their activity *in vivo*, and proteomic approaches for identifying their substrates, inhibitors, and interaction partners *in planta*. Likewise, the book includes protocols for assessing programmed cell death in plants, including the hypersensitive response and ferroptosis—a new form of iron-dependent cell death. Last but not least, we include two chapters on new bioinformatic N-termini annotation and data mining tools for *in silico* analysis of plant proteases and plant protease function using rapidly accumulating public data.

Fascinating reports on the function of plant proteases and plant cell death mechanisms have emerged recently as we have compiled this issue, representative of a scientific field that is becoming established while constantly evolving. Much has been learned, but still the vast majority of the hundreds of proteases encoded in each plant genome have no known functions. In stark contrast to research on programmed cell death in animals, the molecular function of diverse proteolytic enzymes in regulated cell death in plants remains obscure.

We hope that this timely collection of protocols assembled in this book will inspire new studies to address these and other important questions and advance our understanding of plant proteases. We sincerely thank all authors for their insightful contributions, their tremendous efforts in challenging times caused by the COVID-19 pandemic, and their willingness to openly share their "lab secrets" in the notes. It has truly been a pleasure to work with you on this issue.

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Chapter 1

Expression and Purification of the Type I Metacaspase from a Cryptophyte *Guillardia theta*, GtMCA-I

Vida Štrancar, Katarina Petra van Midden, Marina Klemenčič, and Christiane Funk

Abstract

Type I metacaspases are the most ubiquitous of the three metacaspase types and are present in representatives of prokaryotes, unicellular eukaryotes including yeasts, algae, and protozoa, as well as land plants. They are composed of two structural units: a catalytic so-called p20 domain with the His-Cys catalytic dyad and a regulatory p10 domain. Despite their structural homology to caspases, these proteases cleave their substrates after the positively charged amino acid residues at the P1 position, just like the metacaspases of type II and type III. We present a protocol for expression and purification of the only type I protease from a secondary endosymbiosis *Guillardia theta,* GtMCA-I by overexpression of its gene in BL21 (DE3) *E. coli* cells and one-day sequential purification using nickel-affinity, ion-exchange, and size-exclusion chromatography.

Key words Proteolysis, C14, Trypsin-like, Regulated cell death, Algae

1 Introduction

Metacaspases are structural homologues of caspases, one of the best characterized proteases involved in execution of apoptosis in metazoan cells. Caspases and metacaspases are both composed of two structural subunits (domains), which are termed after their respective sizes in caspases: the p20 domain (approximately 20 kDa) and the p10 domain (approximately 10 kDa) [1]. According to the architecture of the two domains, metacaspases can further be divided into three types (I–III).

Type I metacaspases are the most ubiquitous among the three types and can be found in prokaryotes, yeast, algae, as well as higher plants. In this type, the catalytic p20 domain is followed by a regulatory p10 domain without any longer linker in-between (as it is for example the case in type II metacaspases). Metacaspases of green algae, land plants, and fungi can contain an additional

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N-terminal domain [2], although this feature is not present in type I metacaspases of bacteria or secondary endosymbiotic algae, including *Guillardia theta* [3], and is thus most probably a derived trait. Up to now, two structures of type I metacaspases have been resolved: from *Trypanosoma brucei*, TbMC2 [4], and from *Saccharomyces cerevisiae*, Yca1 [5]. Both proteins contain the N-terminal extension: while it is visible in the crystal structure of TbMC2, where it spans the active site and acts as a gatekeeper, it has been removed from the Yca1 protein by limited proteolysis prior to crystallization and is therefore missing from the structure. Five type I metacaspases from another fungus, *Schizophyllum commune*, were also recombinantly produced in *E. coli* [6]. However only ScMC3, which lacks the N-terminal domain, could be produced as a His-tagged protein, while the rest can only be expressed when the small ubiquitin-related modifier (SUMO) sequence is fused via a linker to the N-terminus of each protein.

Up to now, no type I metacaspases from land plants or green algae have been produced recombinantly. Here, we present a protocol for expression and purification of a full-length type I metacaspase from a secondary endosymbiosis *Guillardia theta* (GtMC1) [7], now termed GtMCA-I [8]. This is the only type I metacaspase in this organism and the gene encoding it is highly expressed during normal growth conditions [7]. Using this protocol, C-terminally His-tagged GtMCA-I is produced in BL21(DE3) *E. coli* cells and purified by nickel-affinity chromatography, followed by ion-exchange and size-exclusion chromatography.

2 Materials

2.1 Solutions for Transformation and Protein Expression

1. A glycerol stock of *E. coli* BL21(DE3) cells.
2. pET28b(+) expression plasmid encoding GtMCA-I (*see* **Note 1**).
3. LB broth and LB agar plates containing 50 μg/mL kanamycin (LBK): For LB broth, mix 10 g peptone, 10 g NaCl, 5 g yeast extract and add up to 1 L deionized water. Sterilize by autoclaving (121 °C, 15 psi, 20 min, slow exhaust). When cooled, add 1 mL of sterile 50 mg/mL kanamycin stock solution. Prepare 50 mg/mL kanamycin stock solution by mixing 0.5 g kanamycin with H_2O to a final volume of 10 mL. Filter sterilize and store at −20 °C. For LB agar, add 15 g of agar per 1 L of LB broth before autoclaving. Prepare plates using still warm autoclaved LB agar (approximately 50–60 °C, when bottle can be held in hands). Add 1 mL of sterile 50 mg/mL kanamycin stock solution to the LB agar and gently mix. Pour into sterile Petri dishes. After cooling the plates at room temperature, store at 4 °C.

4. ZYM medium containing 50 μg/mL kanamycin: Prepare stock solutions of 1 M $MgSO_4$, 50 × M, 50 × 5052, ZY medium. For 1 M $MgSO_4$, mix 24.6 g $MgSO_4$ and 87 g deionized water and sterilize by autoclaving (*see* above). For 50 × M, mix 80 g deionized water, 17.8 g Na_2HPO_4, 17.0 g KH_2PO_4, 13.4 g NH_4Cl, and 3.6 g Na_2SO_4 (*see* **Note 2**) and sterilize by autoclaving. For 50 × 5052, mix 73 g deionized water, 25 g glycerol, 2.5 g glucose, and 10 g α-lactose monohydrate and filter sterilize using a 0.20 μm syringe filter. For ZY medium, mix 10 g protein hydrolysate N-Z-Amine, 5 g yeast extract and add up to 1 L deionized water. Sterilize by autoclaving (*see* above). Store all stock solutions at room temperature. For ZYM medium containing 50 μg/mL kanamycin, add 400 mL ZY medium, 800 μL 1 M $MgSO_4$, 8 mL 50 × M, 8 mL 50 × 5052, and 420 μL of sterile 50 mg/mL kanamycin stock solution in sterile conditions (*see* **Note 3**) [9].

2.2 Other Reagents and Equipment for Transformation and Protein Expression

1. Several 1.5 mL tubes, 15 mL glass test tubes, and one 2 L shake flask (*see* **Note 4**). Sterilize by autoclaving (121 °C, 15 psi, 20 min, fast exhaust).
2. Syringes (10 mL) and syringe filters, 0.20 μm and 0.45 μm.
3. Water bath at 42 °C for transformation (also a can be used).
4. Incubator at 37 °C for incubation of plates.
5. Sterile plastic spreader.
6. Plastic sterile Petri dishes.
7. An autoclave with fast and slow exhaust setting.
8. A temperature-controlled shaker and incubator with room for one 2 L flask, which can be set to either 16 °C or 37 °C.
9. A high-speed refrigerated centrifuge (e.g., Sorvall refrigerated centrifuge) with 1 L centrifuge bottles or similar.
10. A spectrophotometer and plastic cuvette for measurement of absorbance at 600 nm.

2.3 Solutions for Protein Purification

Prepare all solutions using ultrapure water with a conductivity of 18 MΩ cm at room temperature and filter through a 0.2 μm filter prior to storage. This aids the long-term storage of buffers, and also reduces the chances of blocking the columns or narrow tubes on ÄKTA (or similar) fast protein liquid chromatography (FPLC) systems. All buffers can be kept at 4 °C or at room temperature until use.

1. IMAC equilibration buffer: 20 mM HEPES, 500 mM sodium chloride, 20 mM imidazole, pH 7.5. Prepare 500 mL of buffer by mixing 2.4 g HEPES, 14.6 g sodium chloride, 0.7 g imidazole, and deionized water to a volume of 490 mL. Adjust the

pH to 7.5 using concentrated 5 M sodium hydroxide. Add the water to 500 mL.

2. IMAC elution buffer: 20 mM HEPES, 500 mM sodium chloride, 250 mM imidazole, pH 7.5. Prepare 100 mL of buffer by mixing 0.5 g HEPES, 2.9 g sodium chloride, and 1.7 g imidazole. Add water to 90 mL and adjust the pH to 7.5. Adjust the volume to 100 mL by adding water.
3. IEX equilibration buffer: 20 mM HEPES, pH 7.5. Prepare 500 mL of buffer by mixing 2.4 g HEPES and 490 mL deionized water. Adjust the pH to 7.5 with sodium hydroxide and then dilute to 500 mL.
4. IEX elution buffer: 20 mM HEPES, 1 M sodium chloride, pH 7.5. Prepare 100 mL of buffer by mixing 0.5 g HEPES, 5.8 g sodium chloride, and 90 mL deionized water. Adjust the pH to 7.5 and then dilute to 100 mL.
5. Gel filtration buffer: 20 mM HEPES, 500 mM NaCl, pH 7.5. Prepare 500 mL of buffer by mixing 2.4 g HEPES, 14.6 g sodium chloride, and 490 mL deionized water. Adjust the pH to 7.5 and then dilute to 500 mL.

2.4 Other Reagents and Equipment for Protein Purification

1. A mechanical device to homogenize *E. coli* cells in IMAC equilibration buffer (cell homogenizer).
2. A mechanical device to disrupt *E. coli* cells (ultrasonic homogenizer).
3. A peristaltic pump at room temperature.
4. Several 50 mL plastic tubes.
5. ÄKTA pure FPLC system (GE Healthcare Life Sciences, Marlborough, Massachusetts, USA) or equivalent.
6. HisTrap™ FF Purification Column (Cytiva) or similar.
7. HiTrap ANX FF (GE Healthcare Life Sciences, Marlborough, Massachusetts, USA) anion-exchange column or similar.
8. Superdex 75 (GE Healthcare Life Sciences, Marlborough, Massachusetts, USA) size-exclusion chromatography column or similar.
9. Filtration unit (bottle top vacuum filter or similar).
10. Amicon filtration unit (Millipore Corp., Temecula, California, USA) equipped with a 10 kDa exclusion membrane or similar.
11. 1.5 mL centrifuge tubes.
12. Ice.
13. 4–12% SDS-PAGE protein gel (or equivalent) with sample loading buffer, running tank, running buffer, and power pack (all optional, necessary only if purity of the protein is to be assessed).

3 Methods

3.1 Transformation and Preparation of an Overnight Culture

1. Warm the LB medium in a water bath or incubator at 37 °C.
2. Place an agar plate upside down in a 37 °C oven. This step enables cultures to be plated out onto a pre-warmed plate, which enhances their growth.
3. Add 1.5 μL of plasmid (concentration at least 10 ng/μL) to 100 μL of chemically competent *E. coli* BL21(DE3) cells in a 1.5 mL tube on ice for 30 min. Do not mix by pipetting up and down as this will damage the cells. Instead, mix gently by flipping the tube with fingers. Be careful not to warm the tubes as this decreases the transformation efficiency.
4. Using a foam floater to keep the top of the tube above water level, place the tubes in a water bath at 42 °C for 45 s. This can also be done in a block heater. Remove and cool on ice for 1 min.
5. Add 0.8 mL of preheated LB media without an antibiotic to the transformation mixture and place in a 37 °C shaking incubator for 30–60 min (approximate duration of 1–2 E. coli replication cycles).
6. Remove the agar plate from the oven and the culture from the shaking incubator.
7. Pipet 200 μL of the transformation mixture in media on the surface of the agar plate, and spread out with a sterile plastic spreader, rotating the plate with the other hand to ensure an even distribution of the mixture.
8. Put a lid on the plate, turn the plate over, and label with a marker pen. Place upside down in the 37 °C oven overnight (~16 h).
9. Add 6 mL of LBK medium to a 15 mL test tube.
10. Pipet 200 μL of the transformation mixture in media in the liquid 6 mL LBK medium. Label the 15 mL tube with a marker pen, place in a shaker, and incubate at 37 °C.
11. Dispose the rest of the tube and spreader as biohazard waste.
12. The next morning, remove the plate from the oven and place at 4 °C in the fridge to store. Use the overnight liquid culture as a starter culture in the next step. Later, the cells from the plate can be used to make a starter culture.

3.2 Overexpression of Soluble GtMCA-I in E. coli

1. Add 400 μL of the overnight culture per 420 mL of ZYM medium containing 50 μg/mL kanamycin in one 2 L shake flask (*see* **Note 3**).

2. Place the flask in a shaking incubator at 250 rpm and 37 °C until optical density of the culture (OD_{600}) reaches a value of approximately 0.8. This usually takes 4–5 h.
3. After that, move the flask to a cooled incubator at 16 °C and continue shaking at 250 rpm overnight (for an additional 18 h) [9].
4. The next day, start by cooling the centrifuge to 4 °C.
5. Harvest the cells by pouring the bacterial culture into 1 L centrifuge bottles. Adjust their weight so that the centrifuge rotor is balanced and spin at 5000 × *g* for 5 min at 4 °C.
6. Pour the supernatant back into the flasks (or any other large container) and decontaminate with 1% (w/v) Virkon or any other laboratory bactericide before discarding.
7. Resuspend the pellet from one flask (*see* **Note 5**) in 30 mL of cold IMAC equilibration buffer.
8. Transfer the resuspended pellet to a 50 mL plastic tube and store at −80 °C until required or proceed immediately with **step 1** of Subheading 3.3 (Lysing Pellet and Extracting Soluble Fraction).
9. Decontaminate the centrifuge bottles with 1% (w/v) Virkon or other laboratory bactericide.

3.3 Lysing Pellet and Extracting Soluble Fraction

For maximum time efficiency, begin Subheading 3.6, **step 1** before moving on to lysing the pellet and extracting the soluble fraction. Although GtMCA-I is a stable protease, all procedures should be performed on ice (4 °C).

1. Put the tube containing the resuspended cells on ice or remove it from −80 °C and thaw it on air. When thawed, place on ice.
2. Lyse the cell suspension by sonicating 3 × 5 min (80% power) on ice using a 130 W sonicator with a 6 mm tip and a pulse cycle of 1 s on, 1 s off. After every 5 min, pause the sonication for 1 min, remove the sample from ice, and mix with a spatula or glass sticki before returning for sonication (*see* **Note 6**).
3. Transfer the lysed solution into a suitable centrifuge tube and spin at ~30,000 × *g* at 4 °C for at least 30 min to remove insoluble debris.
4. Transfer the supernatant to a fresh 50 mL tube and discard the pellet.
5. Using a 10 mL syringe and disposable sterile syringe filters, filter the supernatant from **step 4**, through a 0.45 μm filter into a fresh 50 mL plastic tube. If the filter becomes blocked, it is necessary to use a new filter. Repeat filtration with a 0.2 μm filter, changing filters if required (*see* **Note 7**).

6. Keep the filtered supernatant on ice and proceed directly to Subheading 3.4.

3.4 Nickel Affinity Chromatography

We purify MCAI-His_6 in three steps: via immobilized metal affinity chromatography (IMAC) using Ni-NTA resin followed by ion-exchange chromatography (IEX) with weak anion exchanger and size-exclusion chromatography (gel filtration).

1. Equilibrate the Ni-NTA Cartridge with the IMAC equilibration buffer using the peristaltic pump. If the column was stored in 20% ethanol, wash it first with 10 column volumes of distilled water followed by 10 column volumes of the buffer.
2. Wash the flow path of a chromatography system (e.g., ÄKTA Prime) with equilibration buffer (buffer A inlet) and elution buffer (buffer B inlet).
3. Apply the supernatant to a Ni-NTA Superflow Cartridge equilibrated in IMAC equilibration buffer (*see* **Note 8**) using the peristaltic pump.
4. Connect the column to the ÄKTA system and wash it with IMAC equilibration buffer until a stable baseline is reached (approximately 10 column volumes).
5. Elute bound metacaspase-His_6 with a linear gradient to 100% elution buffer over 10 column volumes in 10 min. Collect fractions of 0.75 mL (Fig. 1a).
6. Select the fractions which contain the eluted protein (or the majority of the eluted protein) and place them on ice.
7. Take a 10 μL sample from each fraction to run on an SDS-PAGE gel. Mix with SDS-PAGE loading buffer and store until running the gel (Fig. 1b).

3.5 Ion-Exchange Chromatography

1. Wash the flow path of a chromatography system with IEX equilibration buffer (buffer A inlet) and IEX elution buffer (buffer B inlet) and equilibrate the HiTrap ANX FF column. If the column was stored in 20% ethanol, wash it first with 10 column volumes of distilled water using the peristaltic pump.
2. Calibrate the tubes of the peristaltic pump in IEX equilibration buffer.
3. Pour 50 mL of the IEX equilibration buffer to a 100 mL beaker and put it on ice.
4. Slowly pipet in the fractions containing the most protein fractions from the IMAC elution. Pipet well to mix. No precipitation should be visible. Connect the IEX column to the peristaltic pump and start loading the diluted fractions onto the IEX column.

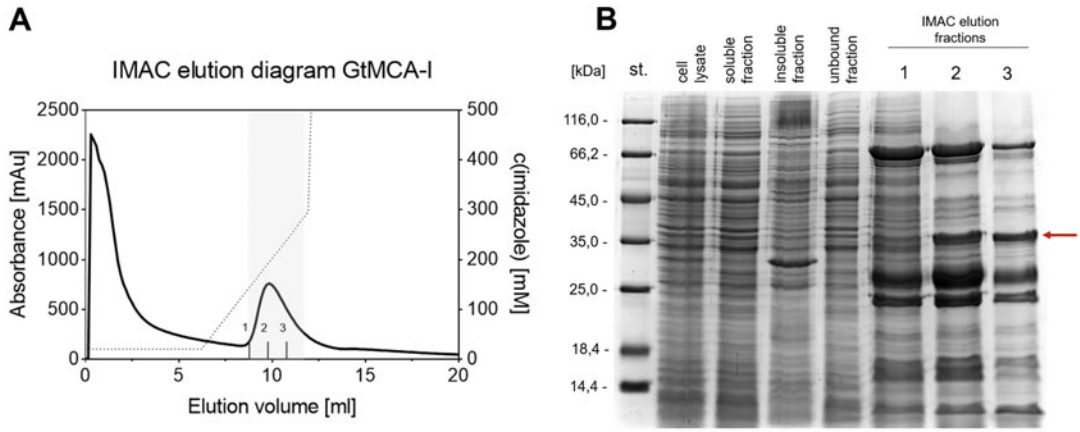

Fig. 1 Nickel affinity purification. (**a**) Elution diagram representing the A_{280} at increasing concentrations of imidazol (gradient: 10 min, flow: 1 mL/min). Fractions 1–3 were chosen for analysis on the SDS-PAGE gel. (**b**) Sample analysis on the 12.5% NaDS-PAGE gel before and after nickel affinity purification. Expected position of the recombinantly expressed GtMCA-I is denoted with a red arrow

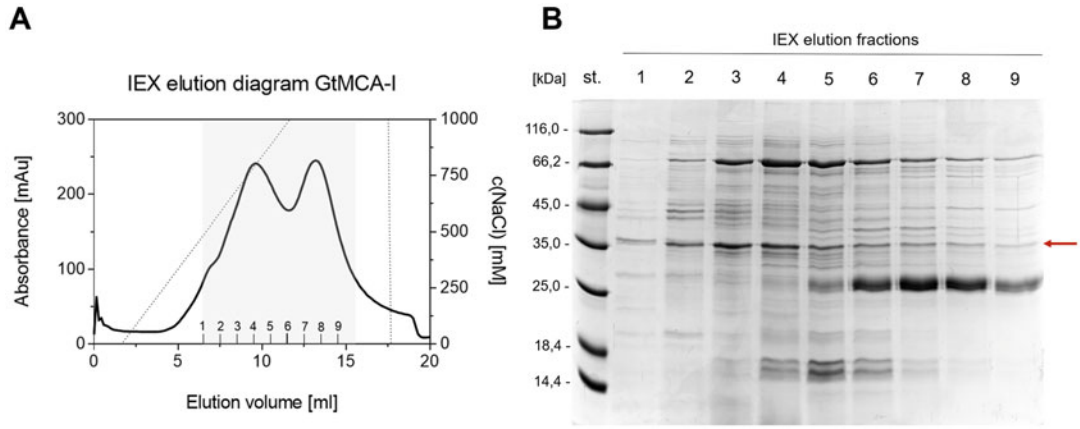

Fig. 2 Ion-exchange chromatography. (**a**) Elution diagram representing the A_{280} at increasing concentrations of NaCl (gradient: 10 min, flow: 1 mL/min). Fractions 1–9 were chosen for analysis on the SDS-PAGE gel. (**b**) Sample analysis on the 12.5% NaDS-PAGE gel after ion-exchange purification. Expected position of the recombinantly expressed GtMCA-I is denoted with a red arrow

5. When all volume is loaded, disconnect the column from the pump and connect it to the ÄKTA system. Wash it with IEX equilibration buffer until a stable baseline is reached (approximately 3 column volumes).
6. Elute bound metacaspase-His_6 with a linear gradient to 100% IEX elution buffer over 10 column volumes in 10 min. Collect fractions of 0.75 mL (Fig. 2a).
7. Select the fractions which contain the eluted protein (or the majority of the eluted protein) and place them on ice.

8. Take a 10 µL sample from each fraction to run on an SDS-PAGE gel (optional). Mix with SDS-PAGE loading buffer and store until running the gel (Fig. 2b).

3.6 Size-Exclusion Chromatography

1. Wash the flow path of a chromatography system with gel filtration buffer (buffer A inlet only) and equilibrate a 25 mL Superdex 75 gel filtration column. This step will take around 2 h, and for maximum time efficiency can be started before lysing the pellet (Subheading 3.3).
2. Pool and concentrate the fractions containing metacaspase-His_6 to 1 mL or 500 µL using an Amicon filtration unit equipped with a 10 kDa exclusion membrane by centrifugation (*see* **Note 9**).
3. Apply 500 µL of concentrated sample onto a Superdex 75 size-exclusion chromatography column, connected to the ÄKTA FPLC system using the 500 µL loop. Use a flow rate of 0.5 mL/min and collect 0.5 mL fractions (Fig. 3a).
4. After size exclusion, collect the fractions containing the most and take a 10 µL sample from each fraction to run on an SDS-PAGE gel (optional). Mix each sample with SDS-PAGE loading buffer and store until running the gel (Fig. 3b).
5. Mix all fractions containing monomeric protein and concentrate back to ~1 mg/mL using an Amicon filtration unit.
6. Measure protein concentration using a UV–Vis spectrometer.
7. Aliquot into 1.5 mL tubes (typically 5, 10, 100 or 200 µL aliquots depending on expected final purpose) and store at −80 °C.

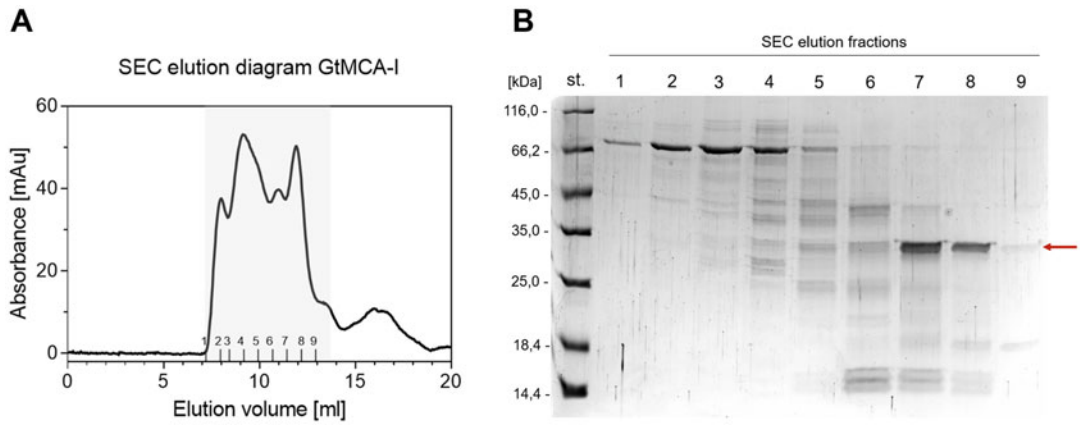

Fig. 3 Size-exclusion chromatography. (**a**) Elution diagram (A_{280}) off GtMCA-I on a Superdex 75 gel filtration column. Fractions 1–9 were chosen for analysis on the SDS-PAGE gel. (**b**) Sample analysis on the 12.5% NaDS-PAGE gel after size-exclusion chromatography. Expected position of the recombinantly expressed GtMCA-I is denoted with a red arrow

4 Notes

1. The gene encoding GtMCA-I was amplified from total *G. theta* mRNA via cDNA in such a way that restriction sites NcoI and XhoI were introduced on the 5′ and 3′ ends, respectively.
2. When preparing 50 × M, salts will most probably not dissolve completely. The mixture can be either warmed in a microwave or directly autoclaved. Autoclaving will heat the solution to a point where all salts dissolve.
3. **Steps 1–3** of Subheading 3.2 (Overexpression of soluble metacaspase-His6 in *E. coli*) should be performed in a sterile workspace, such as in a laminar flow safety cabinet or within the sterile field by a Bunsen burner flame.
4. From 400 mL of culture, usually approximately 3–5 mg of pure protein are obtained. If greater amounts are needed, adjust the number of flasks and consequently the volume of IMAC equilibration buffer can be increased proportionally.
5. There is quite a large amount of biomass after the overnight autoinduction experiment. Resuspension of the cell pellet in IMAC equilibration buffer after cell harvest can be done either manually with a glass stick or with a rotatory cell homogenizer at low speed.
6. When cells are well lysed, a change in color can be observed as it changes from bright brown to darker brown.
7. After sonication and subsequent centrifugation, soluble fraction of cell lysate is firstly filtered through 0.45 μm syringe filter and after with 0.20 μm syringe filter. This step is helpful to remove residual particles prior to chromatography.
8. Equilibrations of columns: all columns are usually stored in 20% ethanol. Before use, each column is firstly washed with 10 volumes deionized water and subsequently 10 volumes equilibration buffer. Appropriate flow for 1 mL columns is 1 mL/min. After elution, columns are washed with 5 volumes elution buffer, 10 volumes deionized water, and 10 volumes 20% ethanol. IMAC can be also performed without chromatography system, using peristaltic pump. Binding and elution profiles can be monitored by SDS-PAGE.
9. We never observed any precipitation when concentrating the GtMCA-I even at high (more than 5 mg/mL) concentrations.

Acknowledgments

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Chapter 2

Expression and Purification of the Type II Metacaspase from a Unicellular Green Alga *Chlamydomonas reinhardtii*

Igor Sabljić, Yong Zou, Marina Klemenčič, Christiane Funk, Jerry Ståhlberg, and Peter Bozhkov

Abstract

Type II metacaspases (MCAs) are proteases, belonging to the C14B MEROPS family. Like the MCAs of type I and type III, they preferentially cleave their substrates after the positively charged amino acid residues (Arg or Lys) at the P1 position. Type II MCAs from various higher plants have already been successfully overexpressed in *E. coli* mostly as His-tagged proteins and were shown to be proteolytically active after the purification. Here we present a protocol for expression and purification of the only type II MCA from the model green alga *Chlamydomonas reinhardtii*. The two-step purification, which consists of immobilized metal affinity chromatography using cobalt as ion followed by size-exclusion chromatography, can be performed in 1 day and yields 4 mg CrMCA-II protein per liter of overexpression culture.

Key words Metacaspase, Cobalt IMAC, Overexpression, Protease

1 Introduction

Type II metacaspases are proteases, which are up to now identified only in the green lineage of eukaryotic organisms: green algae and land plants [1]. Just like type I and type III MCAs, type II MCAs contain a larger p20 domain and a smaller p10 domain. However, only in MCAs of type II, the two domains are separated by a linker domain, which has to be cleaved for the proteases to become active [2–4]. Two main subtypes of type II MCAs were up to now identified: calcium-dependent type II MCAs, ubiquitous in green plants and featuring a longer interdomain linker (>150 aa), and calcium-independent type II MCAs, distributed only in angiosperms and characterized by a shorter linker (<90 aa) [3, 5]. Furthermore, while the calcium-dependent MCAs exhibit optimal activity at neutral or slightly basic pH, the calcium-independent ones are most active at acidic pH [2, 3].

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Two *Arabidopsis thaliana* type II MCAs, AtMCA-IIa and AtMCA-IIf (named according to a new classification [6] and formerly known as AtMC4 and AtMC9, respectively) were the first MCAs to be recombinantly expressed and characterized *in vitro* [2]. Since then, other type II MCAs from *Arabidopsis* [7], Norway spruce (*Picea abies*) [8], wheat (*Triticum aestivum* L.) [9], tomato (*Lycopersicon esculentum*) [10], and tobacco (*Nicotiana tabacum*) [11] were expressed and purified from *E. coli* and methodology for monitoring MCA activity was established [ ]. Molecular mechanisms of type II MCA activation and substrate processing remain poorly understood, largely because of a scarcity of structural data, with AtMCA-IIa being the only type II MCA whose structure is resolved by now [4]. Here, we present the first protocol for expression and purification of an algal type II metacaspase, CrMCA-II from a model green alga *Chlamydomonas reinhardtii*.

2 Materials

2.1 Solutions for Transformation and Protein Expression

1. A glycerol stock of chemically competent *E. coli* BL21 (DE3) cells.
2. pET28b(+) expression plasmid encoding CrMCA-II.
3. 50 mg/mL kanamycin stock solution: Dissolve 0.5 g of kanamycin in 10 mL dH_2O, sterilize by filtering through a 0.22 μm filter, aliquot, and store at −20 °C.
4. LB broth and LB agar plates containing 50 μg/mL kanamycin: Dissolve 25 g LB BROTH HIGH SALT (Tryptone 10 g/L, NaCl 10 g/L, Yeast extract 5 g/L) or 35 g LB AGAR HIGH SALT (Tryptone 10 g/L, NaCl 10 g/L, Yeast extract 5 g/L, Agar 10 g/L) in 1 L deionized water for LB broth and LB agar, respectively. Sterilize by autoclaving at 121 °C for 20 min. When cooled down to approximately 50–60 °C (can be held in hands), add 1 mL of filter-sterilized 50 mg/mL kanamycin stock solution to 1 L LB broth or LB agar. For making LB plates, mix warm LB agar after adding kanamycin gently to avoid bubbles, and pour into sterile Petri dishes. After cooling the plates at room temperature, store at 4 °C and use within 2 months.
5. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose).
6. IPTG: Dissolve 2.38 g of IPTG (Isopropyl β-D-1-thiogalactopyranoside) in 8 mL of sterile dH_2O. Bring to a final volume of 10 mL. Filter sterilize with a 0.22 μm syringe filter. Store this 1 M solution in 1 mL aliquots at −20 °C.

2.2 Other Reagents and Equipment for Transformation and Protein Expression

1. Several 1.5 mL tubes and 2 L or higher volume shake flasks. Sterilize by autoclaving (121 °C, 20 min).
2. Sterile tubes, 15 mL and 50 mL.
3. Syringe and sterilization filters, 0.22 μm and 0.45 μm.
4. Water bath at 42 °C for transformation (a thermo-block can also be used).
5. Incubator at 37 °C for incubation of plates.
6. Sterile plastic spreader.
7. Plastic sterile Petri dishes.
8. An autoclave.
9. A temperature-controlled shaker and incubator with a room for at least four 2 L flasks, which can be set to either 16 °C or 37 °C.
10. A refrigerated centrifuge for centrifugation of larger volumes with the corresponding centrifugation bottles.
11. A high-speed (up to 40,000 × *g*) refrigerated centrifuge with corresponding centrifugation tubes.
12. A spectrophotometer and plastic cuvettes for absorbance measurement at 600 nm.

2.3 Solutions for Protein Purification

Prepare all solutions using distilled water and filter through a 0.45 μm filter (*see* **Note 1**). All solutions can be stored at 4 °C or at room temperature.

1. IMAC binding buffer: 20 mM Tris, 500 mM sodium chloride, pH 7.4. Prepare 500 mL of buffer by mixing 1.2 g Tris, 14.6 g sodium chloride, and deionized water to a volume of 490 mL. Adjust the pH to 7.4 using hydrochloric acid solution. Adjust the volume to 500 mL.
2. IMAC elution buffer: 20 mM Tris, 500 mM sodium chloride, 500 mM imidazole, pH 7.4. Prepare 200 mL of buffer by mixing 0.48 g Tris, 5.8 g sodium chloride, and 4.1 g imidazole. Adjust the volume to 180 mL and adjust pH to 7.4. Finally, adjust the volume to 200 mL.
3. Size exclusion buffer: 20 mM Tris, 100 mM NaCl, pH 7.4. Prepare 500 mL of buffer by mixing 1.2 g Tris, 3.3 g sodium chloride, and 480 mL deionized water. Adjust the pH to 7.4 and the volume to 500 mL.

2.4 Other Reagents and Equipment for Protein Purification

1. Magnetic stirrer.
2. A mechanical device to disrupt *E. coli* cells (cell disruptor).
3. An ÄKTA FPLC system or equivalent equipped with two pumps.
4. HiTrap Chelating HP Column (Cytiva), or equivalent.

5. 0.1 M $CoCl_2$: Dissolve 1.19 g of $CoCl_2$ hexahydrate in 50 mL of dH_2O and mix well.
6. HiLoad 16/600 Superdex 200 pg size-exclusion chromatography column.
7. Filtration unit (bottle top vacuum filter or similar).
8. Vivaspin ultrafiltration unit, or equivalent with a 10 kDa molecular weight cutoff.
9. Ice.
10. 4–20% gradient SDS-PAGE protein gel (or equivalent) with sample loading buffer, running tank, running buffer, and power pack.

3 Methods

3.1 Transformation and Preparation of an Overnight Culture

1. Take chemically competent *E. coli* BL21 (DE3) cells out from −80 °C and put on ice for 20 min.
2. Add 5 ng plasmid to 20 μL of chemically competent *E. coli* BL21 (DE3) cells in a 1.5 mL tube and mix by tapping gently (mixing cells by pipetting will lower the transformation efficiency). Leave the mixture on ice for 30 min.
3. Place the tubes in a water bath at 42 °C for 30 s. Remove and cool on ice for 5 min.
4. Add 250 μL of SOC medium to the transformation mixture and place in a 37 °C shaking incubator for 30–60 min (approximate duration of 1–2 *E. coli* replication cycles).
5. Spin down the cells at the 5000 × *g* for 10 s and discard 220 μL of the supernatant.
6. Mix the pellet with the remaining supernatant. Pipet the mixture onto the surface of the agar plate, and spread out with a sterile plastic spreader to an even distribution of material.
7. Place the plate upside down in the 37 °C incubator overnight (~16 h).
8. Transfer a single colony from the plate to a 3 mL LB medium containing 50 μg/mL kanamycin, in 15 mL sterile tube. Transfer the tube in a shaking incubator and incubate at 200 rpm and 37 °C overnight (~16 h). Store the plate with remaining colonies at 4 °C in the fridge for up to a month.

3.2 Overexpression of Soluble CrMCA-II-His_6 in E. coli

1. Inoculate 500 mL of LB medium containing 50 μg/mL kanamycin, in one 2 L shake flask, with 500 μL of the overnight culture (*see* **Note 2**).

2. Place the flask in a shaking incubator and incubate at 200 rpm and 37 °C until OD_{600} reaches a value between 0.6 and 0.8. This step usually takes 4–5 h.
3. After reaching the desired OD_{600} value, cool down the cell culture by incubation at 4 °C for 30 min.
4. Add IPTG into the cooled cell culture to a final concentration of 0.5 mM, and incubate at 200 rpm and 16 °C overnight (for an additional 18 h).
5. The next day, harvest the cells by pouring the bacterial culture into centrifuge bottles, balance them, and spin at 5000 × *g* for 10 min at 4 °C.
6. Discard the supernatant (*see* **Note 3**), and transfer the cell pellet with a spoon or spatula into a 50 mL plastic tube. Measure the weight of the pellet and store it at −80 °C until required, or proceed immediately with **step 1** of Subheading 3.3 (Cell Lysis and Extraction of the Soluble Fraction).

3.3 Cell Lysis and Extraction of the Soluble Fraction

For maximum time efficiency make sure you prepare the affinity chromatography column (Subheading 3.4, **steps 1–6**), before moving on to lysing the pellet and extracting the soluble fraction. All procedures should be performed on ice (4 °C).

1. By using a spoon or a spatula put the cell pellet (obtained in Subheading 3.2, **step 6**) into a glass beaker and add 5 mL of a precooled IMAC binding buffer per g of cell pellet.
2. Put the beaker on ice and place it on a magnetic stirrer. Stir slowly until the cells are completely homogenized.
3. Lyse the cells by passage through a cell disruptor at 20,000 psi (internal pressure) or equivalent.
4. Remove insoluble cell debris by centrifugation at 38,000 × *g* for 20 min at 4 °C.
5. Collect the clarified supernatant and filter through a 0.45 μm filter into a fresh 50 mL plastic tube and discard the pellet.
6. Keep the filtered supernatant on ice.

3.4 Affinity Chromatography

CrMCA-II-His_6 can be purified in two steps: immobilized metal affinity chromatography (IMAC) using cobalt ions (*see* **Note 4**), followed by size exclusion chromatography. Both steps should be done at 4 °C, and all buffers should be cooled down to 4 °C before use.

For supernatant obtained from 1 L of an overexpression culture (2 × 0.5 L) use a 1 mL HiTrap Chelating HP Column (*see* **Note 5**).

1. If you are using a new HiTrap Chelating HP Column, go to **step 2**, otherwise make sure you are using an affinity column with cobalt ions, and proceed with **step 6**.

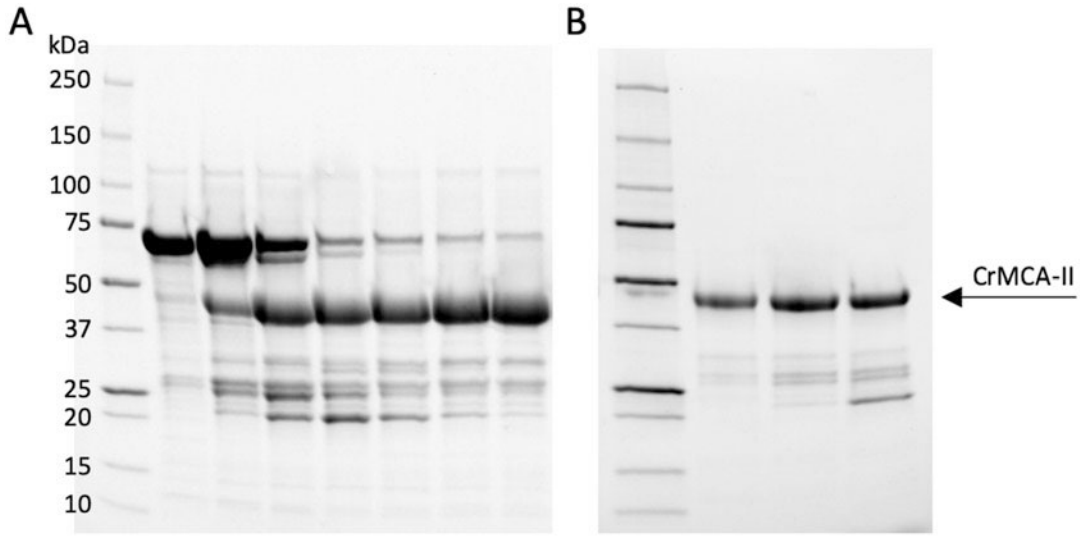

Fig. 1 Purification analysis of CrMCA-II. The 4–20% gradient SDS-PAGE gel of the eluted fractions after affinity chromatography (**a**), and size exclusion chromatography (**b**). The arrow denotes the position of the CrMCA-II

2. Connect the column to an ÄKTA system (or other liquid chromatography system), which was previously washed with filtered distilled water.
3. Wash the column with filtered distilled water, 10 column volumes (CV). For all washing and equilibration steps, the flow rate can be up to 3 mL/min (*see* **Note 6**).
4. Load the column with 0.1 M $CoCl_2$ dissolved in water, 2 CV, flow rate 0.5 mL/min.
5. Wash out excess metal ions with distilled water, 10 CV.
6. Equilibrate the column using IMAC binding buffer, 10 CV (*see* **Note 7**).
7. Apply the supernatant to the column by using 0.5 mL/min flow rate.
8. Wash the column with IMAC binding buffer until a stable baseline is reached (approximately 10 CV).
9. Elute the bound proteins using a linear gradient to 100% elution buffer over 15 CV, flow rate 0.5 mL/min. Collect fractions of 0.5 mL (*see* **Note 8**).
10. Analyze the purity of the eluted fractions on a SDS-PAGE gel (Fig. 1a).

3.5 Size-Exclusion Chromatography

1. Connect the HiLoad 16/600 Superdex 200 pg column to an ÄKTA system (or similar), which was previously washed with filtered distilled water.

2. Equilibrate the column using at least 120 mL of size exclusion buffer (*see* **Note 6**). Since this step takes several hours, consider doing it a day before.
3. Pool and concentrate the fractions with the highest absorbance to 1 mL using a Vivaspin ultrafiltration unit with a 10 kDa molecular weight cutoff.
4. Load the sample to the column using a 1 mL sample loop. Use 0.5 mL/min flow rate and collect 1 mL fractions.
5. Analyze the purity of the fractions on a SDS-PAGE gel (Fig. 1b).
6. Pool all fractions containing the protein and concentrate them using a Vivaspin ultrafiltration unit with a 10 kDa molecular weight cutoff. The final concentration depends on requirements for future experiments.
7. Measure the protein concentration at 280 nm using a UV–Vis spectrometer.
8. Aliquot samples into 1.5 mL tubes and store at −80 °C.

4 Notes

1. Filtering reduces the risk of clogging chromatography columns, valves or tubing on ÄKTA (or similar) fast protein liquid chromatography (FPLC) systems.
2. **Steps 1–4** of Subheading 3.2 (Overexpression of Soluble CrMCA-II-His_6 in *E. coli*) should be performed in a sterile work-space such as in a laminar flow safety cabinet.
3. Decontaminate with 1% (w/v) Virkon or any other laboratory bactericide before discarding. Keep in mind to do the same while washing the labware which was in contact with bacteria.
4. IMAC was tested with nickel and cobalt ions. Nickel ions had lower specificity toward CrMCA-II-His_6, and it was not possible to get the enzyme as pure as it was with cobalt ions.
5. HiTrap Chelating HP Column has binding capacity of 12 mg pure His_6-tagged protein per mL of medium. Overexpression of CrMCA-II in 0.5 L LB medium should yield more than 6 mg of enzyme. In order to completely fill the 1 mL column with the desired enzyme, overexpression should be performed in 1 L medium (2 × 0.5 L). Overloading the column reduces the amount of nonspecific binding to the column, resulting in a much purer enzyme.
6. Columns from various manufacturers have different maximum pressure over the packed bed, resulting in different maximum flow rates. The pressure varies depending on a range of

parameters, such as the characteristics of the chromatography medium and the column tubing used. Do not exceed maximum allowed pressure by increasing the flow.

7. Columns are usually stored in 20% ethanol. Before use, wash the column with filtered deionized water. For IMAC column use 5 CV and for size exclusion use 1 CV.
8. After elution, columns are washed with 5 CV elution buffer, 10 CV deionized water, and 10 CV 20% ethanol.

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Chapter 3

Expression in *Escherichia coli*, Refolding, and Purification of Plant Aspartic Proteases

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Abstract

Aspartic proteases (APs) are widely distributed in plants. The large majority of genes encoding putative APs exhibit distinct features when compared with the so-called typical APs, and have been grouped as atypical and nucellin-like APs. Remarkably, a diverse pattern of enzymatic properties, subcellular localizations, and biological functions are emerging for these proteases, illustrating the functional complexity among plant pepsin-like proteases. However, many key questions regarding the structure-function relationships of plant APs remain unanswered. Therefore, the expression of these enzymes in heterologous systems is a valuable strategy to unfold the unique features/biochemical properties among members of this family of proteases. Here, we describe our protocol for the production and purification of recombinant plant APs, using a procedure where the protein is refolded from inclusion bodies by dialysis. This method allows the production of untagged versions of the target protease, which has revealed to be critical to disclose differences in processing/activation requirements between plant APs. The protocol includes protein expression, washing and solubilization of inclusion bodies, refolding by dialysis, and a protein purification method. Specific considerations on critical aspects of the refolding process and further suggestions for evaluation of the final recombinant product are also provided.

Key words Aspartic proteases, Pepsin-like, Plant, Atypical aspartic proteases, Typical aspartic proteases, Nucellin-like aspartic proteases, Refolding, *E. coli*, Inclusion bodies, Heterologous expression

1 Introduction

Aspartic proteases (APs) of the A1 family (pepsin-like) are widely distributed in plants [1, 2], with the completion of different plant genomes confirming a remarkable diversity of A1 family members [3–5]. These proteases are generally organized into three different groups (typical, atypical, and nucellin-like), based on their domain organizations and active site motifs. Typical plant APs share the general characteristics among members of this family. They are synthesized as pre-proenzymes and, upon maturation, display optimal activity at acidic pH and are inhibited by pepstatin A [1]. A

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unique feature to these plant APs compared to other eukaryotic counterparts is the presence of an extra segment known as the plant-specific insert (PSI), which is very similar to saposin-like proteins and is removed upon maturation [1]. On the other hand, the more recently identified atypical and nucellin-like APs display very distinct features, making them very different from typical APs. This includes the lack of the PSI, higher number of cysteine residues, variable lengths and features at the N terminus, and different sequence signatures of the first catalytic aspartate [2]. Although a relatively small number of atypical and nucellin-like APs have been characterized so far, a remarkably diverse pattern of enzymatic properties, subcellular localizations, and biological functions are observed for these enzymes [2], which likely reflects a higher complexity among non-typical APs. Bearing in mind these differences in domain organization and active site signature, it is not unexpected that the overall catalytic properties might vary significantly between typical and non-typical APs, as well as among non-typical members. Although limited, results available thus far suggest significant differences in optimum pH (ranging from very acidic to pH ~6.5), inhibition profile (broad sensitivity to pepstatin A and varied sensitivity to other classes of inhibitors/compounds), and proteolytic sequence specificity (from broader specificity to strict specificity requirements) among atypical and nucellin-like APs [2]. Moreover, it has been shown that the N-terminal/putative prosegment region might have a different impact on plant typical and non-typical APs. For recombinant cardosin A (a typical AP) autoprocessing of the prosegment domain was required for protease activation, which was accomplished only under acidic conditions [6]. In contrast, recombinant CDR1 and its rice homolog (atypical APs) were shown to be active without the irreversible removal of the prosegment [7, 8]. Chlapsin, a typical AP from *C. reinhardtii* with an atypical N-terminal region, was successfully produced in *E. coli* without this region and comprising only 32 amino acids upstream to the first catalytic aspartate (corresponding to the predicted mature form) [9]. The same has been observed for recombinant At2g42980, also recombinantly produced in its putative mature form (our unpublished data). These different examples illustrate the complex requisites of folding/processing/activation among plant APs, which, combined with their diverse biochemical properties, highlight how little we know (still) about this widespread family of plant proteases and particularly for the non-typical APs.

As many key questions regarding the structure-function relationships of plant APs remain to be answered, expression of these enzymes in heterologous systems has proven to be a valuable strategy to reveal unique features among members of this family of proteases. Various strategies have been published for several plant APs [6–16]. Among them, *Escherichia coli* has been used as a frequent host, although using diverse expression strategies. These

can be divided into procedures where the recombinant protein accumulates in the soluble form, usually using fusion partners [7, 10, 11, 14, 16], and procedures where the protein is refolded from inclusion bodies [6, 8, 9, 13]. We have preferentially adopted this latter method. It was initially developed for human memapsin 2 (also known as beta secretase or BACE1) [17] and was adapted in our laboratory to produce both typical and atypical plant APs [6, 8, 9]. The refolding from inclusion bodies allows for the production of untagged versions of the protease of interest, which can then be purified by standard chromatographic procedures. Indeed, this has allowed us to uncover some of the differences, as mentioned earlier, at the level of plant APs processing/activation (e.g., cardosin A [6], CDR1 [8], chlapsin [9]). Here, we present a detailed workflow for the production of the protease of interest in *E. coli* in the form of inclusion bodies, washing and solubilization of inclusion bodies, protease refolding by dialysis, and purification protocol (Fig. 1).

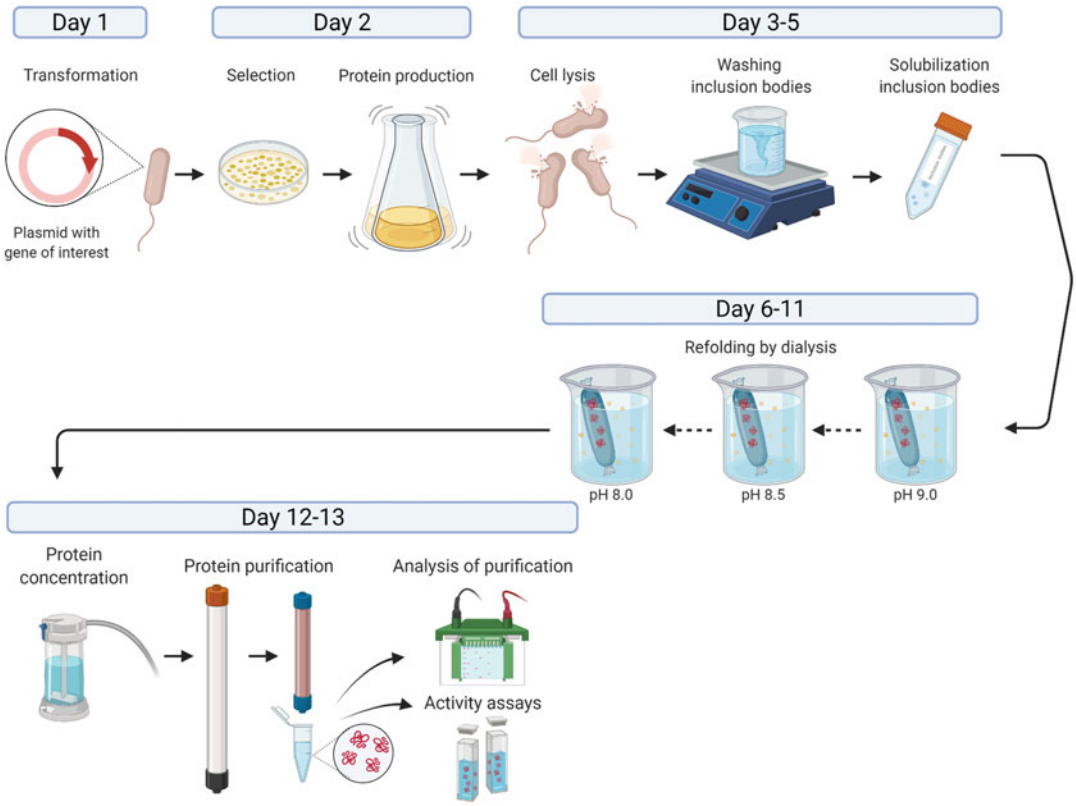

Fig. 1 Timeline and workflow for expression, refolding by dialysis, and purification of plant aspartic proteases. Plasmid transformation, *E. coli* selection, and protein expression (day 1–2). Cell lysis, washing, and solubilization of inclusion bodies (day 3–5). Protease refolding by sequential dialysis at different pH values, includes 48 h incubation at 4 °C after Dialysis Buffer 3 (day 6–11). Protease concentration and purification by size-exclusion chromatography, followed by an ion-exchange chromatography (day 12). Analysis of purified fractions by SDS-PAGE and evaluation of enzymatic activity (day 13). (Created with BioRender.com)

Detailed considerations on each step of the protocol are provided in the following sections.

Nevertheless, it is important to note that the recovery yields of refolding from inclusion bodies may vary between target proteins, and refolding conditions may require some level of optimization for each target protein. In this protocol, we use 8 M urea buffer to solubilize the inclusion bodies, and our refolding buffer has a mixture of oxidizing and reducing agents that allow disulfide bonds to be correctly formed. This is particularly critical in plant non-typical APs, given their higher number of cysteine residues [2]. Other critical aspects in the refolding process include maintaining a low protein concentration in the dialysis membrane to avoid aggregation and keeping a low concentration of the chaotropic agent in the refolding solution and purification buffers to help dissociate aggregates. Furthermore, our protocol includes a preparative size-exclusion chromatography as the first purification step to separate the monodisperse protein from large soluble multimers, which tend to bind tightly to ion-exchange columns [18]. We always recommend assessing the primary structure organization of the target AP and eventually test the expression/refolding of different truncated constructs.

2 Equipment, Consumables, and Reagents

2.1 *Expression in* E. coli, *Washing, and Solubilization of Inclusion Bodies*

1. LB broth, adjusted to pH 7.4 with 1 M NaOH.
2. pH meter.
3. Petri dishes.
4. Magnetic stirrer hotplate.
5. LB-agar: LB media with 15 g/L of agar.
6. Fernbach culture flasks (2.8 L).
7. Sterile syringe filters w/ 0.2-μm cellulose acetate membrane.
8. Ampicillin stock solution: 100 mg/mL ampicillin in water, filtered-sterilized through a 0.2-μm syringe filter. Stock solution can be stored at −20 °C.
9. BL21 Star (DE3) competent *E. coli* (e.g., Invitrogen).
10. Cell spreader.
11. Temperature-controlled incubator.
12. Temperature-controlled incubator shaker.
13. Conical tubes 15 mL.
14. Spectrophotometer (e.g., NanoDrop or equivalent).
15. 0.5 M Isopropyl-β-D-1-thiogalactopyranoside (IPTG) in water.

16. Centrifuge (e.g., Avanti J-26 XPI Beckman Coulter).
17. Fixed-angle rotor for 1 L bottles (e.g., JLA 8.1000, Beckman Coulter).
18. 1 L Polycarbonate centrifuge bottle suited for large centrifuge rotor (e.g., in JLA 8.1000).
19. Fixed-angle rotor for 50 mL tubes (e.g., JA.25.50, Beckman Coulter).
20. Centrifuge polycarbonate bottles with caps (50 mL) for use in smaller rotor (e.g., Beckman Coulter 357002 for use in the JA.25.50 rotor).
21. TN buffer solution (1×): 0.05 M Tris base, 0.05 M NaCl, adjusted with 6 M HCl to pH 7.5.
22. Cell-cracker (e.g., Emulsiflex C3, Avestin) (nitrogen gas line to pressurize the system).
23. TNT buffer solution: TN buffer solution (1×) with 1% Triton X-100 (*see* **Note 1**).
24. 8 M Urea buffer: 8 M urea, 0.1 M Tris base, 1 mM glycine, 1 mM EDTA, adjusted with 1 M NaOH to pH 10.5. Store at room temperature (*see* **Note 2**).
25. Conical tubes 50 mL.
26. β-Mercaptoethanol
27. Roller mixer.

2.2 Refolding by Dialysis

1. Ultracentrifuge (e.g., Optima L-100 XP, Beckman Coulter).
2. Fixed-angle ultracentrifuge rotor for 12 mL tubes (e.g., 90 Ti, Beckman Coulter).
3. Spectrophotometer (e.g., NanoDrop or equivalent).
4. 10 kDa MW cutoff dialysis membrane (flat width: 45 mm, diameter: 29 mm).
5. 8 M Urea buffer (*see* **Note 2**).
6. Dithiothreitol (DTT) (powder) to have a final concentration of 10 mM.
7. L-Glutathione reduced (GSH) (powder) to have a final concentration of 1 mM.
8. L-Glutathione oxidized (GSSG) (powder) to have a final concentration of 0.1 mM.
9. β-Mercaptoethanol.
10. Magnetic stirrer hotplate.
11. Dialysis buffer 1: 20 mM Tris base, adjusted with 6 M HCl to pH 9.0.

12. Dialysis buffer 2: 20 mM Tris base, 0.4 M urea, adjusted with 6 M HCl to pH 8.5.
13. Dialysis buffer 3: 20 mM Tris base, 0.4 M urea, adjusted with 6 M HCl to pH 8.0.

2.3 Protease Purification

1. Stirred concentration cell (e.g., Merck Millipore Amicon) (use clean compressed air or nitrogen gas to pressurize the cell).
2. 10 kDa cutoff regenerated cellulose ultrafiltration membrane discs (e.g., Ultracel 10 kDa, 65.5 mm, Millipore).
3. Ultracentrifuge (e.g., Optima L-100 XP, Beckman Coulter).
4. Fixed-angle ultracentrifuge rotor for 12 mL tubes (e.g., 90 Ti, Beckman Coulter).
5. Vacuum filtration unit.
6. pH meter.
7. Chromatography Buffer A: 20 mM Tris base, 0.4 M urea, adjusted with 6 M HCl to pH 8.0. Filter solution through 0.2-μm filter (vacuum filtration unit).
8. Chromatography Buffer B: 20 mM Tris base, 0.4 M urea, 1 M NaCl, adjusted with 6 M HCl to pH 8.0. Filter solution through 0.2-μm filter (vacuum filtration unit).
9. Fast Performance Liquid Chromatography System (e.g., Cytiva ÄKTA lab-scale systems, BioRad NGC Chromatography System).
10. Gel filtration chromatography column (e.g., HiLoad 26/60 Superdex 200 prep grade, Cytiva, or equivalent).
11. Ion exchange chromatography column (e.g., Mono Q 5/50 GL, Cytiva or equivalent).
12. Gel electrophoresis equipment.

3 Methods

3.1 Expression in E. coli, Washing, and Solubilization of Inclusion Bodies

(Day 1)

1. Prepare 2 L LB media, dispense to Fernbach flasks (1 L per flask), and autoclave.
2. Prepare LB-agar and autoclave. After sterilization, let the media cool down before adding the appropriate antibiotic (e.g., ampicillin: 1:1000 dilution).
3. Pour LB agar plates (100 μg/mL ampicillin), let plates solidify, and store at 4 °C.
4. Transform BL21 Star (DE3) competent cells with the plasmid of interest (e.g., coding sequence of aspartic protease cloned into pET-23 a,d vector) (*see* **Note 3**).

5. Spread the cells onto LB agar (amp) plates, and incubate plates at 37 °C for 18 h.

(Day 2)

6. Confirm colony formation on LB agar plates. Using a bacterial cell spreader, harvest all transformed colonies in 3 mL of LB and transfer to a 15 mL Falcon.
7. Repeat the process with an additional 2 mL of LB.
8. Inoculate 2 mL of this pre-inoculum per 1 L of LB media in the Fernbach flasks. Add ampicillin to a final concentration of 100 μg/mL ampicillin.
9. Incubate the culture at 37 °C with agitation (150 rpm), until reaching an O.D. at 600 nm of 0.6–0.7.
10. Induce protein expression by addition of IPTG to a final concentration of 0.5 mM.
11. Incubate the culture at 37 °C with agitation (150 rpm) for 3 h.
12. Harvest the cells by centrifugation for 20 min at 3000 × *g*, at 8 °C.
13. Resuspend the pellet in 10 mL TN buffer per liter of culture.
14. Freeze the pellet at −20 °C (for at least 18 h) (*see* **Note 4**).

(Day 3)

15. Thaw the cell pellet in TN buffer at room temperature and proceed to cell lysis by passing the cells through an Emulsiflex homogenizer (3 passes at ~25,000 psi). In the end, the solution should not be viscous (*see* **Note 5**).
16. Dilute the cell lysate in 400 mL of TN buffer. Leave with agitation at 4 °C for 18 h (*see* **Note 6**).

(Day 4)

17. Centrifuge for 25 min at 7500 × *g*, at 4 °C, discard the supernatant.
18. Resuspend the pellet in 400 mL TNT buffer. Leave with agitation at 4 °C for 18 h (*see* **Note 6**).

(Day 5)

19. Centrifuge for 25 min at 7500 × *g*, at 4 °C, discard the supernatant. The pellets will have a pinkish color (*see* **Note 7**).
20. Add 10 mL of 8 M urea buffer to the pellet and keep it on ice for 20 min, before resuspension. This incubation step will help to release the pellet. The solubilized inclusion bodies will turn transparent but with a pinkish-brown color.

21. Transfer the solution with the inclusion bodies to a conical tube and adjust the volume to 20 mL with 8 M Urea buffer.
22. Add β-mercaptoethanol to a final concentration of 7 mL/L (*see* **Note 8**).
23. Incubate for 18 h at 4 °C with agitation (using a roller mixer). In the end, the solution should be homogeneous.

3.2 Refolding by Dialysis

(Day 6)

1. Ultracentrifuge the solubilized inclusion bodies for 20 min at 144,000 × *g* at 4 °C. A gelatin-like pellet should be visible corresponding to aggregated protein. Discard the pellet and keep the supernatant on ice.
2. Determine the protein concentration of the solubilized inclusion bodies at two distinct dilutions in water (e.g., 1:100, 1:200) in the NanoDrop (*see* **Note 9**).
3. Assuming a protein concentration of approximately 0.5 mg/mL in the dialysis membrane (**step 4**, this section) (*see* **Note 10**), take the volume equivalent to ~50 mg of solubilized inclusion bodies. Dilute to a final volume of 10 mL in 8 M urea buffer. Add DTT, GSH, and GSSG (*see* **Note 11**). Add β-mercaptoethanol to a final concentration of 7 mL/L (*see* **Note 8**). Mix gently. The solution should turn pinkish in color when β-mercaptoethanol is added.
4. Dilute 10× in 8 M urea buffer and immediately proceed to dialysis.
5. Perform dialysis with 10 kDa MW cutoff membranes overnight against 2 L Dialysis Buffer 1 at 4 °C, with gentle agitation. Distribute approximately 50 mL of the diluted sample per dialysis membrane.

(Day 7)

6. Transfer the two membranes to 2 L Dialysis Buffer 2 and maintain overnight at 4 °C, with gentle agitation.

(Day 8)

7. Transfer the two membranes to 2 L Dialysis Buffer 3 and maintain overnight at 4 °C, with gentle agitation.

(Day 9)

8. Stop the agitation. Keep at 4 °C for 48 h (*see* **Note 12**).

3.3 Protease Purification

(Day 12)

1. Concentrate the 100 mL of dialyzed sample by pressure-driven filtration in stirred concentration cell up to a volume of 10–15 mL.

2. Ultracentrifuge the concentrated sample for 20 min at 144,000 × *g*, at 4 °C, to remove large insoluble protein aggregates. Discard the pellet. Save an aliquot of the supernatant for a quality control SDS-PAGE analysis.
3. Equilibrate gel filtration column with Chromatography Buffer A using the FPLC system.
4. Apply the supernatant onto a gel filtration column previously equilibrated in Chromatography Buffer A. Run the chromatography at a flow rate of 2 mL/min and collect fractions of 10 mL (example in Fig. 2a) (*see* **Note 12**).
5. Pool the fractions corresponding to the non-aggregated forms of the protease (approximate molecular weight estimated by standard protein elution volumes) (*see* **Note 13**). Save an aliquot for a quality control SDS-PAGE analysis (example in Fig. 2c).
6. Using the FPLC system, equilibrate anion-exchange Mono Q column with chromatography Buffer A.
7. Apply the pooled sample to the equilibrated anion-exchange Mono Q column.
8. Elute the protein with a linear gradient of NaCl using chromatography Buffer B at a flow rate of 0.75 mL/min. This protocol is usually performed using the following steps at 0.75 mL/min: 3.75 Column Bed Volumes (CBV) of Chromatography Buffer A; continuous gradient (0–50% Chromatography Buffer B) in 22.50 CBV; continuous gradient (50–100% Chromatography Buffer B) in 3.75 CBV; 3.75 CBV of Chromatography Buffer B; continuous gradient (100–0% Chromatography Buffer B) in 6 CBV; 3.75 CBV of Chromatography Buffer A (example in Fig. 2b) (*see* **Note 14**).
9. Collect fractions of 1 mL. Save an aliquot of purified eluted protein for SDS-PAGE analysis.

(Day 13)

10. Proceed to evaluation of enzymatic activity of purified recombinant protease by testing different substrates [e.g., oxidized insulin β-chain, fluorogenic substrates such as: (7-methoxycoumarin-4-acetic acid) (MCA)Lys-Lys-Pro-Ala-Glu-Phe-Phe-Ala-Leu-Lys-(2,4-dinitrophenyl)(DNP)] (*see* **Note 15**). If possible, evaluate the monodispersity of the protein by analytical size-exclusion-chromatography calibrated with appropriate molecular weight markers.

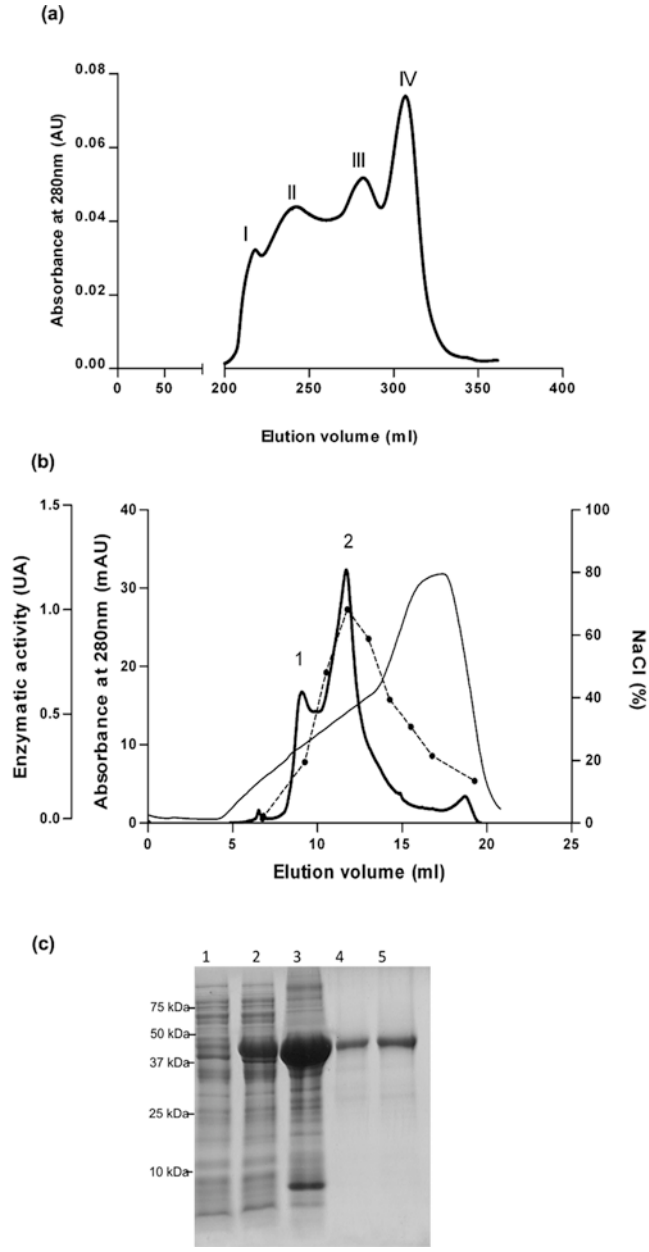

Fig. 2 Purification of recombinant rchlapsin. (**a**) Size-exclusion chromatography of the refolded rchlapsin on a Sephacryl S-300 column. The column was pre-equilibrated with 20 mM Tris–HCl pH 8.0 supplemented with 0.4 M urea (buffer A). Elution was performed at a 0.5 mL/min flow rate and the fractions were detected at 280 nm. (**b**) Ion-exchange chromatography of fraction IV, corresponding to nonaggregated rchlapsin. The fraction was applied onto a Mono-Q column and eluted using a linear gradient of 0–0.5 M NaCl, in buffer A. The enzymatic activity was tested toward the fluorogenic peptide (MCA)Lys-Leu-His-Pro-Glu-Val-Leu-Phe-Val-Leu-Glu-Lys(DPN) and is represented by a discontinuous line. (**c**) SDS-PAGE analysis of the expression and purification

4 Notes

1. The solution should be stirred until Triton X-100 is completely dissolved.
2. To prepare 8 M urea buffer, use a stirrer hotplate. Heat the solution until complete dissolution of reagents (do not overheat the solution), turn off the heat, and let the solution cool down before adjusting the pH. Store at room temperature.
3. Although APs have been successfully produced in *E. coli* with the prosegment domain [6, 8] (whose autoprocessing was observed in typical APs [6], but not in atypical ones [7, 8]), there is also the example of *C. reinhardtii* chlapsin [9] that was produced in its mature form through deletion of the N-terminal sequence up to 35 amino acids to the first catalytic aspartate. Therefore, given the differences at the level of the N-terminal region of plant APs—comprising different sizes for the putative prosegment domain or putative transit peptides [2]—it is recommended to evaluate the primary structure organization of the target AP and test the expression/refolding of different truncated constructs.
4. If no cell cracker is available, cell lysis can alternatively be performed by freeze/thaw cycles. In this case, lysozyme should be added to the resuspended cells at 100 μg/mL before freezing at −20 °C.
5. If performing cell lysis by freeze/thaw cycles, after thawing the pellet add DNase 1 to a concentration of 0.012 mg/mL and $MgCl_2$ to 20 mM. Keep the solution on ice, with occasional stirring until the viscosity is lost. Take in consideration that the efficiency of cell lysis by freeze/thaw cycles is lower than that obtained by using the cell cracker.
6. If necessary, the duration of this step may be reduced to 2 h. Longer washing times help cleaning up the inclusion bodies.
7. If the pellets have a jelly-like (loose) consistency, this may indicate contamination with high molecular weight DNA. To

Fig. 2 (continued) process, stained with Coomassie Brilliant Blue. Lane 1 total protein extract before induction, lane 2, total protein extract after induction, lane 3 inclusion bodies, lane 4 fraction IV from size exclusion chromatography, lane 5, fraction 2 from Mono Q. (Reprinted by permission from Spinger Nature: Spinger, PLANTA, Chlapsin, a chloroplastidial aspartic proteinase from the green algae *Chlamydomonas reinhardtii,* Carla Malaquias Almeida, Cláudia Pereira, Diana Soares da Costa, Susana Pereira, José Pissarra, Isaura Simões, Carlos Faro, Copyright © 2012, Springer-Verlag)

avoid this, make sure that the viscous nucleic acids are removed upon cell lysis (or by treatment with DNaseI).

8. β-Mercaptoethanol is toxic, harmful, and vapors are irritant. Should be always handled in a fume hood.
9. Other protein quantification method can be used. However, we recommend assaying several dilutions as the presence of high concentrations of urea and reducing agents may cause a noticeable interference in the quantification method.
10. The protein concentration should be kept at the lowest concentration that is feasible to reduce aggregation.
11. DTT, GSH, and GSSG should be prepared fresh just before use. The mixture of reducing and oxidizing agents aims to allow disulfide shuffling to contribute to the correct formation of disulfide bonds.
12. This step is important to separate the monodisperse form of the protease from soluble multimers that tend to bind tightly to ion-exchange columns (example in Fig. 2a). The chaotropic agent is maintained in the buffer to dissociate aggregates.
13. The incubation time at 4 °C after dialysis will vary from protein to protein. At least two different refolding times should be tested to evaluate the recovery yield of the non-aggregated form of the protease.
14. In this step, an anion-exchange or cation-exchange column should be selected depending on the theoretical pI of the target protease. Buffer must be adjusted accordingly. The chaotropic agent is maintained in the buffer to dissociate aggregates.
15. We recommend testing different pH values, substrates, and incubation conditions when assessing protease activity, as it has been shown that the enzymatic properties (optimum pH, specificity) of atypical or nucellin-like plant APs may vary significantly from those of typical APs [1, 2].

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Chapter 4

Production of Functional Plant Legumain Proteases Using the *Leishmania tarentolae* Expression System

Elfriede Dall, Andreas Licht, and Hans Brandstetter

Abstract

Plant proteases of the legumain-type are key players in many processes along the plant life cycle. In particular, legumains are especially important in plant programmed cell death and the processing and maturation of seed storage proteins within the vacuole. Plant legumains are therefore synonymously called vacuolar processing enzymes (VPEs). Because of their dual protease and cyclase activities, plant legumains are of great interest to biotechnological applications, e.g., for the development of cyclic peptides for drug design. Despite this high interest by the scientific community, the recombinant expression of plant legumains proved challenging due to several posttranslational modifications, including (1) the formation of structurally critical disulfide bonds, (2) activation via pH-dependent proteolytic processing, and (3) stabilization by varying degrees of glycosylation. Recently we could show that LEXSY is a robust expression system for the production of plant legumains. Here we provide a general protocol for the recombinant expression of plant legumains in Leishmania cells. We further included detailed procedures for legumain purification, activation and subsequent activity assays and additionally note specific considerations with regard to isoform specific activation intermediates. This protocol serves as a universal strategy for different legumain isoforms from different source organisms.

Key words Vacuolar processing enzyme, Legumain, Asparaginyl endopeptidase, *Leishmania tarentolae*, Recombinant expression, Cysteine protease, Ligase, Autocatalytic activation, Transpeptidation

1 Introduction

Proteases are critical regulators of all biological processes along the plant life cycle, including plant growth, senescence, germination, and fruit ripening (see Martinez M. et al. for a Review) [1]. Plant proteases are divided into different families, according to their mechanism of action and evolutionary relationship. In particular, the cysteine proteases of the C13 family, also called legumains, play key roles in many physiological processes [2]. Legumains are primarily expressed in seeds and vegetative organs where they are the main mediators of the processing and maturation of seed storage proteins and in plant programmed cell death [3–7]. *Arabidopsis*

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thaliana produces four functional legumain isoforms and other plants even up to eight variants [3]. Due to their primary localization to the vacuole, they are similarly referred to as vacuolar processing enzymes (VPEs) [8, 9]. Plant legumains are structurally similar to the mammalian caspases and show a strict preference for cleaving after asparagine and, to a lesser extent, aspartate residues [10, 11]. Therefore, they are also termed the Asparaginyl Endo-Peptidases (AEPs). Structural and functional analysis showed that plant legumains are synthesized as inactive proenzymes consisting of a caspase-like catalytic domain (AEP domain) and a C-terminal death domain-like prodomain (LSAM, legumain stabilization and activity modulation domain) that are connected by an activation peptide (Fig. 1a) [11–14]. Full activation of prolegumain proceeds via the auto-proteolytic release of the prodomain at acidic pH, resulting in AEP activity. Additionally, we identified a two-chain intermediate state that occurs along the activation pathway, where cleavage between the AEP and the prodomain occurred but both domains remain non-covalently bound [12]. Activity of plant legumains is further regulated by a latent, enzymatically inactive, dimer state, which was however so far only reported for specific isoforms (e.g., AtLEGγ). On top of their well-established protease activity, plant legumains encode peptide ligase and cyclase activities [15–20]. Plants frequently produce cyclic peptides as a first line of defense against pathogens [15, 18, 21, 22]. Cyclization of these peptides is primarily catalyzed by legumains [23]. Therefore, plant legumains became of particular biotechnological interest, e.g., for protein engineering or the synthesis of cyclic peptides for drug design. However, their recombinant production proved challenging due to manifold posttranslational modifications: (1) The proenzyme is stabilized via three conserved disulfide bonds, (2) activation to the mature AEP form proceeds via proteolytic processing of the prodomain, and (3) stability and full function of the distinct legumain forms, including the active AEP form, depend on glycosylation. Depending on the organism and the isoform, the catalytic AEP domain encodes at least one, but up to four N-glycosylation sites. Expression protocols are available for some plant legumains using *E. coli* SHuffle® cells [15]. However, missing glycosylation renders these recombinant proteins less stable and may even affect their dimerization, activation, and activity profiles. To assess the physiologically relevant legumain forms, it is inevitable to use a eukaryotic expression host. Meanwhile the Leishmania-based expression system LEXSY proved to be a robust choice for the production of plant legumains. *Leishmania* are a family of unicellular, eukaryotic parasites, which switch hosts between sand flies (*Phlebotominae*) and mammals (e.g., dogs, humans) during their life cycle (Fig. 1b). Contrary to other Leishmania, which cause leishmaniasis in mammals, *Leishmania tarentolae* changed its host to lizards during evolution and is no longer able to infect

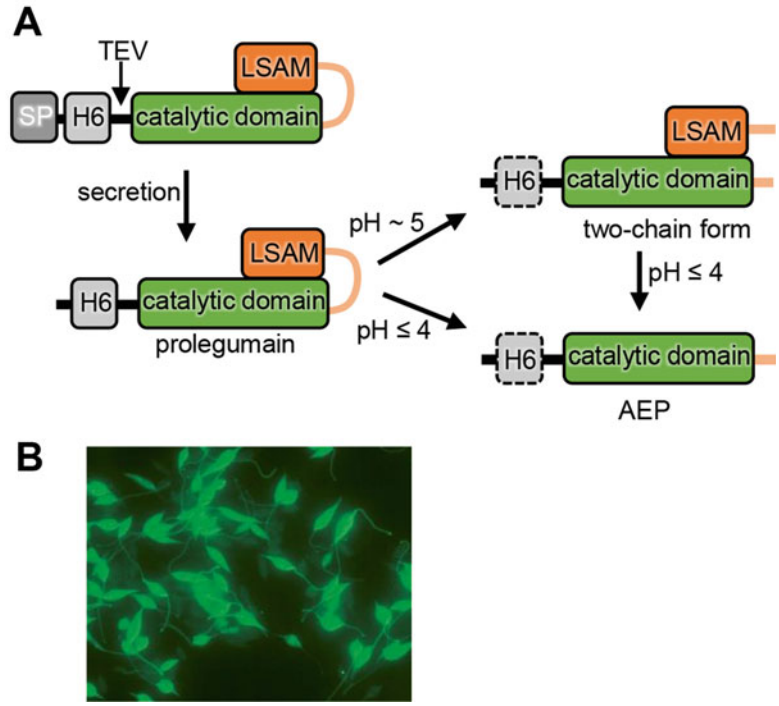

Fig. 1 (**a**) Schematic representation of a plant legumain expression construct, and its subsequent activation. Plant legumains are expressed as secreted proenzymes by Leishmania cells. Activation to the mature protease is triggered by shifting pH to the acidic. *SP* signal peptide, *H6* His_6-tag, *TEV* Tobacco Etch Virus (TEV) protease recognition sequence, *LSAM* legumain stabilization and activity modulation domain, *AEP* asparaginyl endopeptidase. During pH-triggered auto-activation, we frequently observed removal of the N-terminal His_6-tag by legumain, which is indicated by a dashed line surrounding H6. Cleavage occurs after an asparagine residue within the TEV recognition sequence. (**b**) *Leishmania tarentolae* cells expressing enhanced green fluorescence protein

mammals [24]. Despite this host change, *L. tarentolae* kept the mammalian-like glycosylation pattern that can be found in other Leishmania species. These modifications together with its ability for cultivation in suitable media independent from host cells make *L. tarentolae* a very interesting candidate as a protein expression host [25]. The biotech company Jena Bioscience has developed the eukaryotic expression system LEXSY harnessing *L. tarentolae* (Fig. 1) [26]. LEXSY offers simple handling comparable to prokaryotic expression systems, while at the same time providing a eukaryotic protein expression and folding machinery as well as a broad spectrum of posttranslational modifications like disulfide formation, N- and O-glycosylation, acetylation, or myristoylation. The generation time of Leishmania cells in liquid culture is ca. 6–8 h, which is relatively short for a eukaryotic host. Contrary

to other parasites, Leishmania can be cultivated in inexpensive medium like Terrific-Broth (TB) and Brain Heart Infusion (BHI) and are also suited for growth in a bioreactor [27], allowing protein yields of up to 500 mg/L of culture. Using this expression system we could express and purify mg-amounts of *Arabidopsis thaliana* prolegumain isoforms β and γ. These recombinant proteins proved to be functional, i.e., capable of autocatalytic activation, were active in different enzymatic activity assays, had superior long-term stability properties, and were of appropriate homogeneity and quality for successful protein crystallization experiments. Detailed protocols for the expression of *Arabidopsis thaliana* prolegumain isoforms have been published recently [11, 12, 28]. Here we present a simple protocol that may serve as a general basis for the recombinant expression of plant legumains in LEXSY (Fig. 2). We further provide protocols for their purification, activation to the mature AEP form and subsequent activity assays, which are conveniently implemented in any molecular biology lab. Additionally, we discuss specific considerations with regard to isoform specific activation intermediates.

2 Materials

2.1 Preparation of Expression Construct (Cloning)

1. Standard equipment for cloning
2. Standard reagents for cloning
3. pLEXSY-sat2.1 expression vector (Jena Bioscience, Cat. No. EGE-274)
4. Plasmid containing coding sequence of protease of interest
5. DNA gel extraction kit (e.g., Qiagen)
6. PCR tubes
7. P1442 forward insert sequencing primer (5'-CCGACTGCAA CAAGGTGTAG-3′)
8. A264 reverse insert sequencing primer (5'- CATCTATAGA GAAGTACACGTAAAAG-3′)
9. Restriction enzyme SwaI or SmiI
10. Restriction enzyme reaction buffer (supplied with the restriction enzyme)

2.2 Transfection of Leishmania Cells

1. LEXSY P10 host (Jena Bioscience; Cat. No. LT-101)
2. 50 ml Suspension culture flasks
3. Sterile serological pipette tips
4. BHI medium (autoclaved: 15 min, 121 °C, *see* **Note 1**)
5. 500× Pen-Strep (PS) stock solution: 10.000 units of penicillin and 10.000 μg of streptomycin per ml, sterile-filtered

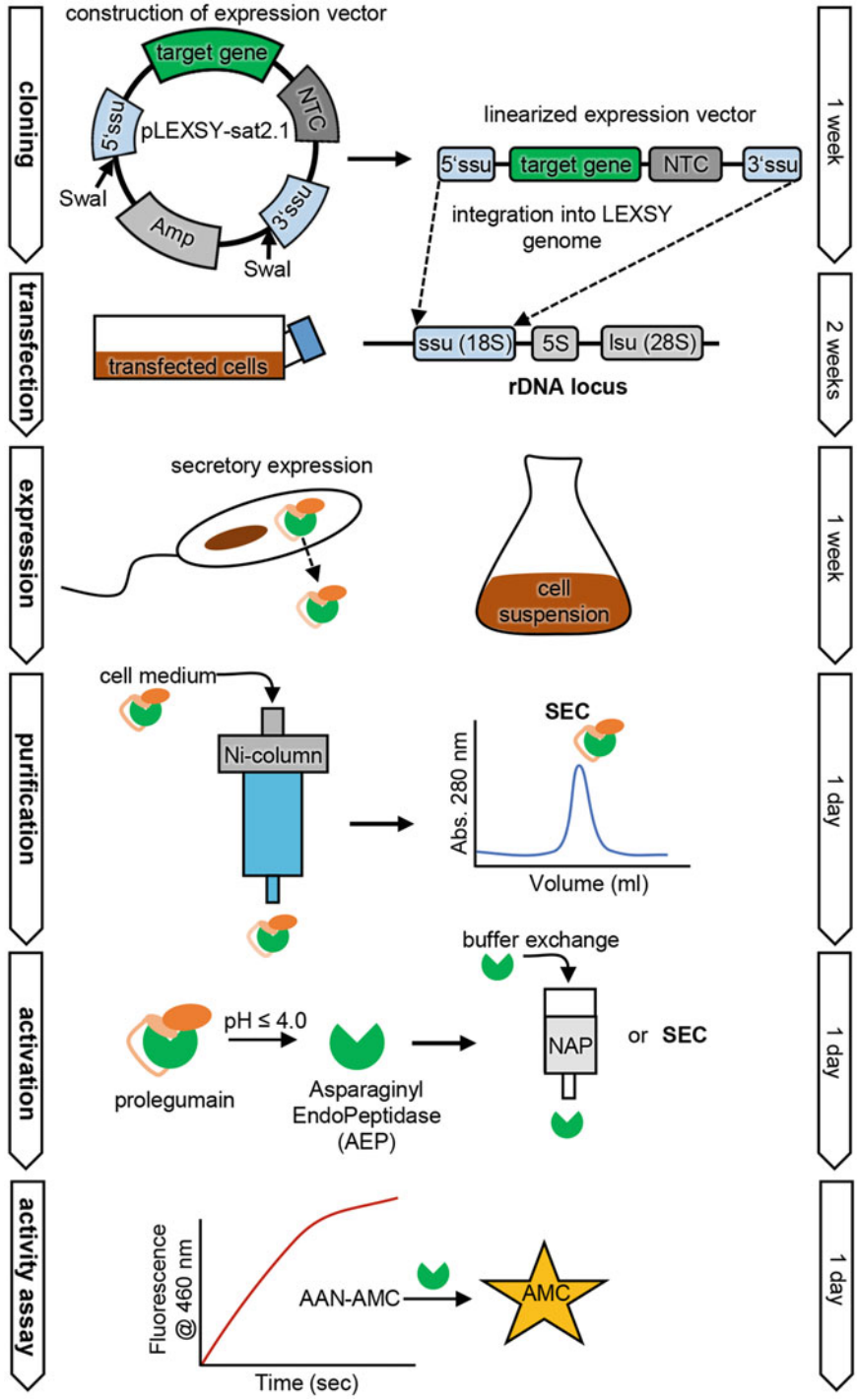

Fig. 2 Workflow and Timeline of recombinant expression of plant legumains in Leishmania cells. The coding sequence of the target protease is inserted into the pLEXSY-sat2.1 expression vector (1 week). The expression cassette is linearized by digesting with SwaI and stably integrated into the Leishmania genome by electroporation and subsequent recombination into the rDNA locus. Stably transfected cells are selected by the addition of the selection marker Nourseothricin (2 weeks). Stably transfected cells are expanded to larger suspension cultures. The recombinant protein is constitutively expressed and secreted to the cell culture medium

6. 200× Hemin stock solution: 0.25% porcine Hemin in 50% triethanolamine, sterile-filtered, stored in the dark
7. Incubator at 26 °C
8. Inverse microscope
9. Electroporation device (e.g., BioRad GENE PULSER Xcell with PC and CE modules)
10. Electroporation cuvettes with long electrodes (0.4 mL must fit between the electrodes)
11. 1000× Nourseothricin stock solution: 100 mg/mL Nourseothricin, sterile-filtered

2.3 Preparation of Glycerol Stock

1. 80% Glycerol, autoclaved
2. Sterile cryovials 2 mL
3. Freezing Container (e.g., Mr. Frosty™, ThermoFisher Scientific)

2.4 Large-Scale Expression

1. 650 mL Suspension culture flasks
2. Expression flasks (2 L)
3. Shaking cell incubator (with window covered to protect the inside from light)
4. Centrifuge (with capacity to fit 1 L bottles)
5. Schott bottle (1 L)

2.5 Purification

1. Ni^{2+}-NTA resin
2. Gravity flow columns
3. pH meter
4. Gravity flow loading buffer: 20 mM Hepes pH 7.0 and 300 mM NaCl
5. Elution buffer: 20 mM Hepes pH 7.0, 300 mM NaCl, 250 mM imidazole
6. Reaction tubes 15 or 50 mL
7. Gel electrophoresis equipment
8. SDS-PAGE gels
9. Spin filtration columns with 3 kDa MW cutoff

Fig. 2 (continued) (1 week). The cell culture medium is harvested and applied to a Ni^{2+}-column for affinity purification utilizing a His_6-tag. Subsequently, eluted protein is further purified by size exclusion chromatography (SEC) (1 day). To activate legumain, pH is shifted to the acidic (pH $\leq$4.0) and activated protein is further buffer exchanged using a NAP™ column or subjected to SEC, if the activation was incomplete (1 day). Success of activation is further assessed using the legumain specific Z-Ala-Ala-Asn-AMC (AAN-AMC) fluorescence substrate (1 day)

10. Reaction tubes 0.5 and 1.5 mL
11. FPLC system
12. S200 or S75 size exclusion chromatography (SEC) column
13. SEC buffer: 20 mM Hepes pH 7.0 and 100 mM NaCl

2.6 Activation

1. 1 M citric acid, pH adjusted to 4.0
2. 2 M dithiothreitol (DTT)
3. Temperature-controlled benchtop incubation shaker
4. MMTS stock solution: 500 mM S-methyl Methanethiosulfonate (MMTS)
5. desalting column (e.g., NAP-5 column, cytiva)

2.7 Activity Assay

1. AAN-AMC stock solution: 50 mM Z-Ala-Ala-Asn-AMC substrate (AAN-AMC, Bachem) in DMSO
2. Substrate solution: 50 mM citric acid pH 5.5, 100 mM NaCl, 2 mM DTT, and 100 μM AAN-AMC substrate
3. Fluorescence plate reader
4. 96-well plates (fitting to the respective fluorescence plate reader)

3 Method

3.1 Preparation of Expression Construct (Cloning)

1. Insert the coding sequence of your protease of interest into the pLEXSY-sat2.1 expression vector using standard cloning procedures (*see* **Notes 2** and **3**). Our plant legumain expression constructs typically carry an N-terminal His_6-tag followed by a TEV recognition sequence, which we introduced after the XbaI restriction enzyme cleavage site by using an extended primer for PCR amplification (*see* **Note 4**). If the expression construct should carry an N-terminal His_6-tag, use XbaI and NotI restriction sites, if it should harbor a C-terminal His_6-tag, use XbaI and KpnI sites (*see* **Note 5**).
2. Confirm success of cloning by DNA sequencing using the P1442 (forward) and A264 (reverse) sequencing primers.
3. Digest 10 μg of plasmid with 20 U of SwaI in reaction buffer that was supplied with the enzyme for 1 h at 30 °C (*see* **Note 6**).
4. Mix with DNA loading buffer and run on a 1% agarose gel.
5. If the SwaI digestion works out as expected, two bands will be visible: the upper band will be 4800 bp + X bp (X = size of your insert) in size (Fig. 2).

6. Cut out the upper band and extract the DNA following the protocol of your preferred gel extraction kit. Elute the DNA in a maximum of 30 µL of ddH_2O (*see* **Note 7**).
7. Freeze DNA if not needed immediately.

3.2 Transfection of Leishmania Cells

1. Thaw frozen Leishmania P10 cells on ice.
2. Transfer 10 mL BHI medium to a 50 mL cell culture flask (*see* **Notes 8** and **9**).
3. Add 50 µL of PS and 20 µL of Hemin to the medium (*see* **Notes 10** and **11**).
4. Transfer the entire content of the vial (1.6 mL) of thawed Leishmania cells to the medium.
5. Incubate at 26 °C (dark, flask positioned flat), for 4 days or until culture gets cloudy, i.e., $OD_{600} > 2$. Frequently check under a microscope if the cells are vital by monitoring increase in cell density (Fig. 1b).
6. Passage dense culture 1:10 to a new cell culture flask containing 10 mL BHI + PS + Hemin (*see* **steps 2** and **3**) and incubate for 3 days (flask positioned upright).
7. Passage culture 1:5 to new cell culture flask containing 10 mL BHI + PS + Hemin (*see* **steps 2** and **3**) and incubate overnight (24 h, flask positioned flat).
8. On the next day, check if the cells are dense (OD_{600} between 1 and 2) and vital.
9. Spin cells for 3 min at 2000 × *g* and 20 °C.
10. Carefully remove and discard half of the supernatant.
11. Carefully resuspend pellet in remaining medium and put on ice for 10 min.
12. In the meantime, thaw 30 µL of the gel-extracted, linearized DNA and precool an electroporation cuvette on ice.
13. Transfer 350 µL cold cells to the DNA, mix gently, and subsequently transfer the mix to the electroporation cuvette.
14. Electroporate at 450 V, 450 µF (*see* **Note 12**).
15. Incubate cuvette on ice for exactly 10 min.
16. In the meantime, prepare 10 mL BHI medium in a 50 mL cell culture flask and supplement with PS and Hemin (*see* **steps 2** and **3**).
17. After 10 min, transfer cells into medium using a 1 mL or 100 µL pipette.
18. Incubate cells at 26 °C (flask positioned flat) for 16–24 h.

19. For polyclonal selection, add 10 μL of Nourseothricin (= selection marker) and continue incubation at 26 °C (*see* **Notes 13** and **14**).
20. After 2 days, transfer 2 mL of the culture into 10 mL BHI medium (=1:5 dilution) supplemented with PS, Hemin, and Nourseothricin and incubate at 26 °C (flask positioned flat).
21. Frequently check the cells under the microscope. After 5–7 days, the 1:5 diluted cells will appear cloudy/dense.
22. Once the cells of the 1:5 dilution are dense, do a 1:10 dilution in 10 mL BHI medium supplemented with PS, Hemin, and Nourseothricin and incubate at 26 °C for 4 days (flask positioned standing).
23. Maintain the stable culture as continuous 10 mL suspension culture. Passage the stable culture twice a week (1:10, e.g., Monday and Friday; *see* **Note 15**).

3.3 Preparation of Glycerol Stock

1. Passage dense culture 1:5 in BHI medium supplemented with PS, Hemin, and Nourseothricin and incubate for 24 h at 26 °C (flask positioned flat).
2. On the day when cryo-stocks shall be prepared, check that the cells are dense and vital ($OD_{600} > 3$; *see* **Note 16**).
3. Put 1.2 mL of an 80% glycerol solution (autoclaved) in a 15 mL Falcon tube.
4. Add 3.6 mL of cells and gently mix by inverting.
5. Transfer 1.6 mL to three 2 mL sterile cryovials.
6. Incubate for 10 min at 20 °C.
7. Transfer to a freezing container that was pre-equilibrated at 4 °C.
8. Transfer freezing container to −80 °C freezer and leave inside overnight.

3.4 Large-Scale Expression

1. Inoculate 200 mL of BHI medium supplemented with 1 mL PS and 400 μL Hemin with 10 mL of dense Leishmania culture ($OD_{600} \sim 2.0$) in a 650 mL cell culture flask (*see* **Note 17**).
2. Incubate at 26 °C, lying flat, for 3 days.
3. After 3 days, prepare four non-baffled, 2 L expression flasks containing 500 mL of autoclaved BHI medium.
4. Add 2 mL PS, 1 mL Hemin, and 50 mL of pre-culture to the expression flasks.
5. Incubate at 26 °C, dark, shaking at 140 rpm for 48 h until cells reach an $OD_{600} = 3.5–4.5$ (*see* **Notes 18** and **19**).
6. Harvest medium by centrifugation at 4000 rpm and 4 °C for 10 min.

7. Carefully transfer the supernatant containing the target protein to two 1 L Schott bottles using a 50 mL serological pipette (*see* **Note 20**).

3.5 Purification

1. Load two gravity flow columns with 5 mL Ni^{2+}-beads per column (column volume ca. 50 mL each).
2. Wash beads two times with 50 mL dH_2O and once with 50 mL loading buffer composed of 20 mM Hepes pH 7.0 and 300 mM NaCl (gravity flow, *see* **Note 21**).
3. Add ca. 40 mL of medium to the beads, close the column, mix by inverting and transfer the slurry to the Schott bottles containing the medium.
4. Add a magnetic stirrer to the medium and incubate with the Ni^{2+}-beads, gently stirring at 4 °C for 1 h.
5. Harvest the Ni^{2+}-beads back into the columns, by gravity flow.
6. Some beads will stick to the inside of the glass bottle. Wash them out by adding approx. 50 mL of loading buffer, shake well, and pour the wash into the columns.
7. Empty the columns by gravity flow and repeat this step four times.
8. Elute the bound protein by applying 5 mL of elution buffer containing 20 mM Hepes pH 7.0, 300 mM NaCl, and 250 mM imidazole. Carefully mix the beads with the elution buffer and incubate for 5 min at 4 °C on a horizontal shaker or by manually mixing it from time to time.
9. Collect the elutions in a 15 mL falcon tube and put on ice.
10. Repeat **steps 7** and **8** one more time.
11. Finally, add 30 mL of elution buffer to the beads, mix gently, incubate for 5 min, and collect elutions in a 50 mL falcon tube.
12. Check on SDS-PAGE if the purified protein migrates at the expected height (Fig. 3). It may happen though that the protein concentration of the elutions is too low to get a clear signal on SDS-PAGE. If that is the case, analyze your protein by western blot or after concentrating it (**steps 13–15**; *see* **Notes 22–25**).
13. Concentrate the elutions (80 mL in total) at 4 °C to a final volume of approx. 1.5 mL using a spin filter device with 3 kDa MW cutoff. Start with elution number 3 (*see* **step 11**), as it has the lowest protein concentration. Pool all elutions in the same spin filter device.
14. When all elutions were collected in the spin filter, concentrate the protein solution to approx. 1.5 mL. Subsequently do a buffer exchange by filling the spin filter with SEC buffer and

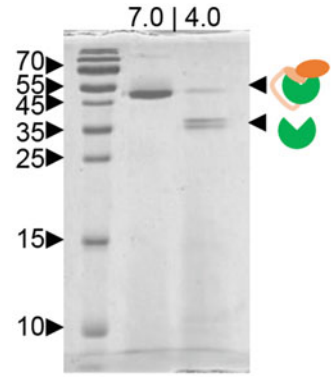

Fig. 3 SDS-PAGE gel showing *A. thaliana* prolegumain isoform β at pH 7.0 and after activation at pH 4.0. The double band observed after activation (pH 4.0) corresponds to heterogenous cleavage within the TEV-recognition sequence and subsequent removal of the N-terminal His_6-tag

concentrating down to approx. 1.5 mL. Repeat this step twice. Finally concentrate the protein down to 0.5–1 mL.

15. Transfer the concentrated protein to a 1.5 mL tube.
16. Load the concentrated protein on an S200 or S75 size exclusion column pre-equilibrated in SEC buffer.
17. Collect the peak fractions and analyze them on SDS-PAGE.
18. Pool the fractions containing the prolegumain and concentrate them using a spin filter device to 1–2 mg/mL final concentration.
19. Determine protein concentration by measuring the UV_{280} signal.
20. Store the concentrated protein in aliquots of, e.g., 20 μL in thin-walled PCR tubes at −20 °C.

3.6 Activation

1. Dilute the proenzyme to 0.5 mg/mL in SEC buffer (ideally, the final volume should be ≥0.5 mL; *see* **Note 26**).
2. Add citric acid stock solution (pH 4.0, 1 M) to achieve a final concentration of 100 mM (*see* **Notes 27** and **28**).
3. Add DTT to a final concentration of 2 mM (stock: 2 M, *see* **Note 29**).
4. Incubate at 30 °C for 20 min.
5. Check the progress of activation by loading samples after different time points of incubation on SDS-PAGE. Add 10 mM MMTS to your samples before loading them on SDS-PAGE (*see* **Note 30**). Activation is finished when the prodomain (LSAM domain) was completely cleaved and disappears on SDS-PAGE (band at around 10–15 kDa, *see* **Notes 31** and **32** and Fig. 3).

6. Stop activation by addition of 4 mM MMTS.
7. Concentrate the activated protein to a final volume of 0.5 mL using an Amicon (cutoff: 3 kDa).
8. Pre-equilibrate a NAP-5 column in desalting buffer containing 20 mM citric acid pH 4.0 and 100 mM NaCl.
9. Load 0.5 mL of activated and concentrated protein onto the NAP-5 column and let it migrate into the column.
10. Elute protein by applying 1 mL of desalting buffer. Collect the elution in a 1.5 mL tube.
11. Determine protein concentration by measuring the UV_{280} signal.
12. Concentrate protein to 1–20 mg/mL concentration, depending on downstream applications, using an Amicon (cutoff: 3 kDa).
13. Store the concentrated protein in aliquots of, e.g., 20 µL in thin-walled PCR tubes at −20 °C (*see* **Note 33**).

3.7 Activity Assay

1. Prepare the substrate solution composed of 50 mM citric acid pH 5.5, 100 mM NaCl, 2 mM DTT, and 100 µM of AAN-AMC substrate.
2. Pre-incubate the substrate solution at 25 °C.
3. Dilute the enzyme in desalting buffer (pH 4.0) to a final concentration of 600 nM.
4. Measure substrate turnover at 25 °C in a fluorescence reader (e.g., Tecan Plate reader) after mixing 63 µL of substrate solution with 7 µL of enzyme solution in a 96-well plate. Excitation wavelength: 380 nm, emission wavelength: 460 nm.

4 Notes

1. BHI medium should not be autoclaved too long (15 min at 121 °C max.), otherwise the cells will not grow properly. If growth problems are encountered, point autoclaved or sterile filtered BHI can be tested as an alternative.
2. For some of our constructs it proved beneficial in terms of expression yield to do a codon optimization toward the Leishmania codon usage table. As in our hands codon optimization never reduced the expression yield, we recommend to optimize the sequence, if possible.
3. In case the protein sequence contains an N-terminal ER or vacuolar sorting signal, we recommend to remove that sequence from the expression construct. By doing so, we could increase the expression yield of some of our constructs.

4. In principle both SalI and XbaI can be exploited as 5′ cutting restriction enzymes to insert the target sequence after the leishmanial signal peptide for secretory expression. However, in our experience it proved beneficial in terms of expression yield to use the XbaI site. If SalI is used, attention has to be paid to not disrupt the signal peptidase cleavage site, which is located in between the SalI and XbaI cleavage sites. Specifically, the GCT GGC sequence has to be added directly after the SalI cleavage site to maintain the signal peptidase cleavage site.
5. As a default, the pLEXSY-sat2.1 plasmid encodes a C-terminal His_6-tag for affinity purification. However, for most of our plant legumain expression constructs we rather use an N-terminal His_6-tag followed by a TEV-cleavage site which we introduced in the expression vector between signal peptide and the 5′-end of the actual expression construct. To remove the C-terminal His_6-tag, we used NotI as 3′ cutting enzyme and introduced a stop codon before the NotI restriction enzyme cleavage site.
6. We recommend doing the SwaI digest at 30 °C for no longer than 1 h. Incubation at 37 °C may result in unspecific cleavages, due to the high Star activity of SwaI.
7. When linearizing Leishmania expression vectors for transfection, purification after SwaI digestion is in principle not necessary. The salt from the restriction enzyme buffer will not affect transfection efficiency. If problems with contamination are encountered, ethanol precipitation of the digested plasmid can help. As we so far did not yet test transfection with un-purified, linearized vector, we are however hesitant to recommend it.
8. Handling Leishmania cells does not require a laminar flow sterile bench. However, we recommend to handle cells and media next to a flame.
9. In principle, Leishmania cells grow well in BHI, TB, as well as several types of insect cell medium. Although TB medium is cheaper and works great for expression runs, BHI is recommended for strain maintenance, preparation of glycerol stocks, and for preparing cells for transfection, as it gives more reproducible results.
10. Penicillin and Streptomycin can be added to Leishmania medium during growth to prevent contamination with bacteria. These antibiotics do not affect Leishmania cells.
11. Leishmania cells require Hemin in the medium, since they cannot synthesize it themselves. Because Hemin is light sensitive, Leishmania should always be cultivated in the dark.

12. Leishmania cells can only be transfected by electroporation or a particle gun. Normal transfection reagents will not work.
13. In principle, it is recommended to do a clonal selection after transfecting Leishmania cells with a new expression construct. Typically, the expression levels when integrating the protein of interest into the genome are comparable between different clones, so screening ca. 10 clones is usually enough to find a suitable clone. However, as we did not observe significant differences in expression levels between different expression clones for our legumain constructs, we rather go for polyclonal expression.
14. When plating Leishmania, the agar concentration in the plate should be 1% and the plates must not be dried for more than 10 min.
15. Stable cultures may be passaged up to 20 times without significant loss in expression yield.
16. When preparing glycerol stocks, it is beneficial if the cells have a relatively high density ($OD_{600} > 3$). Low cell density in the glycerol stocks will result in a non-viable stock.
17. To reduce costs, we recommend to leave out the selection antibiotic (Nourseothricin) during large-scale expression. In our experience, leaving out the selection antibiotic did not adversely affect the expression yield.
18. When growing Leishmania in a fermenter, the oxygen saturation should be kept at ca. 20%. Too much aeration will inhibit growth.
19. The maximum of protein expression is usually reached 2–3 days after inoculation.
20. After spinning down Leishmania cells, the cell pellet is rather soft and cells easily detach. Be careful not to disturb the cell pellet when removing the medium.
21. All buffers used during purification should be pre-cooled to 4 °C.
22. Leishmania cells have many proteins with internal multi-histidine patches, which can give background bands in western blots when using anti-His antibodies. To prevent this, blocking with 4% milk powder in TBS is recommended. If the expression level of the target protein is low, block with TBS + 2% BSA instead, to allow detection. These multi-histidine proteins can also bind to Ni^{2+}-NTA agarose columns if only little target protein is expressed and can be removed by stringent washing.
23. When purifying secretory proteins, Hemin can sometimes inhibit protein binding to Ni^{2+}-NTA agarose columns. In such cases, concentrating the supernatant or removing the

Hemin with decontamination bags with activated charcoal can greatly improve the protein yield.

24. Sometimes secretory proteins get stuck in the ER or Golgi apparatus. These proteins are usually glycosylated and in most cases also had their signal peptide cleaved off. The proteins are typically folded correctly and also active and can then be prepared from cell pellets.
25. If encountering low expression levels with a secretory protein, switching to the protein's native signal peptide instead of the leishmanial one can help. Most eukaryotic secretion signals are recognized by Leishmania cells.
26. For some applications it may be necessary to remove the N-terminal His_6-tag using TEV protease. Since TEV protease requires neutral pH for cleavage, and the prolegumain is typically more stable at neutral pH as compared to the active enzyme, we recommend to cleave off the His_6-tag from the proenzyme, rather than the active enzyme. The TEV-recognition sequence (ENLYFQ|G) harbors a surface exposed asparagine, and may therefore also serve as a substrate to legumain. During auto-activation, we observed removal of the His_6-tag for some of our constructs (Fig. 3).
27. Some legumain isoforms, like, e.g., *A. thaliana* legumain isoform γ (AtLEGγ), are expressed as enzymatically latent dimers, where dimerization is mediated by LSAM – LSAM' interactions. This dimer formation is critically dependent on pH and protein concentration. Activation of proAtLEGγ, i.e., release of the prodomain, will efficiently work only at low pH (pH 4.0), where the dimer dissociates to monomeric two-chain legumain and the prodomain is released. Monomeric, two-chain AtLEGγ can be generated upon incubation at pH 4.5.
28. Two-chain legumain is typically generated by incubation of the proenzyme at pH approx. 4.5–5.0. Success of activation needs to be monitored over time by SDS-PAGE. The optimal pH depends on the respective legumain isoform.
29. Different legumain isoforms require slightly different conditions for activation, e.g., different concentrations of DTT or incubation times. In some cases, it may also be beneficial to use β-mercaptoethanol instead of DTT. These parameters have to be optimized for the respective legumain isoform. However, as a starting condition we recommend to use 2 mM DTT and 20 min incubation at 30 °C.
30. In our hands, it frequently happens that the legumains degrade themselves when mixed with SDS-PAGE loading buffer, especially when it is a reducing buffer containing DTT. In case legumain looks degraded on SDS-PAGE, add 10 mM MMTS to the SDS-PAGE loading buffer.

31. If after activation the LSAM domain is still visible on SDS-PAGE, it is likely forming a stable complex with the AEP domain and thereby (partly) blocking its activity. Therefore, it is critical to monitor the progress of activation on SDS-PAGE and/or by using the fluorescently labeled AAN-AMC peptide substrate.
32. If the activation was incomplete, i.e., the proenzyme and/or the prodomain are still visible on SDS-PAGE, load the reaction on a S75 or S200 size exclusion column pre-equilibrated in buffer composed of 20 mM citric acid pH 4.0 and 100 mM NaCl to separate the proenzyme and the two-chain form from the fully activated enzyme. Analyze fractions by SDS-PAGE and concentrate those containing the active enzyme.
33. For some legumain isoforms it proved beneficial to add 0.5–2 mM MMTS to the storage buffer of the activated enzyme. MMTS will inhibit auto-degradation of the active enzyme. MMTS can be removed any time later by adding 5 mM DTT.

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Chapter 5

Purification of His-Tagged Proteases from the Apoplast of Agroinfiltrated *N. benthamiana*

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Abstract

Protein expression in plants by agroinfiltration and subsequent purification is increasingly used for the biochemical characterization of plant proteins. In this chapter we describe the purification of secreted, His-tagged proteases from the apoplast of agroinfiltrated *Nicotiana benthamiana* using immobilized metal affinity chromatography (IMAC). We show quality checks for the purified protease and discuss potential problems and ways to circumvent them. As a proof of concept, we produce and purify tomato immune protease Pip1 and demonstrate that the protein is active after purification.

Key words Recombinant protein, Agroinfiltration, Protease, Protein purification, *Nicotiana benthamiana*, His-tag, Immobilized metal affinity chromatography

1 Introduction

Plant expression systems have emerged as suitable alternatives to classic protein expression platforms [1]. Over recent years, transient expression of recombinant proteins in *Nicotiana benthamiana* using Agrobacterium-mediated transformation has become very popular among plant researchers. Active serine proteases from tomato have already been purified after being produced in agroinfiltrated *N. benthamiana* [2, 3] presenting this platform as a suitable alternative for the production of plant proteases.

Here we describe a way to produce secreted His-tagged proteases in *N. benthamiana* and how to purify them via immobilized metal affinity chromatography (IMAC), which uses immobilized metal ions to purify histidine-containing proteins from aqueous solutions. To illustrate the overall strategy, we use the tomato papain-like-cysteine protease Pip1 as an example (Fig. 1). A C-terminally His-tagged version of Pip1 is cloned into an overexpression vector and transformed into *Agrobacterium*

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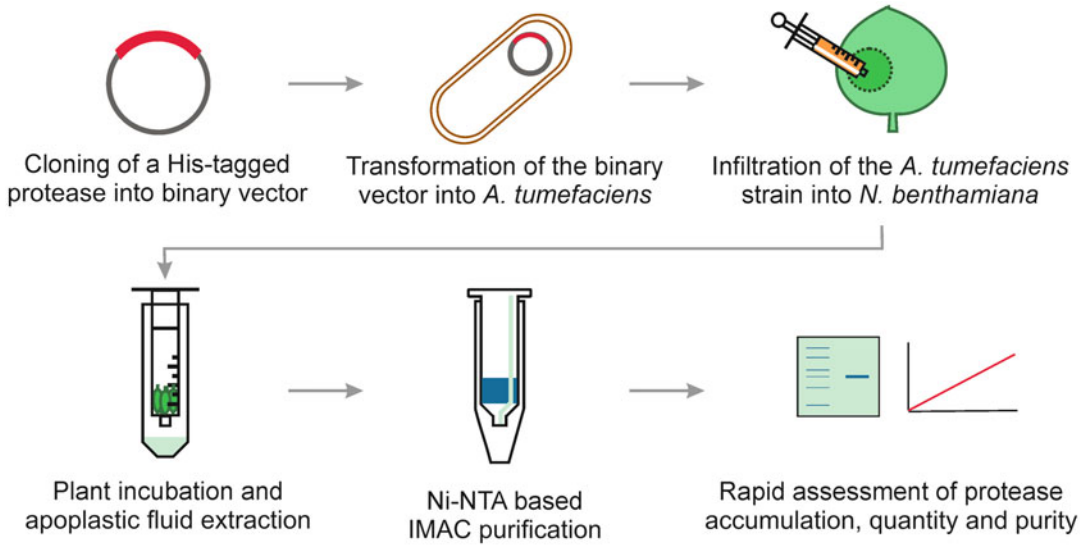

Fig. 1 Procedure for production and purification of proteases in *N. benthamiana*.The open reading frame encoding Pip1-His is cloned into a binary vector suitable for agroinfiltration. *A. tumefaciens* carrying the vector is infiltrated into leaves of *N. benthamiana*. Five to six days after infiltration, apoplastic fluid of these agroinfiltrated leaves is collected using the infiltration-centrifugation technique. His-tagged proteins are purified from the apoplastic fluid via IMAC. Quality controls of pure proteases include visualization of the eluted sample in protein gels and assessment of protease activity using fluorescent substrates

tumefaciens. The Agrobacterium strain is then used for syringe agroinfiltration of *N. benthamiana*. After 4–6 days, apoplastic fluid is extracted from the agroinfiltrated plants using the infiltration-centrifugation technique. Pip1-His is then purified from the apoplastic fluid by IMAC using Ni-NTA resin and concentrated. The purity and activity of Pip1-His is assessed on protein gels and with fluorescent substrate assays (Fig. 1). Using this one-week strategy, it is possible to produce ~1 mg of pure Pip1-His from 8 mL apoplastic fluid collected from six *N. benthamiana* plants.

2 Materials

2.1 Plant Materials

1. Ca.100 *Nicotiana benthamiana* seeds.
2. 260 mL Plastic pots.
3. Soil. We use a 75% soil 25% vermiculite mixture. Soil Levington Advance M2 pot and bedding medium nutrient compost, pH 5.3–5.8 (including 180 mg/mL N, 90 mg/mL P, 299 mg/mL K).

2.2 Binary Plasmid and Agrobacterium Strain

1. In this example, the binary expression vector corresponds to pJK083 [10] (pL2M-P19-2x35S::*Nt*PR1a-Pip1ΔSP-6xHIS) (Fig. 1 and *see* **Note 1**).
2. *Agrobacterium tumefaciens* strain GV3101 pMP90 [4] (*see* **Note 2**).

2.3 Agroinfiltration of Nicotiana benthamiana

1. Sterile LB medium (10 g/L Bacto-Tryptone, 5 g/L Bacto-Yeast extract, 10 g/L NaCl).
2. Antibiotics. 50 mg/mL Kanamycin A (Melford) stock in water, 50 mg/mL Gentamicin sulfate (Melford, G38000) stock in water, 50 mg/mL Rifampicin (Melford) stock in DMSO (*see* **Note 3**).
3. 1 M Acetosyringone (Sigma-Aldrich) stock in DMSO.
4. Infiltration buffer (10 mM $MgCl_2$, 10 mM 2-(4-morpholino) ethanesulfonic acid (MES)-KOH, pH 5.6).
5. 50 mL Centrifuge tubes.
6. Spectrophotometer cuvettes (Supelco).
7. 1 mL Syringe without needle.
8. 70% Ethanol.
9. Spectrophotometer Jenway 7200.

2.4 Apoplastic Fluid Isolation

1. 1 L Glass beaker.
2. Ice.
3. 1 L MilliQ water.
4. Pebbles, 10 of each 3–5 cm diameter.
5. Vacuum desiccator (Thermo Scientific) with pump (Welch).
6. 50 mL Centrifuge tubes.
7. 20 mL Plastic syringes without needle.
8. Centrifuge with swinging-bucket rotor and able to maintain a temperature of 4 °C (e.g., Allegra X-15R benchtop centrifuge (Beckman Coulter)).

2.5 Protein Gel Electrophoresis and Western Blotting

1. 1 M Tris pH 6.8: Tris-(hydroxymethyl)-aminomethane (Sigma-Aldrich).
2. 1.5 M Tris pH 8.8.
3. 10% APS: 400 mM ammonium persulfate, $(NH_4)_2S_2O_8$ (Sigma-Aldrich) (*see* **Note 4**).
4. 30% Acrylamide/Bis (Serva).
5. 15% Resolving gel: for one gel, mix the following components in a clean glass beaker: 1.6 mL water; 1.75 mL 1.5 M Tris pH 8.8; 70 µL 10% SDS; 70 µL 10% APS; 3.5 mL 30% acrylamide/bis solution; and finally, 28 µL TEMED. Pour the gel between

the gel plates and overlay the surface with isopropanol. After 15 min, the resolving gel is polymerized. Pour off the isopropanol and dry the gel surface with Whatman paper.

6. 6% Stacking gel: For one gel, mix the following components in a glass beaker on ice: 2 mL water; 378 μL 1.0 M Tris pH 6.8; 30 μL 10% SDS; 30 μL 10% APS; 6 mL 30% acrylamide/bis solution; and finally 3 μL TEMED. Fill the gel cassette with stacking gel solution and add the comb. Incubate the gel at room temperature for 15 min. In case storage is needed, wrap the cassette in wet tissue paper and store in a plastic bag at 4 °C for a few days.
7. 10× SDS running buffer (248 mM Tris, 2 M glycine, 35 mM SDS).
8. Anti-C-His-HRP antibody (Thermo Fisher) (*see* **Note 5**).
9. Trans blot turbo transfer system (Biorad) or similar.
10. ImageQuant LAS 400 imager (GE Healthcare) or similar.

2.6 Ni-NTA Purification an Analysis of Proteases

1. Ni-NTA resin (Qiagen) (*see* **Note 6**).
2. Purification buffer (50 mM Tris/HCl pH 7.4; 150 mM NaCl) (*see* **Notes 7** and **8**).
3. Washing buffer (50 mM Tris/HCl pH 7.4; 150 mM NaCl, 50 mM imidazole) (*see* **Note 8**).
4. Elution buffer (50 mM Tris/HCl pH 7.4; 150 mM NaCl, 250 mM imidazole) (*see* **Note 8**).
5. Mini bio spin chromatography columns (Biorad) (*see* **Notes 9** and **10**).
6. 2 mL Microcentrifuge tubes (*see* **Note 7**).
7. 15 mL Centrifuge tubes (*see* **Note 7**).
8. Amicon Ultra centrifugal filter (Sigma-Aldrich) (*see* **Note 11**).
11. Coomassie staining solution (5% aluminum sulfate 14–18 hydrate $Al_2(SO_4)_3$ $18H_2O$; 10% ethanol; 0.02% w/v Coomassie G 250 (Sigma-Aldrich), 8% v/v phosphoric acid) (*see* **Note 12**).
12. Coomassie distaining solution (10% ethanol, 8% phosphoric acid).

2.7 Fluorescent Substrate Assay

1. Reaction buffer, in this case for cysteine proteases (50 mM sodium acetate pH 5; 10 mM DTT) (*see* **Note 13**).
2. 100 mM Fluorescent substrate Z-Leu-Arg-AMC (Bachem) (*see* **Note 14**) dissolved in DMSO.
3. Black 96-well plate with clear bottom (Costar).
4. Infinite microplate reader M200 (TECAN).

3 Methods

3.1 Plant Seedling and Growth

1. Grow *Nicotiana benthamiana* in a growth chamber with 60% relative humidity and 16:8 light:dark cycles at 26 °C (day) and 22 °C (night).
2. Use 5-week-old *Nicotiana benthamiana* plants with some fully expanded leaves for agroinfiltration (Fig. 2).

3.2 Generation of Expression Vector and Transformation of Agrobacterium tumefaciens

1. Pip1-His was expressed from binary plasmid pJK083 [10] (Fig. 1, *see* **Note 15**).
2. The plasmid was transformed into *Agrobacterium tumefaciens* GV3101(pMP90) by freeze-thaw transformation [5].

3.3 Bacterial Culture and Suspension Preparation

1. Pre-cultures should be prepared 2 days prior to agroinfiltration. In a 50 mL centrifuge tube, inoculate 10 mL of LB medium supplemented with 25 mg/L rifampicin, 50 mg/L gentamycin, and 50 mg/L kanamycin with *A. tumefaciens* carrying the tagged protease expression plasmid (*see* **Notes 3** and **16**).
2. Incubate the precultures for 24 h at 28 °C under constant agitation at 220 rpm.
3. Harvest *A. tumefaciens* cells via centrifugation for 10 min at 4000 × *g* and resuspend in 10 mL infiltration buffer.
4. Measure and adjust the OD_{600} to 0.5 by diluting the cell suspension in infiltration buffer. Prepare 2 mL cell suspension/leaf.

Fig. 2 Healthy five-week-old *Nicotiana benthamiana* plant used for agroinfiltration. The plant should have two or three fully expanded leaves with >5 cm diameter and not be flowering. Light, water, temperature, and soil can drastically affect *N. benthamiana* growth

5. Add acetosyringone to a final concentration of 100 μM and incubate the cultures in the dark at 28 °C under constant agitation at 220 rpm for 1 h.

3.4 Syringe Infiltration and Plant Incubation

1. Use 5-week-old *N. benthamiana* plants for agroinfiltration (Fig. 2, *see* **Note 17**).
2. Fill a 1 mL needless syringe with the bacterial culture and hand-infiltrate the leaves via the abaxial (lower) side with a needless syringe through a tiny wound made with a plastic micropipette tip (*see* **Note 18**).
3. Incubate the agroinfiltrated plants in a greenhouse or growth chamber with 60% relative humidity and 16:8 light:dark cycles at 26 °C (day) and 22 °C (night).

3.5 Apoplastic Fluid Isolation

1. Before isolating apoplastic fluids, water the plants and cool enough MilliQ water on ice (*see* **Note 19**).
2. Detach de infiltrated leaves and rinse them with water to remove any dirt from the surface of the leaves (*see* **Note 20**).
3. Add the leaves, 100 mL ice, and 500 mL MilliQ water to the 1 L beaker and submerge the leaves into the ice-cold water with a pebble.
4. Place the beaker in a vacuum desiccator and apply vacuum until no more bubbles are released from the leaves (*see* **Note 21**).
5. Slowly release the vacuum (*see* **Note 22**).
6. Carefully dry the surface of the infiltrated leaves with tissue paper (*see* **Note 23**).
7. Stack 4–6 leaves, roll them up as a cigar, and carefully slide them into a 20 mL syringe.
8. Place the 20 mL syringe in a 50 mL centrifuge tube without lid.
9. Centrifuge for 25 min at 1000 × *g* and 4 °C using slow acceleration and de-acceleration.
10. Remove the syringes, close the centrifuge tubes, and precipitate Agrobacterium cells present in the apoplastic fluid by centrifugation at >4000 × *g* for 5 min.
11. Retrieve the supernatant (*see* **Note 24**).

3.6 Assessment of Protein Expression by Western Blot

This is an optional step to assess the presence of any tagged protein in apoplastic fluids prior purification.

1. Separate the proteins by SDS-PAGE on a protein gel with the desired acrylamide percentage.
2. Transfer the proteins from the gel onto a PVDF membrane using the Trans blot turbo transfer system.

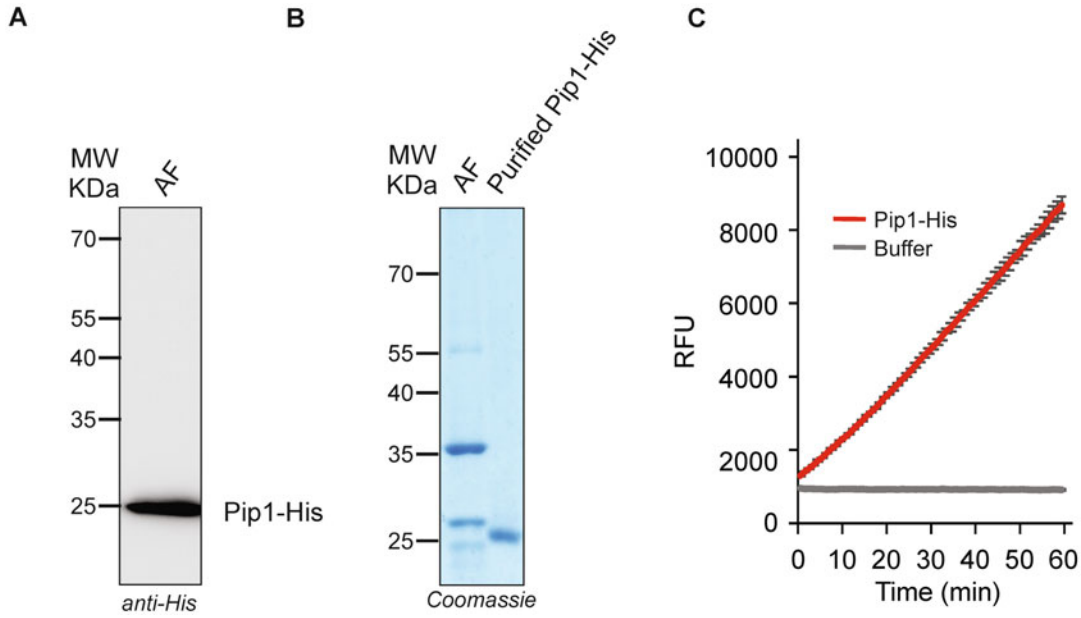

Fig. 3 Assessment of Pip1-His accumulation, purity, and activity. (**a**) Apoplastic fluid from Pip1-His expressing *N. benthamiana* plants was separated on a protein gel. Immunoblotting was performed with a peroxidase-conjugated anti-His antibody on the separated apoplastic fluid. (**b**) Coomassie stained SDS-PAGE gel of apoplastic fluid of Pip1-His expressing *N. benthamiana* leaves and purified Pip1-His. (**c**) Protease activity of Pip1-His monitored by Z-Leu-Arg-AMC hydrolysis in the elution sample. Error bars represent SD of three replicates. *RFU* relative fluorescence units

3. Evaluate the accumulation of the His-tagged protease by immunodetection with an anti-C-His antibody following the manufacturer's instructions (Fig. 3a).

3.7 IMAC Purification

1. Make a holder for the columns by cutting the bottom of 2 mL microcentrifuge tube and placing it in 15 mL centrifuge tubes. Place the centrifuge tube on either ice or in the cold room (*see* **Note 25**).
2. Open the lower end of the mini spin column by snapping of the tip, place the tip in a 2 mL microcentrifuge tube, add 500 μL MilliQ water, and centrifugate for 1 min at maximum speed. This step will ensure the subsequent flow of liquids by gravity.
3. Add 200 μL of Ni-NTA resin to the column (*see* **Note 26**).
4. Equilibrate the resin with 4 mL (20 column volumes) ice-cold purification buffer (50 mM Tris/HCl pH 7.4; 150 mM NaCl) (*see* **Note 27**).
5. Apply all the apoplastic fluid on the column (*see* **Note 28**).
6. Wash the column with 8 mL (40 column volumes) purification buffer containing 50 mM imidazole (50 mM Tris/HCl pH 7.4; 150 mM NaCl; 50 mM imidazole) (*see* **Note 29**).

7. Elute the proteins from the column with 3 mL (15 column volumes) purification buffer containing 250 mM imidazole (50 mM Tris/HCl pH 7.4; 150 mM NaCl; 250 mM imidazole) (*see* **Note 30**).
8. Optional: concentrate your protease using Amicon ultra-centrifugal filters by adding the elution fraction to the filter and centrifuging at 4 °C. The centrifugation speed will depend on the ultra-centrifugal filter used and the time will depend on the desired final concentration (*see* **Note 11**).
9. Flash-freeze aliquots in liquid nitrogen and store at −80 °C (*see* **Note 31**).

3.8 Assessment of Protein Quantity and Purity by Coomassie Staining

1. Separate the proteins in the concentrated sample by gel electrophoresis. Load at least 1 μg protein for it to be clearly visible with Coomassie staining (*see* **Note 32**).
2. After protein gel electrophoresis, carefully transfer the gel into a staining tray filled with MilliQ water.
3. Wash the gel three times with MilliQ water for 10 min on a horizontal shaker (*see* **Note 33**).
4. Shake the Coomassie solution before use to resuspend the colloidal particles. Incubate the gels in Coomassie solution by agitation on a shaker for 2–12 h (*see* **Note 34**).
5. Discard the Coomassie solution and rinse the gels twice with Milli-Q water (*see* **Note 35**).
6. Remove sticking dye particles from the staining dish with a lint-free paper towel and distain for 10–60 min.
7. Finally rinse the gels twice with MilliQ water: the gels will reach their original thickness (the alcohol-acid media shrinks the gel) and neutralization in water enhances color intensity of Coomassie. After staining and scanning you can keep the gels in the fridge for several years.
8. A single band of the expected size visible on the gel is indicative of successful purification (Fig. 3b).

3.9 Assay Protease Activity Using a Fluorogenic Substrate

1. Dilute the fluorogenic substrate in reaction buffer (50 mM Sodium acetate pH 5, 10 mM DTT) to a final concentration of 40 μM. Of this diluted substrate, 50 μL is needed for each sample (*see* **Note 36**).
2. Add 50 μL of sample (*see* **Note 37**) in a black 96-well microtiter plate with clear bottom. Use 50 μL buffer as negative control.
3. Set the plate reader to record every minute for 30 min at an excitation wavelength of 360–380 nm and emission wavelength of 440–460 nm.

4. Shake the plate for 15 s in the plate reader and start the fluorescence measurements.
5. Add 50 μL of diluted substrate to the samples and measure fluorescence immediately. An increase in the relative fluorescent units detected in the sample over time is indicative of proteolytic cleavage of the substrate by an active protease (Fig. 3c).

4 Notes

1. Any binary expression vector for Agrobacterium-mediated transformation can be used. It is important to ensure that the protein is tagged and secreted and that the tag does not influence the activity of the protease. If the expression vector does not include the silencing suppressor P19 [6], a second culture of Agrobacterium carrying an expression vector encoding P19 or other silencing inhibitor should be co-infiltrated to improve protein production (Fig. 4).
2. The hypervirulent Agrobacterium strain AGL1 [7], which can further increase levels of recombinant proteins, can be used as an alternative.
3. The antibiotics and their concentrations will depend on the strain of Agrobacterium and binary plasmid used.
4. The ammonium persulfate solution can be stored at 4 °C for 1 week, or in aliquots at −20 °C for longer periods.
5. Any suitable anti-His antibody can be used. If the protease is C-terminally His-tagged, it is recommendable to use a C-term anti-His antibody. A good alternative is the His antibody HRP (C. term) from Miltenyi Biotec.
6. Other Ni-NTA products are also suitable for His-tagged protein purification. Good alternatives include NTA magnetic beads.
7. If the purified protein would be used for mass spectrometry, it is advised to use mass spectrometry quality reagents and materials.
8. The quantity of imidazole must be adjusted for the purification of your protein of interest. Some purification buffers include 10 mM imidazole to wash the column. The concentration of imidazole in the washing buffer should be enough to wash all impurities from the sample without eluting the protein of interest. The concentration of imidazole in the elution buffer should be enough to elute all protein from the column.
9. Larger columns to scale up the procedure are commercially available, e.g., from Biorad.

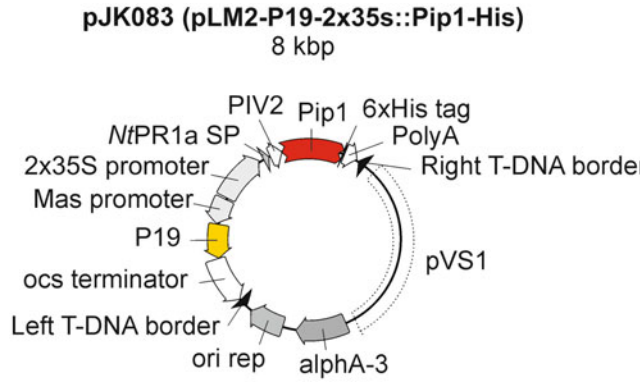

Fig. 4 Schematic representation of the Pip1-His expression plasmid [10] pJK083. The plasmid is divided into two functional parts separated by the left and right T-DNA borders (black triangles). To the left, sequences required for efficient protein expression in plants: 2x35S promoter: cauliflower mosaic virus 35S promoter with a duplicated enhancer region; *Nt*PR1a SP: N-terminal transit peptide of tobacco PR1a ensures efficient secretion of the protease; PIV2: engineered version of the second intron (IV2) of the *ST-LS1* gene of potato, to prevent gene expression in bacteria; Pip1: coding sequence of Pip1 without signal peptide and stop codon. This region can be replaced by the coding sequence of the protease of interest; 6xHis: six histidine purification tag to facilitate IMAC-based protein purification; PolyA: polyadenylation signal of cauliflower mosaic virus. In addition to the Pip1 expression cassette, a second expression cassette comprising the *A. tumefaciens*-derived mannopine synthase (*Mas*) promoter and the *A. tumefaciens*-derived octopine synthase (*ocs*) terminator allows for the production of P19 silencing inhibitor of the tomato bushy stunt virus (yellow). P19 enhances protein expression in plants. To the right of the T-DNA are features required for propagation in both *E. coli* and *Agrobacterium*. These include origin of replication (pVS1) for propagation in *A. tumefaciens*; aminoglycoside phosphotransferase (*aph(3′)-Ia*), which confers resistance to kanamycin; and high-copy origin of replication (*ori rep*) from pUC for propagation in *E. coli*. T-DNA borders are both from the nopaline C58 T-DNA

10. The only purpose of the column is to retain the resin while allowing gravity flow and therefore columns can be self-made instead of purchased. Thus, a self-made column alternative can be made from a needles syringe without plunger and using a piece of Whatman paper to prevent the resin leaking through the syringe.
11. It is recommended to choose an ultra-centrifugal filter with a molecular weight (MW) cutoff that is 2–3 times smaller than the MW of the protein of interest. Amicon ultra-centrifuge filters with a MW cutoff of 10 and 30 kDa are also available from Sigma-Aldrich.
12. Prepare the colloidal Coomassie solution by adding the components in the following order: first dissolve aluminum sulfate

in MilliQ water, add ethanol, homogenize, and then add Coomassie G-250 to the solution. Let Coomassie dissolve for ~10 min and slowly add phosphoric acid. Finally fill up with MilliQ water and store in a bottle wrapped in aluminum foil at room temperature.

13. The optimal reaction buffer would depend on the class of protease to be tested. For instance, cysteine proteases require reducing agents and metalloproteases are inactivated by chelators.
14. The fluorescent substrate would depend on the class of protease to be tested. A good alternative for a general fluorescent substrate is FITC-casein (Thermo Scientific).
15. The Pip1 coding region, obtained originally from tomato cDNA via PCR, was assembled into the vector via Golden Gate cloning [8]: pJK083 [10] (pL2M-P19-2x35S::*Nt*PR1a-Pip1ΔSP-6xHIS) was generated by combining pJK002 (pL0M-SP-*Nt*PR1a-PIV2) [9]; EC15259 (pL0M-C2-6xHis); pJK268 (pL1V2-P19-F2); pJK007 (pL0M-C1-Pip1ΔSP) [10]; pICH51288 (pL0M-P5U-2x35S-TMV); and pICH41414 (pL0M-3UT-T35S) in a BsaI Golden Gate reaction.
16. It is best practice to first streak out the glycerol stock of the Agrobacterium strain on a LB agar plate supplemented with antibiotics. After 1 day incubation, use a single colony to inoculate the pre-culture.
17. Whereas all leaves from *N. benthamiana* are able to express recombinant proteins upon agroinfiltration, expression levels are much higher in younger leaves, though unexpanded leaves are difficult to infiltrate. A good rule of thumb is to take the two youngest, fully expanded leaves.
18. [11] provides a good explanatory video for infiltration.
19. Watering plants at least 30 min before apoplastic fluid isolation opens the stomata, which increases access of water into the leaves under vacuum.
20. Manipulate the leaves carefully to prevent damage since this leads to contamination of the apoplastic fluid with cellular contents. Also, please beware that water used to rinse agroinfiltrated leaves contain genetically modified bacteria. This should be collected and autoclaved.
21. It is important that the leaves stay submerged using pebbles. Carefully shake the exicator on the table to remove larger bubbles.
22. Leaves become water soaked while the vacuum is released. Infiltrated regions become dark green. Repeat the vacuum procedure if the leaves are only partially infiltrated.

23. Any water left on the surface of the infiltrated leaves will dilute the apoplastic fluid. Tissue paper used to soak surface water contains genetically modified organisms and should be autoclaved.
24. It is important to maintain apoplastic fluids at low temperature to avoid degradation of the protease.
25. Maintaining low temperature during protein purification prevents degradation of the protease.
26. The amount of Ni-NTA resin should be adjusted to the amount of protein expected to be purified. The binding capacity of the resin is up to 50 mg/mL and therefore up to 500 μL resin could be used for the proposed setup.
27. Some protocols include incubation of the apoplastic fluid with the Ni-NTA resin at 4 °C while gentle shaking for up to 16 h. Although this might increase the yield of certain proteins, Pip1 could not be purified this way because of its self-degrading activity.
28. You can collect and pass the apoplastic fluid a second time over the column in case too much protease is left in the flow through after the first run.
29. This washing step may need to be optimized for each protein. Parameters to modify are the imidazole concentration and wash duration. The aim is to make sure the protein does not elute prematurely and that there are no more impurities eluting from the column after washing. This can be tested by analyzing the flow through samples on protein gels stained with Coomassie.
30. At this stage you can measure the protein concentration using systems as, e.g., Bicinchoninic acid (BCA) (Sigma-Aldrich) or Qubit (Thermofisher) assays.
31. The storage protocol must be optimized for each protease, as it will depend on protease stability, storage time, and intended applications after storage. Some proteases lose activity upon freezing when not stored in the right buffer.
32. At this stage it is advisable to not only visualize the concentrated elution sample but also the input, and wash samples as these will be indicative of steps that might require further optimization as well as steps where an error could have been made.
33. Insufficient washing reduces detection sensitivity because the remaining SDS disturbs the binding of Coomassie to the protein.
34. Occasionally turn the gels during the staining procedure so they are stained evenly from both sides. After 10 min you can

see first protein spots appearing. About 80% of the maximum staining is reached in 2 h of incubation. Overnight incubation is recommended for nearly 100% staining. In some cases, when huge amounts of protein are stained, the solution becomes bright blue. In this case, the staining solution should be refreshed.

35. You can reuse the staining solution several times as long as blue particles are still remaining; otherwise discard it according to your laboratories toxic waste disposal.
36. The amount of substrate needed will depend on the amount of protease to be tested and the measuring time.
37. This could correspond either to pure protease of diluted pure protease.

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Chapter 6

Identification of Cognate Protease/Substrate Pairs by Use of Class-Specific Inhibitors

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Abstract

Many proteins are regulated post-translationally by proteolytic processing. This includes plant signaling peptides that are proteolytically released from larger precursor proteins. The proteases involved in the biogenesis of signaling peptides and in regulation of other proteins by limited proteolysis are largely unknown. Here we describe how protease inhibitors that are specific for a certain class of proteases can be employed for the identification of proteases that are responsible for the processing of a given target protein. After having identified the protease family to which the processing enzyme belongs, candidate proteases and the GFP-tagged target protein are agro-infiltrated for transient expression in *N. benthamiana* leaves. Cleavage products are analyzed on immuno-blots and specificity of cleavage is confirmed by co-expression of class-specific inhibitors. For the identification of processing sites within the target protein, cleavage product(s) are purified by immunoprecipitation followed by polyacrylamide gel electrophoresis and analyzed by mass spectrometry.

Key words Agroinfiltration, Immunoprecipitation, *Nicotiana benthamiana*, Precursor processing, Protease inhibitor, Pull-down, Substrate specificity, Subtilase, Transient expression

1 Introduction

Proteases are critically involved in the regulation of plant growth and development. They contribute to the post-translational regulation of other proteins by site-specific processing. Among the targets of proteolytic regulation are precursors of enzymes (zymogens) that need to be processed for activation. Examples include pectin methylesterases [1] and different classes of peptidases [2–5]. Further, proteolytic cleavage of plasma membrane-resident receptor kinases is emerging as a mechanism in the regulation of intracellular signaling [6]. Membrane-anchored transcription factors depend on proteolytic cleavage and on release from the membrane to exert their gene-regulatory function in the nucleus [7]. Likewise, the C-terminal end of EIN2 (ETHYLENE INSENSITIVE 2) needs to be proteolytically released from the ER membrane to exert its

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nuclear function in ethylene signaling [8]. Proteases also are indispensable for plant immunity, as they generate danger signals by cleaving foreign and host proteins to form microbe- and damage-associated molecular patterns (MAMPs and DAMPs), respectively [9, 10]. Finally, proteases are required for the maturation of immune-modulatory phytocytokines, growth factors, and peptide hormones by site-specific processing of the respective precursor proteins [9, 11].

It thus appears that virtually all aspects of plant life are subject to proteolytic regulation. However, the specific proteases responsible for regulatory cleavage of cognate target proteins are largely elusive. The identification of such enzymes is challenging because of the abundance of proteases in plant genomes (887 proteases in 41 clans are listed, e.g., for Arabidopsis in the MEROPS database, release 12.3) [12], because of their frequently low expression levels, and, most importantly, because of genetic and/or functional redundancy [13–16].

We recently developed a genetic approach to overcome functional redundancy by tissue-specific expression of protease inhibitors. We hypothesized that if a group of proteases acts redundantly in the activation of a certain target protein, inhibition of these proteases by a class-specific protease inhibitor would result in a phenotype resembling the loss-of-function phenotype of the target protein. To test this proposition, the subtilase (SBT) inhibitors E XTRACELLULAR P ROTEINASE INHIBITOR (EPI) 1a and EPI10 from the pathogenic oomycete *Phytophthora infestans* were expressed in transgenic Arabidopsis plants under control of the *IDA* (*INFLORESCENCE DEFICIENT IN ABSCISION*) promotor. *IDA* codes for the precursor of a peptide that acts as a signal for the abscission of floral organs. Confirming a role of SBTs in IDA maturation, floral organs did not abscise in the EPI-expressing transgenics, and abscission was restored upon treatment with the mature IDA peptide [17]. The same approach has since been used to confirm involvement of SBTs in the formation of CLE and CLE-like peptides [18, 19], in the activation of the tomato immune protease Rcr3 [4], and in parasitic plants for haustorium development [20]. As an alternative to EPIs, we employed AtSPI-1 (Arabidopsis subtilisin propeptide-like inhibitor 1; [21]) for the in vivo inhibition of SBTs. AtSPI-1 is broadly active against plant SBTs with an inhibition constant almost three orders of magnitude below that of EPIs [21]. The formation of haustoria was delayed to a similar extent in *Phtheirospermum japonicum* expressing the structurally unrelated AtSPI-1 (inhibitor family I9) or EPI10 (family I1), indicating that SBT activity is required for haustorium development [20]. The use of class-specific protease inhibitors for loss-of-function analysis can be extended to other protease families. For example, the *P. infestans* cystatin-like effectors EpiC1 and EpiC2B, and the tomato cystatin SlCYS8, inhibit numerous papain-like

cysteine proteases and can be used to overcome functional redundancy in this family [4, 22–24].

Once the protease family responsible for processing the target protein of interest has been identified, class-specific protease inhibitors can also be employed for the subsequent identification of the specific enzymes involved. With dissociation constants in the low nanomolar or even picomolar range [17, 21], the SBT-inhibitor complexes are sufficiently stable for affinity enrichment. Provided that the inhibitors are equipped with an affinity tag, the complex can be pulled down from the tissue of interest, followed by the identification of the protease by mass spectrometry. Alternatively, cognate protease/substrate pairs can be identified by transient co-expression in the presence or absence of class-specific inhibitors in *Nicotiana benthamiana*. The approach is presented here in Subheading 3.1. Subsequent identification of the cleavage site by mass spectrometry is described in Subheading 3.2.

For identification of protease(s) responsible for the processing of a given target protein, the GFP-tagged substrate and protease candidates are agro-infiltrated for transient expression in *N. benthamiana* leaves. Cleavage of the target protein by co-expressed protease(s) is analyzed on immuno-blots of leaf extracts in comparison to control extracts from empty vector-infiltrated leaves (Subheading 3.1). Exemplary results are shown in Fig. 1a. In this experiment we analyzed processing of the C-terminally GFP-tagged IDA precursor (*see* **Note 1**) by candidate SBTs that are known to be expressed in the abscission zone of Arabidopsis floral organs (Fig. 1a; [17]). Specificity of SBT-mediated processing can be confirmed by co-expression of class-specific EPIs, as shown for the processing of the CLE40 precursor by SBT1.4 in Fig. 1b. In this experiment, cleavage of the GFP-tagged CLE40 precursor was observed upon co-expression with SBT1.4. This cleavage was inhibited when EPI10 was co-expressed with the other two constructs by simultaneous agro-infiltration into *N. benthamiana* leaves (Fig. 1b) [19]. In Subheading 3.2 we describe how to use immunoprecipitation followed by mass spectrometry to identify GFP-tagged cleavage products generated by the processing protease. Exemplary results for the processing of the TWISTED SEED1 (TWS1) precursor by the subtilase ABNORMAL LEAF SHAPE 1 (ALE1) are shown in Fig. 1c [25].

2 Materials

2.1 Equipment

1. Bead beater for leaf protein extraction (e.g., TissueLyzer LT, Qiagen); with 2 mL microfuge tubes and 5 mm stainless steel beads.

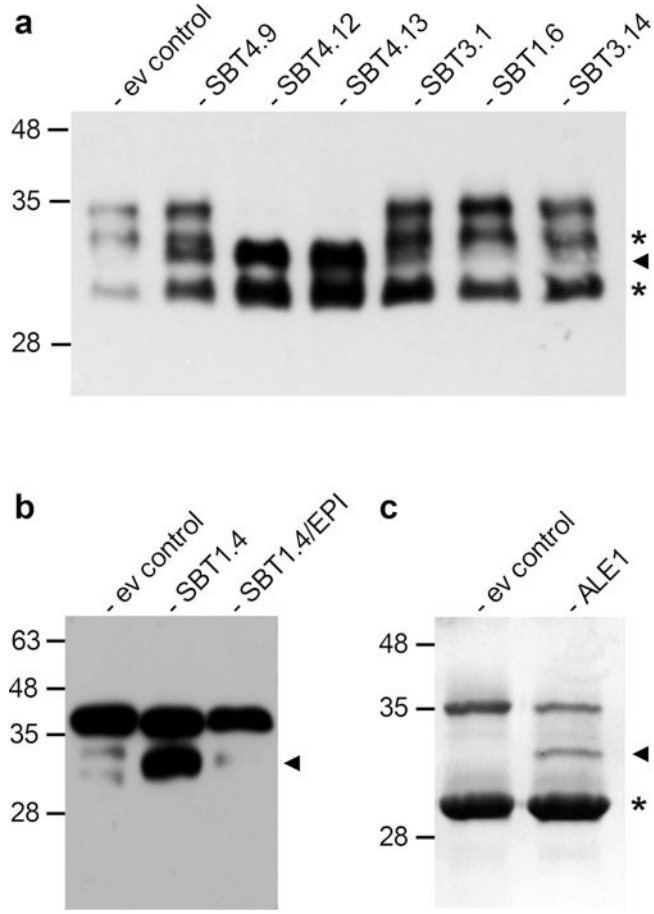

Fig. 1 Processing of peptide precursors by SBTs. (**a**) proIDA is cleaved by SBT4.12 and 4.13. proIDA-GFP was co-expressed in *N. benthamiana* leaves with candidate abscission zone SBTs or the empty vector (ev) as control. Leaf protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The blot was developed with a polyclonal GFP antiserum followed by enhanced chemiluminescence (ECL) detection. Asterisks mark unspecific cleavage products generated by *N. benthamiana* cell wall proteases. The cleavage product generated specifically by SBT4.12 and SBT4.13 is marked by an arrowhead. (**b**) Cleavage of the CLE40 precursor by SBT1.4 is inhibited by EPI10. GFP-tagged proCLE40 was co-expressed in *N. benthamiana* leaves with SBT1.4 or the ev control by agro-infiltration. Leaf protein extracts were analyzed as in (**a**). The cleavage product generated by SBT1.4 is marked by an arrowhead. SBT1.4-mediated cleavage of proCLE40 is inhibited upon co-expression of EPI10. (**c**) The TWS1 precursor is processed by ALE1. proTWS1-GFP was co-expressed in *N. benthamiana* leaves with ALE1 (SBT2.4) or the ev control. Leaves were harvested 5 days after infiltration. Leaf protein extracts were subjected to immunoprecipitation by GFP-Trap. The precipitate was analyzed by SDS-PAGE. ALE1-specific and -unspecific cleavage products are marked by an arrowhead and asterisk, respectively. For cleavage site identification, the specific band was cut out from the Coomassie-stained gel, subjected to in-gel tryptic digestion, and resulting peptides were analyzed by mass spectrometry as described in Subheading 3.2

2. Cork borer, 7 mm.
3. Incubation shaker for microfuge tubes.
4. Chemiluminescence imaging system.
5. High resolution tandem mass spectrometer (e.g., Q-Exactive HF-X, Thermo) linked to a nano-high performance liquid chromatography (nano-HPLC) system (e.g., Ultimate 3000 RSLCnano, Thermo) via an electrospray ionization source (e.g., EASY-Nano Flex, Thermo).
6. Trap (e.g., C18 PepMap100, 300 μm, 100 Å, 5 μm × 5 mm, Thermo) and analytical (e.g., NanoEase M/Z HSS C18 T3, 1.8 μm 100 Å 75 μm × 250 mm, Waters) reverse-phase chromatography columns suitable for the used nano-HPLC system.
7. Personal computer with software package for peptide identification and quantification from MS/MS data (e.g., MaxQuant [26], or Scaffold (Proteome Software, Portland, OR)).
8. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) system.
9. Semi-dry blotting system with power supply.
10. Vacuum concentrator.

2.2 Plant Material, Constructs, Bacteria, and Media

1. *Nicotiana benthamiana* plants (seeds from, e.g., Agroscience, Neustadt, Germany) six to eight weeks old (*see* **Note 2**).
2. *Agrobacterium tumefaciens*, strain C58C1 (Rif^R, Tet^R) or GV3101 (Rif^R, $Gent^R$).
3. *Agrobacterium tumefaciens*, strains C58C1 or GV3101 with p19 suppressor of silencing (Kan^R, Rif^R, Tet^R) [27].
4. Expression constructs for the substrate and protease of interest, and for the class-specific inhibitor. We use pART27 [28] as the binary vector for expression of substrate and protease, and pGreen0229 [29] for the EPI inhibitor. Other binary vectors are equally possible. Avoid using the same vector for all three constructs.
5. 100 μg/mL Rifampicin; 1000× stock in DMSO.
6. 25 μg/mL Tetracycline; 1000× stock in 70% (v/v) ethanol.
7. 100 μg/mL Spectinomycin; 1000× stock in ddH_2O.
8. 50 μg/mL Kanamycin; 1000× stock in ddH_2O.
9. 50 μg/mL Gentamycin; 1000× stock in DMSO.
10. LB (Lysogeny Broth)-medium: add 10 g tryptone, 5 g yeast extract, and 5 g NaCl to 1 L H_2O. For solid media, add 15 g/L agar. Autoclave for 20 min. Cool down to about 60 °C before adding antibiotics from 100× stocks and pouring plates.

2.3 Transient Expression in N. benthamiana

1. Infiltration buffer: 10 mM MES pH 5.6, 10 mM $MgCl_2$, 0.15 mM acetosyringone.
2. 15 mL Culture tubes.
3. 1 mL PP/PE syringes.
4. Extraction buffer: 50 mM Tris/HCl pH 7.5, 100 mM NaCl. For method described in Subheading 3.1, add 0.5% (v/v) Triton X-100, 10 mM β-mercaptoethanol, and 10 μL/mL Protease-Inhibitor-Cocktail for plants (Sigma-Aldrich; Steinheim, Germany). For mass spectrometric method described in Subheading 3.2, exclude Triton X-100 and β-mercaptoethanol.
5. Bradford protein quantification assay.

2.4 SDS-PAGE

1. 40% (w/v) Acrylamide/bisacrylamide (29:1) stock solution.
2. Stacking gel buffer: 0.5 M Tris/HCl pH 6.8, 0.4% (w/v) sodium dodecylsulfate (SDS).
3. Separating gel buffer: 1.5 M Tris/HCl pH 8.8, 0.4% (w/v) SDS.
4. 10% (w/v) Ammonium persulfate (APS; *see* **Note 3**).
5. N,N,N′,N′-Tetramethylethylenediamine (TEMED).
6. 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.
7. Running buffer: 25 mM Tris/HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS.
8. Pre-stained protein marker.

2.5 Immunoblotting and Detection

1. Nitrocellulose membrane. For proteins below 25 kD use 0.2 μM Polyvinylidene difluoride (PVDF) membrane instead.
2. Blotting paper (1 mm).
3. Anode buffer 1:48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% (v/v) methanol.
4. Cathode buffer: 48 mM Tris, 39 mM glycine, 1.3 mM SDS.
5. 20x Tris-buffered saline (TBS): 0.5 M Tris/HCl pH 7.4, 3 M NaCl.
6. TBS-T: 1x TBS containing 0.02% (v/v) Tween-20.
7. Blocking buffer: 6% (w/v) skimmed milk powder in TBS-T.
8. Primary antibody: rabbit polyclonal anti-GFP (Merck, A11122).
9. Secondary antibody: goat anti-rabbit IgG, peroxidase conjugate (Calbiochem).

10. 90 mM Coumaric acid stock solution: 74 mg coumaric acid dissolved in 5 mL DMSO, store at −20 °C, keep away from light.
11. 250 mM Luminol stock solution: 443 mg 3-aminophtalhydrazide dissolved in 10 mL DMSO, store at −20 °C, keep away from light.
12. ECL (enhanced chemiluminescence) solutions. Solution A: 100 mM Tris/HCl pH 8.5, 198 μM coumaric acid, 1.25 mM luminol. Add 5 mL 1 M Tris/HCl pH 8.5 to 40 mL ddH_2O. Add 110 μL coumaric acid stock solution and 250 μL luminol stock solution. Make up to 50 mL with ddH_2O. Solution B: 3% (v/v) H_2O_2. Add 100 μL 30% H_2O_2 (Carl Roth GmbH) to 900 μL ddH_2O. Store at 4 °C, keep away from light. Mix 15 mL solution A with 45 μL solution B immediately prior to use. Alternatively, commercial solutions can be used according to manufacturer instructions.

2.6 Immunoprecipitation and Mass Spectrometry

1. Anti-GFP antibody coupled to magnetic beads (e.g., GFP-Trap® agarose beads, Chromotek).
2. Mass spectrometry-compatible colloidal Coomassie staining solution (e.g., Roti-Blue, Carl Roth, Germany), mix 10 mL 5x stock solution (vortex before use) with 30 mL ddH_2O and 10 mL methanol; add a stir bar and leave on the magnetic stirrer for several hours.
3. Destaining solution: 20% (v/v) methanol.
4. Proteomics grade trypsin, 1 μg/μL in 50 mM acetic acid (*see* **Note 4**).
5. Acetonitrile, LC-MS grade.
6. Solutions for in-gel digest: 40 mM NH_4HCO_3; 100 mM NH_4HCO_3; 100 mM NH_4HCO_3, 10 mM 1,4-dithiothreitol (DTT); 100 mM NH_4HCO_3, 55 mM iodoacetamide (*see* **Note 5**). Prepared from 1 M NH_4HCO_3 and 1 M DTT stock solutions.
7. 50% (v/v) Formic acid, MS grade.
8. 0.1% (v/v) trifluoroacetic acid, eluent additive, LC-MS grade.
9. HPLC solvent A: 0.1% (v/v) formic acid.
10. HPLC solvent B: 0.1% (v/v) formic acid, 80% (v/v) acetonitrile.

3 Method

3.1 Identification of Protease-Substrate Pairs by Co-Expression with Potential Substrates or Class-Specific Inhibitors in N. benthamiana

3.1.1 Transient Expression by Agro-Infiltration of N. benthamiana

1. For the identification of protease-substrate pairs, co-express the substrate protein fused to GFP with candidate proteases. For the confirmation of cleavage specificity, co-express the class-specific protease inhibitor with the other two constructs. The p19 suppressor of silencing is always co-infiltrated. For each of the individual *A. tumefaciens* strains carrying expression constructs for the substrate-GFP fusion, the protease, the class-specific inhibitor, and the p19 silencing suppressor, streak out several single colonies on LB plates with appropriate antibiotics (100 μg/L rifampicin and 25 μg/L tetracyclin for C58C1, 50 μg/L gentamicin instead of tetracyclin for GV3101, 100 μg/L spectinomycin for the binary expression vector (pART27) and 50 μg/L kanamycin for the p19 construct). Grow 48 h at 28 °C.
2. Wash off the bacteria from the plates in 10 mL infiltration buffer, collect in 15 mL culture tube.
3. Spin down at 1000 × *g* for 10 min, discard supernatant.
4. Resuspend cells in 1 mL infiltration buffer, determine optical density (OD) at 600 nm.
5. Prepare bacterial infiltration solution by mixing the strains carrying the expression constructs (C58C1/pART27) and the p19 silencing suppressor (C58C1/p19) at an OD_{600} ratio of 0.35:0.5 in a total volume of 50 mL infiltration buffer. The dilution factor Y for p19 is $OD_{600(p19)}/0.5 = Y$. The dilution factor Z for expression construct is $OD_{600(pART27)}/0.35 = Z$. To prepare X mL of Infiltration solution add X/Y mL bacteria carrying the p19 silencing suppressor and X/Z mL bacteria carrying the expression construct.
6. Use a blunt 1 mL syringe to inject the bacterial infiltration solution into *N. benthamiana* leaves by gently pressing the syringe (no needle) onto the abaxial side, supporting the leaf with one finger on the opposite side.
7. Leaf material is harvested at 3–5 days (*see* **Note 6**) after infiltration. Use a cork borer to collect 12 7-mm leaf discs into a 2 mL microfuge tube, add a 5 mm stainless steel bead, flash freeze in liquid nitrogen. Homogenize the frozen leaf material in bead beater for 1.5 min.
8. Add 250 μL ice-cold extraction buffer, mix for another 30 s in the bead beater.
9. Centrifuge for 5 min, 4 °C, 15000 × *g* to remove cellular debris.

10. Collect the supernatant, use the Bradford assay to determine the protein concentration of the leaf extract.
11. Mix 20 µg leaf protein extract in 15 µL of ddH_2O with 5 µL 4x loading buffer, heat to 95 °C for 5 min, chill on ice, and spin down. Store on ice, or at −20 °C, until further analysis by SDS-PAGE.

3.1.2 SDS-PAGE and Western Blot

1. Mix 6.75 mL acrylamide solution with 4.5 mL separating gel buffer and 6.75 mL ddH_2O. Add 180 µL 10% APS and 18 µL TEMED, mix well and cast the separating gel (15%; volumes are sufficient for two mini gels; about 8 × 7 cm; 1.5 mm thick) using a mini gel casting system or glass plates and spacers. Overlay with isopropanol. Allow the gel to polymerize for at least 1 h. Remove the water. 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.
2. Remove the sample comb, place the gel into the electrophoresis cell, add running buffer, and rinse the wells with running buffer.
3. Carefully load the samples into the wells, using a yellow-tip pipette.
4. Run the gel at 120 V until the dye front leaves the gel.
5. While the gel is running, cut 12 sheets of blotting paper to the size of the gel. Soak 6 sheets of blotting paper in anode buffer and 6 sheets in cathode buffer.
6. Cut a piece of nitrocellulose or PVDF membrane to the size of the gel, rinse in ddH_2O, and soak in anode buffer for 10 min.
7. Stop electrophoresis; dismantle the gel assembly, remove the upper plate, cut off the stacking gel.
8. Assemble the blotting stack in the following order: anode, 6 sheets of blotting paper soaked in anode buffer, membrane, gel, 6 sheets of paper soaked in cathode buffer, cathode. Make sure that no air bubbles are trapped.
9. Transfer the proteins onto the membrane during 90 min at 25 V. Limit the current to a maximum of 5.5 mA/cm^2 for a single gel to avoid excessive heating.
10. After the transfer, the membrane is transferred to blocking solution for at least 1 h (*see* **Note 7**) to minimize unspecific binding of the antibodies to the membrane.
11. Incubate the membrane with the primary antibody (anti-GFP, 1:10000 in blocking buffer) for at least 1 h.

12. Wash the membrane three times for 5 min with TBS-T to remove unbound primary antibody.
13. Incubate the membrane with the secondary antibody (anti-rabbit IgG peroxidase conjugate; 1:10000 in blocking buffer) for 1 h.
14. Wash membrane three times for 5 min with TBS-T.
15. Add 200 μL ECL solution and spread evenly.
16. Record chemiluminescence using X-ray film, a CCD camera, or a suitable imaging system according to the manufacturer's instructions.

3.2 Immunoprecipitation of Cleavage Products and Identification by Mass Spectrometry

1. Aiming at identification of the cleavage site, co-express the GFP-tagged substrate with the cognate protease in *N. benthamiana* as described above (Subheading 3.1.1, **steps 1–9**). Triton X-100 and β-mercaptoethanol should be omitted from the extraction buffer used in **step 8**.
2. For immunoprecipitation, resuspend GFP-Trap beads gently by pipetting up and down (cut off the tip of the pipet tip). Use 15 μL of the slurry for a 4 mg protein equivalent of leaf extract.
3. Equilibrate the beads three times in extraction buffer by adding 500 μL of extraction buffer to 15 μL of the slurry in a 1.5 mL reaction tube; mix gently, do not vortex.
4. Spin down the beads at 2500 × *g* for 2 min at 4 °C. Discard the supernatant.
5. Add leaf protein extract (4 mg of total protein) and incubate for 1 h at 4 °C on a rotating wheel.
6. Collect the beads by centrifugation (2500 × *g*, 2 min, 4 °C) and wash three times with 500 μL ice-cold extraction buffer. Transfer beads to a new tube during the last washing step. Remove the supernatant after the final centrifugation step.
7. Add 70 μL 4x SDS-PAGE loading buffer; heat to 95 °C for 5 min; spin as before to sediment beads. Recover the supernatant and analyze by SDS-PAGE (15%, *see* Subheading 3.1.2).
8. After electrophoresis (**step 7** of Subheading 3.1.2), place the gel (*see* **Note 8**) in a dish containing approximately 100 mL Coomassie-staining solution. Place on a rocking shaker at room temperature for 1 h.
9. Remove the Coomassie-staining solution and replace by destaining solution; change several times until proteins are visible as blue bands against a clear background.
10. Transfer the gel into a clean dish with ddH_2O; equilibrate for at least 30 min at room temperature.

11. Use a scalpel to cut out desired protein bands from the submerged gel; cut the band into small pieces and transfer to a 1.5 mL microfuge tube (*see* **Note 9**).
12. Add 200 μL ddH_2O, agitate at 1000 rpm for 5 min at room temperature.
13. Discard the supernatant; add 150 μL acetonitrile, agitate at 1000 rpm for 10 min at room temperature.
14. Discard the supernatant, add 100 μL 100 mM NH_4HCO_3, 10 mM DTT, agitate at 1000 rpm for 30 min at 56 °C.
15. Discard the supernatant; add 150 μL acetonitrile, agitate at 1000 rpm for 10 min at room temperature.
16. Discard the supernatant; add 85 μL 100 mM NH_4HCO_3, 55 mM iodoacetamide; incubate for 20 min in the dark.
17. Discard the supernatant; add 150 μL 100 mM NH_4HCO_3, agitate at 1000 rpm for 10 min at room temperature.
18. Discard the supernatant; add 150 μL acetonitrile; agitate at 1000 rpm for 10 min at room temperature.
19. Discard the supernatant; place tubes on ice; add 250 ng trypsin in 25 μL 40 mM NH_4HCO_3; incubate on ice for 30 min.
20. Check the liquid level; if necessary, add 40 mM NH_4HCO_3 until gel pieces are covered; incubate at 37 °C overnight.
21. Add 2 μL 50% formic acid, vortex, spin down, recover the supernatant into a new microfuge tube (*see* **Note 10**).
22. Evaporate to dryness in a vacuum centrifuge, resuspend the residue in 15 μL 0.1% (v/v) formic acid, or 0.1% (w/v) trifluoroacetic acid.
23. Analyze sample by nano-LC-ESI-MS/MS (the exact procedure will depend on the specific equipment available). Inject the sample for separation by reversed phase HPLC at 35 °C. Perform gradient elution at a flow rate of 300 nL/min solvent A using a 30 min gradient of 2–55% solvent B. For analysis on an Orbitrap Q Exactive system, use a resolution of 60,000 at $m/z = 200$ for detection of survey spectra ($m/z = 300–1800$). Collect data-dependent MS/MS spectra for the 20 most abundant peptide precursors using high energy collision dissociation (HCD) fragmentation at a resolution of 15,000 with normalized collision energy of 27. Use lock-mass ions from ambient air for internal calibration.
24. For data analysis, use MaxQuant or a similar software. Set carbamidomethylation of cysteine as fixed modification, oxidation of methionine, hydroxylation of proline, tyrosine sulfation, phosphorylation of serine, threonine and tyrosine residues as variable modifications. Do not specify a certain enzyme or the number of missed cleavages, as you search for

peptides resulting from one cleavage by trypsin, the other cleavage by the protease under study. In the example shown in Fig. 1c, we analyzed processing of the TWS1 precursor by the protease ALE1. proTWS1 carries a C-terminal GFP tag. Hence, the cleavage product captured by GFP-Trap (arrowhead in Fig. 1c) is the cleavage product C-terminal of the processing site. The peptide that defines this cleavage site is characterized by a C-terminal Lys or Arg residue resulting from tryptic cleavage, and a non-tryptic cleavage site (generated by ALE1) at its N-terminal end (*see* **Note 11**).

4 Notes

1. Other tags are possible. However, protein-tags are preferred over small epitope tags because they add size to the cleavage product, thus facilitating detection by SDS-PAGE/western blot. We recommend GFP, because highly specific antibodies are available for detection on western blots with little unspecific background. Further, GFP-tagged cleavage products can be purified easily by GFP-trap (Chromotek) using immobilized anti-GFP nanobodies for immuno-precipitation.
2. We grow the plants on seeding substrate (Plagron Seeding and Cutting Soil), at 25 °C and 16/8 h day/night cycle. Once plants reach 2 weeks of age, they are watered with 1.48 g/L N (20%) P (20%) K (20%) universal fertilizer. Expression levels are much reduced in flowering plants. Therefore, plants should be used before they start to bolt.
3. Prepare freshly, or use frozen aliquots.
4. If the protease of interest cleaves at Lys or Arg residues, and if Lys or Arg residues are present close to the cleavage site of the protease of interest, trypsin may not be suitable. Alternative proteases have to be chosen that do not cleave nearby, in order to generate peptides that are amenable to MS analysis. Alternatives include chymotrypsin, LysC, LysN, AspN, GluC, and ArgC [30].
5. Solutions containing iodoacetamide should be prepared freshly and used immediately.
6. The optimum time of co-expression needs to be determined experimentally. It is expected that the abundance of the processing product increases over time. If this is the case, 5 days after infiltration is the best time for harvest. However, there are many proteases in the apoplast of *N. benthamiana* leaves and, depending on the precursor protein that is being analyzed, these proteases may generate unspecific cleavage products, as also seen in Fig. 1a. If such unspecific degradation is observed,

earlier time points may be more suitable, in order to increase the relative abundance of the band of interest compared to the unspecific degradation products.

7. Use a rocking shaker for incubation **steps 10–14**.
8. Make sure to always wear gloves when handling the gel, the tools and dishes, and while cutting out the bands of interest.
9. Use high quality microfuge tubes (e.g., the original Eppendorf tubes) to avoid any plasticizer leaking into the sample, which is detrimental to subsequent MS analysis.
10. If the Coomassie-stained bands are rather faint, it may be advisable to do a second elution of the gel fragments. Add 60 µL 66% acetonitrile/1.7% acetic acid; incubate for 15 min at 37 °C; recover the supernatant and combine with the first eluate from **step 21**.
11. If the processing product (the band of interest) and its precursor are similar in size and thus not well separated by SDS-PAGE, you may also detect peptides that originate from the precursor rather than the processing product. However, these "contaminating" peptides will be much lower in abundance and can thus be distinguished. In order to do that, you will have to quantify the peptides on basis of their peak area in the extracted ion chromatogram.

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Chapter 7

Peptide Backbone Modifications for the Assessment of Cleavage Site Relevance in Precursors of Signaling Peptides

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Abstract

The physiological relevance of site-specific precursor processing for the biogenesis of peptide hormones and growth factors can be demonstrated in genetic complementation experiments, in which a gain of function is observed for the cleavable wild-type precursor, but not for a non-cleavable precursor mutant. Similarly, cleavable and non-cleavable synthetic peptides can be used in bioassays to test whether processing is required for bioactivity. In genetic complementation experiments, site-directed mutagenesis has to be used to mask a processing site against proteolysis. Peptide-based bioassays have the distinctive advantage that peptides can be protected against proteolytic cleavage by backbone modifications, i.e., without changing the amino acid sequence. Peptide backbone modifications have been employed to increase the metabolic stability of peptide drugs, and in basic research, to investigate whether processing at a certain site is required for precursor maturation and formation of the bioactive peptide. For this approach, it is important to show that modification of the peptide backbone has the desired effect and does indeed protect the respective peptide bond against proteolysis. This can be accomplished with the MALDI-TOF mass spectrometry-based assay we describe here.

Key words Bioassay, Loss-of-function analysis, MALDI-TOF mass spectrometry, N-methylation, Peptide hormone biogenesis, Proteolysis, Subtilase

1 Introduction

Subcellular targeting and protein assembly, the function of enzymes, receptors, and transcription factors, as well as the formation of growth factors and peptide hormones from larger precursor proteins may all depend on the activity of specific processing proteases [1–3]. Proteases are thus involved in virtually all aspects of plant growth and development. Despite their relevance, the physiological role of proteases is still poorly understood, and only few proteases have been linked to specific substrates in vivo [4]. To understand protease function, it is essential to identify their

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physiological substrates, and this may well be the most challenging task in protease research [4, 5]. A given substrate protein may be cleaved by several proteases in vitro, but "the fact that they can does not mean that they do" [5]. Here we describe how backbone-modified synthetic peptides can be used in bioassays to demonstrate that cleavage by a certain protease and the respective cleavage site are physiologically relevant.

Genetic complementation experiments were used to confirm the importance of site-specific processing of the IDA (Inflorescence Deficient in Abscission) precursor for formation of the bioactive IDA peptide as a signal for the abscission of floral organs in *Arabidopsis* [6]. The abscission defect of the *ida* mutant was rescued by transformation with the wild-type *IDA* precursor, but not by a site-directed mutant that cannot be cleaved by the processing protease [6]. The experiment showed that the processing site is required for IDA maturation. Similarly, transgenic plants expressing an "uncleavable" variant of the Golven1 (GLV1) precursor, in which the Site-1-Protease (S1P) cleavage site was masked by alanine-substitutions, lacked the gain-of-function phenotype of wild-type *GLV1* overexpressors, attesting to the physiological relevance of the cleavage site for GLV1 maturation [7]. Confirming S1P as the protease relevant for GLV1 maturation in vivo, the *GLV1* gain-of-function phenotype was suppressed in the *s1p* mutant [7].

Following the same rationale, cleavable and non-cleavable variants of synthetic peptides can be used to verify the physiological relevance of a processing site in bioassays [6, 8]. We used this approach to show that processing of the phytosulfokine (PSK) precursor by phytaspase-2 (SlPhyt2) at an aspartic residue in position (P)1 (immediately upstream) of the cleavage site is required in vivo for the production of PSK to induce flower drop in tomato [9]. Treatment with a synthetic N-terminally extended PSK peptide induced flower drop in wild-type inflorescences, whereas a SlPhyt2-resistant peptide with an Asp-to-Ala substitution in P1 was inactive. The experiment showed that P1 Asp is required for bioactivity. Further, using plants lacking SlPhyt2 in our bioassay, the cleavable and uncleavable precursor peptides were both inactive, indicating that PSK formation requires both the cleavage site Asp and SlPhyt2 in vivo [9].

The caveat to Ala substitution as a means of masking protease cleavage sites is the possibility that changes to the amino acid sequence may affect bioactivity in ways other than impairing protease cleavage. Peptide backbone modifications that affect proteolysis with only minimal changes to peptide structure offer an interesting alternative. The interaction of peptides with other proteins is largely governed by their side chains, while the peptide backbone itself may not be essential for high-affinity interactions [10]. Replacing the amide backbone by peptide bond lookalikes holds great potential for drug development, because such peptidomimetics often show

improved metabolic stability, bioavailability, and pharmacokinetics compared to the natural peptide [11]. Of the many backbone modifications that render a peptide bond recalcitrant to proteolysis [10, 11] we found methylation of the amide nitrogen particularly useful, since N-methylated peptides can easily be obtained by solid phase custom synthesis. Methylation of the backbone nitrogen to form a tertiary amide bond significantly slows down, or even completely abolishes, hydrolysis by serine proteases [12]. This is because tertiary amide bonds lack the hydrogen on the reacting nitrogen atom of secondary amides that is required to stabilize the high-energy transition state for nitrogen inversion [12, 13]. Since nitrogen inversion is indispensable for proton transfer from His of the catalytic triad to the leaving nitrogen, the bond is no longer cleaved [14, 15]. Other peptide bond surrogates, obtained, e.g., by reduction of the carbonyl to an alcohol, or substitution of the amide nitrogen by oxygen or sulfur, may be equally effective, also for different classes of proteases.

We used N-methylation of backbone nitrogens to increase the metabolic stability of the tomato wound signaling peptide systemin [16]. Further, using an extended IDA peptide N-methylated at the scissile bond in abscission bioassays, we showed that cleavage at this site is required for bioactivity [6]. Finally, to verify the importance of the suspected precursor processing site of the systemin peptide for activation of the wound signal, we used synthetic systemin (A VQSKPPSKRDPPKMQTD) derivatives with a C-terminal four-amino-acid extension (NNKL) corresponding to the genuine C-terminus of the systemin precursor. Processing at Asp18 rendered the C-terminally extended systemin peptide bioactive in a cell culture bioassay [17]. Activity was much reduced when the processing site was protected against proteolysis by N-methylation (Fig. 1a), indicating that cleavage at the C-terminus is necessary for bioactivity (Fig. 1b; [17]).

If suitable bioassays exist, this approach can easily be adopted for other peptide hormones to address the question whether processing at a certain site, by a certain protease is required for bioactivity. However, the efficacy of peptide backbone modifications (here N-methylation) in preventing cleavage at the putative processing site by the protease in question needs to be confirmed experimentally. To this end, a synthetic peptide comprising the expected cleavage site and its N-methylated derivative are digested in vitro with the purified processing protease. Digests can then be analyzed by HPLC [16] or, more conveniently, by mass spectrometry. In this chapter, we describe an assay for proteolytic cleavage of a synthetic peptide and its N-methylated derivative, including a protocol for the analysis of processing efficiency by MALDI-TOF mass spectrometry. The result of such an experiment is shown in Fig. 1c, confirming that cleavage between Asp18 and Asn19 of a C-terminally extended systemin derivative by tomato phytaspase-1 (SlPhyt1) is abolished by N-methylation of the respective peptide bond.

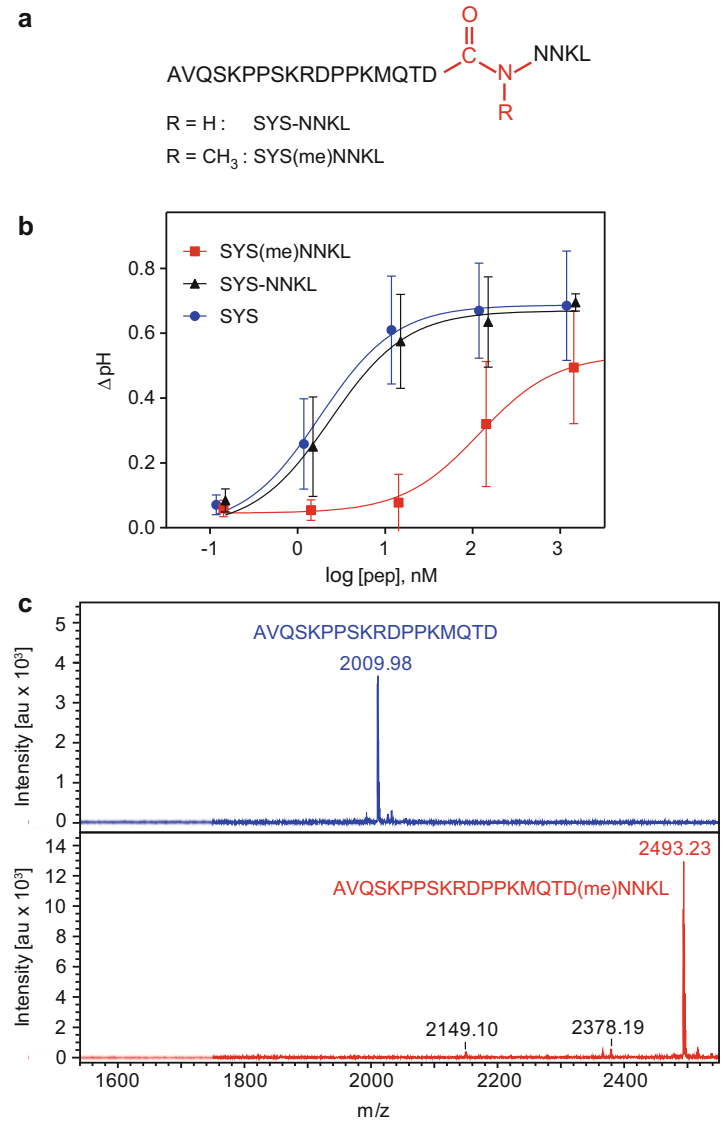

Fig. 1 N-methylation renders C-terminally extended systemin inactive and resistant to cleavage by SlPhyt1. (**a**) Sequence of the C-terminally extended systemin peptide. The peptide bond linking D18 to N19 is shown in red. In the N-methylated peptide, the hydrogen at the amide nitrogen is replaced by a methyl group. (**b**) Cell culture bioassay comparing the activity of systemin with C-terminally extended and N-methylated derivatives. Dose response curves indicate reduced activity for the C-terminally extended peptide methylated at the Asp18-Asn19 bond (SYS (me)NNKL; $n = 4$) compared to the non-methylated form (SYS-NNKL; $n = 4$) and systemin (SYS; $n = 5$). Panel (**b**) was reproduced from Beloshistov et al. 2018 with permission from John Wiley and Sons. (**c**) Cleavage of the Asp18-Asn19 bond of the C-terminally extended systemin derivative by SlPhyt1 is blocked by N-methylation. SYS-NNKL (2 μM, upper panel, blue) and SYS(me)NNKL (2 μM, lower panel, red) were digested with SlPhyt1 as described in Subheading 3.1 and analyzed by MALDI-TOF mass spectrometry as described in Subheadings 3.2 and 3.3 (the 2 h time point of a digest with 400 ng protease is shown). Ion intensities are shown on the Y-axis as a function of the mass-to-charge ratio (m/z) on the X-axis. Masses are indicated for the systemin peptide resulting from cleavage of SYS-NNKL (blue) and for uncleaved SYS(me)NNKL (red)

2 Materials

2.1 Peptide Digest

1. Custom-synthesized synthetic precursor peptides comprising the putative processing site, with and without N-methylation (*see* **Note 1**). In the experiment described, we used a C-terminally extended systemin peptide (AVQSKPPSKRDPP KMQTD /NNKL; C-terminal extension underlined; processing site indicated by /) as a specific case example.
2. Candidate processing protease, i.e., recombinant SlPhyt1 in the experiment described here (*see* **Note 2**).

2.2 Sample Preparation

1. α-cyano-4-hydroxy-trans-cinnamic acid (HCCA, ≥99.0% purity by HPLC).
2. Water (LC-MS grade).
3. Acetonitrile (LC-MS grade).
4. Trifluoroacetic acid (TFA, LC-MS grade).
5. Matrix solution: 5 mg/mL HCCA in 50% acetonitrile (v/v), 0.1% TFA, dissolve at room temperature. Assist the dissolving process by occasional vortexing. HCCA should dissolve completely within a few minutes.
6. Peptide calibration standard (e.g., Peptide calibration standard II, Bruker Daltonics) (*see* **Note 3**).
7. Stainless steel MALDI-TOF target.
8. Standard biochemistry equipment, including Eppendorf tubes, glass containers, and adequate pipettes for small volumes.

For optional purification step:

9. C18 reversed-phase solid phase extraction tips (ZipTip with 0.2 μL C18 resin, or self-made StageTips).
10. Acetic acid (LC-MS grade).
11. Vacuum concentrator.

2.3 MALDI-TOF Analysis

1. Reflector-MALDI-TOF mass spectrometer (Autoflex III from Bruker Daltonics was used here; other instruments can be used).

2.4 Data Analysis

1. Data format conversion tool (vendor software; open-source alternatives exist).
2. Protein database search engine or protein digest calculation tool (e.g., Mascot Server; open-source alternatives exist).

3 Methods

3.1 Peptide Digest

1. Prepare two reactions in microfuge tubes, one for the methylated peptide and one for the non-methylated peptide. Reaction conditions depend on the protease under study. For SlPhyt1 that was used here, the reaction mixture contained 2 μM of either the extended systemin peptide or its N-methylated derivative, and 400 ng of the purified protease in 40 μL 50 mM NaH_2PO_4/Na_2HPO_4, 10 mM NaCl, pH 5.5 at 25 °C (*see* **Note 4**).
2. Take aliquots at 20 min, 1 h, and 3 h, and stop the reaction by addition of 1% (v/v) TFA (*see* **Note 4**).

3.2 Sample Preparation

1. Prepare samples by mixing with equal volume of matrix solution using dried droplet preparation: Take 1 μL of the peptide digest and mix with 1 μL of matrix solution directly on a stainless steel target. Allow the samples to air dry.
2. Mix equal volumes of peptide calibration standard and matrix solution in an Eppendorf tube.
3. Deposit the peptide calibration standard close to the sample spot (*see* **Note 5**). Pipette 0.5 μL of the calibration mix onto the stainless steel target. Allow the calibration mix to air dry.
4. Optional: Purify sample by stage tip purification: Adjust sample pH to 2.0 by addition of 10% (v/v) TFA (*see* **Note 6**).
5. Activate C18 StageTip with 100 μL methanol.
6. Equilibrate with 100 μL 0.5% (v/v) acetic acid.
7. Load the sample.
8. Wash the StageTip three times with 100 μL 0.5% acetic acid.
9. Elute peptides in two successive steps, each with 40 μL of 80% (v/v) acetonitrile.
10. Evaporate the solvent to dryness using a vacuum concentrator.
11. Resuspend the residue in 5 μL 0.1% (v/v) TFA.

3.3 MALDI-TOF Analysis

Analyze samples and peptide calibration standards by MALDI-TOF mass spectrometry. For this purpose, Reflector-MALDI-TOF instruments from different vendors (Bruker, AB Sciex, Shimadzu) can be employed (*see* **Note 7**).

1. Insert the steel target with prepared samples and peptide standards into your MALDI-TOF instrument.
2. Peptide samples are analyzed in the reflector mode using positive ionization. Select an appropriate reflector-instrument method that covers the mass range from 700 to 3500 m/z.

3. Calibrate the MALDI-TOF mass spectrometer using the peptide calibration standard. Peptide signals should have a good signal-to-noise ratio (S/N) and clearly resolved isotope patterns. The intensity of the monoisotopic peak of each peptide should be at least five times higher than the baseline. Adjust laser intensity and detector gain if S/N ratio is not sufficient. Typical settings for an Autoflex III MALDI-TOF instrument (Bruker Daltonics) are:
   - Laser intensity: 30%.
   - Laser frequency: 200 Hz.
   - Reflector Detector Gain: 1800 V.
   - Reflector voltage: 21 kV.

   For the peptide calibration standard 200 laser shots usually are sufficient to obtain a spectrum with good S/N ratio. Load the peptide calibration mass list and calibrate the instrument.
4. Measure samples of the peptide digests using the calibrated instrument method. Usually, laser intensity and reflector voltage have to be increased for sample measurements since concentration of the peptide calibration standard is much higher compared to sample peptides. In addition, higher number of laser shots have to be summed up to achieve a sufficient S/N ratio for the sample peptides (*see* **Note 8**).
5. Save sample spectra including m/z peak lists in the corresponding vendor data format.

3.4 Data Analysis

1. Open the sample spectra in the appropriate vendor's data analysis software (for example, FlexAnalysis 3.4. for Bruker Daltonics instruments). The software automatically generates a peak list from the spectrum by extracting the m/z values of monoisotopic peptide signals, which can be edited manually to remove contaminants and artifacts (small organic molecules, sodium or potassium ion adducts) (*see* **Note 9**).
2. Generate a sequence database in FASTA format using the amino acid sequences of the peptide or protein targets of the protease of interest (i.e., the sequence of the extended systemin peptide, which was the only target peptide in the experiment described here). Depending on the protein database search engine used (Mascot, Sequest, X!Tandem, etc.) raw data have to be converted to specific formats that can be used by the search engines (*see* **Note 9**).
3. Upload the peptide peak list for a Mascot database search using BioTools 3.2. software (Bruker Daltonics) (*see* **Note 10**). If just a single target protein or peptide is analyzed (the extended systemin peptide in the experiment described here), an in silico digest of the protein can be performed in the sequence editor

of the BioTools software rather than using a search engine. The calculated mass lists of the in silico digest can then be matched to the spectrum in BioTools.

Typical settings for a database search of a MALDI-TOF analysis of a protease digest are:

- Enzyme: no enzyme.
- Missed cleavages: 0.
- Mass tolerance: 50 ppm.
- Variable Modifications: Oxidation of methionine residues, methylation of modified amino acid (here: asparagine).

4. Compare retrieved results from database search with expected cleavage specificity of the protease of interest. m/z values of product peptides should match the expectation within the specified mass accuracy. Results can be further validated by recording MS/MS spectra of product peptides.

4 Notes

1. N-methylated peptides obtained by Fmoc-chemistry-based solid-phase synthesis are available from most vendors. High quality and purity peptides should be used (>95% purity). The C-terminally extended systemin peptide and its N-methylated derivative that were used here were obtained from PepMic (Suzhou, China).
2. The protease to be used for the peptide digest assay depends on the specific research question. We addressed the role of SlPhyt1 in C-terminal maturation of systemin. SlPhyt1 was expressed as a C-terminally His-tagged fusion protein in *Nicotiana benthamiana* and purified from cell wall extracts by metal chelate affinity as described [18].
3. The peptide calibration standard is a mixture of peptides with known m/z values, which is necessary for external calibration of the MALDI-TOF mass spectrometer. The peptide standard should cover a mass range between 700 and 3500 m/z.
4. The buffer composition and pH may have to be adjusted to match the requirements of the protease under investigation. Also, the amount of protease in the assay may have to be adjusted accordingly. Since the catalytic rate for the protease with the given peptide substrate is usually not known, we recommend varying reaction time and/or protease concentration. Therefore, prepare a larger reaction volume, take aliquots after different time intervals (e.g., 20 min, 1 h, and 3 h), and stop the reaction by addition of TFA. Alternatively, set up several reactions in parallel including different amounts of the

protease. All samples are then analyzed by MALDI-TOF mass spectrometry to determine the optimum reaction time (or protease concentration) at which cleavage of the non-methylated peptide is near to completion.

5. The external calibration of the MALDI-TOF mass spectrometer works best if calibration spots are located close to sample spots. On a standard steel target, one calibration spot is usually placed in the center of four surrounding sample spots. This calibration spot is then used for external calibration of these four samples, leading to a mass accuracy of the MALDI-TOF mass spectrometer <50 ppm. Be careful not to contaminate the samples with the peptide calibration standard when pipetting the calibration solution onto the steel target. This would lead to signal suppression of the sample peptides since the concentration of peptides is usually much higher in the standard compared to the samples.
6. MALDI-TOF mass spectrometry can cope with a certain amount of non-volatile buffers or salts up to concentrations of 50 mM. If only a few low intensity signals are observed in the MALDI-TOF spectra, buffer substances or salts might suppress ionization of peptides. If many signals are observed that show an m/z difference of 21.99 Da (sodium ion adduct) or 37.95 Da (potassium ion adduct), the salt concentration in the samples is too high. In these cases, new samples have to be prepared, purified, and concentrated using C18 ZipTips or StageTips [19]. This optional procedure is described in Subheading 3.2 **steps 4–11**.
7. For analysis of the peptide digests, the reflector mode of the MALDI-TOF instrument should be used to improve resolution and mass accuracy, since the linear mode of MALDI-TOF mass spectrometers does not offer sufficient resolution and mass accuracy. Also, settings for MALDI-TOF analyses may differ for different MALDI-TOF mass spectrometers. The settings described in this protocol should be adjusted to the actual MALDI-TOF mass spectrometer used for sample analysis.
8. Increasing the laser intensity to high levels is not suitable to improve signal intensity for peptides of low abundance, since the signals of highly abundant peptides would reach saturation. Thereby, resolution and mass accuracy for highly abundant peptides would be compromised. Instead, several single measurements (200 laser shots each) are summed up to enable the detection of low abundance peptides. A sum spectrum of 2000–3000 laser shots is usually sufficient to include also low abundant peptides in the data analysis.
9. Alternatively, open-source tools like ProteoWizard (ProteoWizard: Home (sourceforge.net) can be used to convert vendor

data formats into open formats like mzML or mzXML. These open formats can be used for data analysis with freeware database search engines like OpenMS (https://www.openms.de/) or the Trans-Proteomic Pipeline (http://tools.proteomecenter.org/wiki/index.php).

10. 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 experiments described here, because the "no enzyme" function is only available in the licensed version of Mascot.

Acknowledgments

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Chapter 8

Detection of Apoplastic Protease Inhibitors Using Convolution Activity-Based Protein Profiling

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Abstract

Activity-based protein profiling (ABPP) is a powerful tool in biological chemistry to monitor protein activity using chemical probes that bind covalently and irreversible to active site of enzymes such as proteases. To date, there are three different ways to experimentally use ABPP: comparative, competitive, and convolution ABPP. Here we use and describe the convolution ABPP approach, a method used to detect changes in protease inhibitor abundance in different proteomes. We have applied this method to monitor the activity of *Lolium perenne* apoplastic cysteine proteases during the interaction with the fungal endophyte *Epichloë festucae*. We describe the method to isolate apoplastic fluids from infected and uninfected *L. perenne* ryegrass leaves and the protocol to perform a convolution ABPP experiment. Furthermore, we report how to quantify and analyze fluorescent gels obtained from the ABPP labeling.

Key words Convolution ABPP, Activity, Proteases, PLCP, Apoplast, Ryegrass, Endophyte

1 Introduction

Papain-like cysteine proteases (PLCPs) are involved in many plant processes including development, senescence, and plant immunity. The peptidase database MEROPS [1] classifies PLCPs into the clan CA with the two families C1A (apoplastic) and C1B (cytosolic). Apoplastic plant PLCPs are divided into nine subfamilies [2]. This subdivision is based on functional and structural characteristics as well as phylogeny. PLCPs are produced as inactive proteases called zymogens which contain an N-terminal signal peptide for secretion and an auto-inhibitory domain. The removal of both signaling peptide and auto-inhibitory domain releases ca. 25–35 kDa active protease with the catalytic triad formed by Cys, His, and Asn. Some PLCPs also contain a C-terminal granulin domain, with a yet unknown function [2].

Generally, PLCP activity is regulated post-translationally rather than transcriptionally. To analyze PLCP activity, activity-based

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protein profiling (ABPP) can be used. ABPP uses chemical probes, called activity-based probes (ABPs) that bind covalently and irreversible to the active site of proteins and thereby allow to monitor the availability of active sites. At first, ABPP has been used to compare protein activity between biological replicates and/or treatments. This form of ABPP is referred to as comparative ABPP [3]. Additionally, the competitive ABPP has been developed, in which proteomes were pre-incubated with putative inhibitor molecules followed by ABP labeling. Inhibitors are thought to compete for binding to the same proteins as ABPs. If the inhibitor is bound to the protease, it reduces the availability of active sites for ABP binding and thereby reduces the detected signal in the ABPP blot. A reduction in signal intensity is used as an indication of the inhibitory capacity of potential inhibitors. Therefore, this method has been widely used to discover inhibitor molecules [3]. More recently, a convolution ABPP approach has been used to determine if free inhibitor molecules are present in a given proteome [4]. In principle, a convolution ABPP uses two proteomes, one of them containing putative inhibitors (E+) and the mock proteome (E−). The proteomes are combined before labeling (BL) and after labeling (AL; Fig. 1a). The signal intensities in AL represent the average of the signal intensities of each E− and E+ proteome. Any reduction in signal intensity of BL compared to AL indicates the presence of excess inhibitor in proteome E+ (Fig. 1b).

In this chapter, we show that the convolution ABPP approach can be used to study plant endophyte interactions, more specifically the interaction of the cool season grass L*olium perenne* and the fungal endophyte *Epichloë festucae*. We describe the procedure of a convolution ABPP to analyze the PLCP activity in the apoplastic fluid of uninfected (E-) and *E. festucae* infected (E+) *L. perenne* leaves. In here, the ABP MV202, a fluorescent derivative of the PLCP-specific probe DCG-04, was used [2]. Using convolution ABPP we showed that the activity of apoplastic papain-like cysteine proteases (PLCPs) in *Lolium perenne* is reduced during the interaction with the endophyte *Epichloë festucae*. This reduced PLCP activity is caused by a PLCP inhibitor present in excess in the apoplastic fluid of *E. festucae* infected *L. perenne* plants [5].

2 Materials

All solutions should be prepared with distilled or desalted water (MiliQ water) unless otherwise stated.

2.1 Apoplastic Fluid Isolation

1. Desiccator.
2. Beaker.

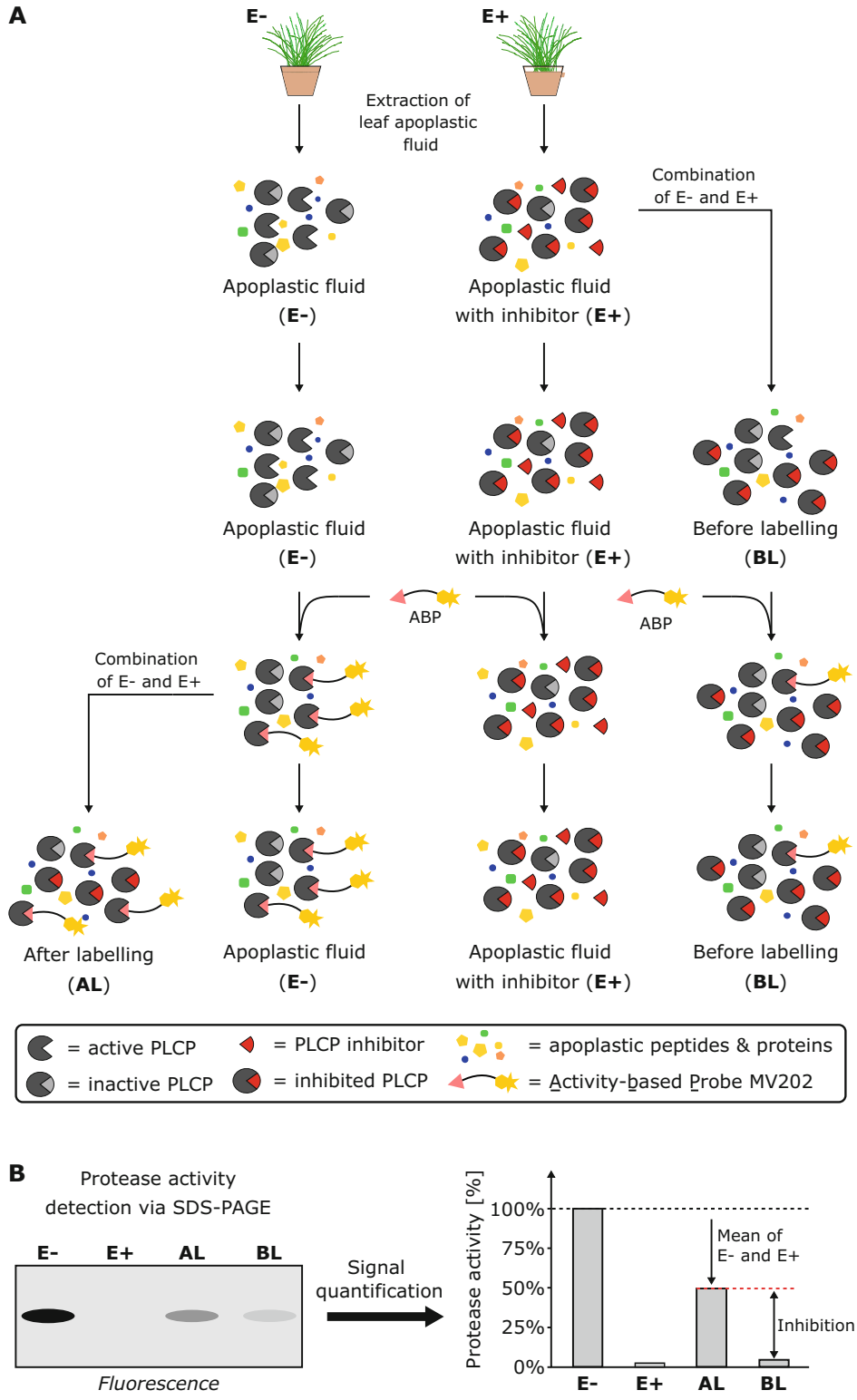

Fig. 1 Convolution ABPP workflow. (**a**) Illustration of a convolution ABPP procedure. Apoplastic fluid of *E. festucae* infected (E+) and uninfected *L. perenne* plants is extracted. To test if an excess of a PLCP

3. Centrifugation tube with sieve and removable bottom (*see* **Note 1**).
4. 4 °C Centrifuge fitting the centrifugation tubes.

2.2 Preparation of Polyacrylamide Gels for Electrophoresis

1. Polyacrylamide gel equipment: Gel cassette, comb, spacers.
2. 30% Acrylamide solution.
3. Sodium dodecyl sulfate (SDS): 10% in water.
4. 1 M Tris–HCl, adjusted to pH 6.8.
5. 1.5 M Tris–HCl, adjusted to pH 8.8.
6. 10% Ammoniumpersulfate (APS).
7. Tetramethylethylenediamine (TEMED).
8. Isopropanol.
9. 12% Resolving gel composition: 12% Acrylamide, 375 mM Tris–HCl pH 8.8, 1% (w/v) SDS, 1% (w/v) APS, 0.04% (v/v) TEMED.
10. 6% Stacking gel composition: 6% acrylamide, 125 mM Tris–HCl pH 6.8, 1% (w/v) SDS, 1% (w/v) APS, 0.1% (v/v) TEMED.

2.3 Convolution Activity-Based Protein Profiling

11. 1 M Dithiothreitol (DTT).
12. 1 M Sodium acetate (NaAcO), adjusted to pH 6.
13. Dimethyl sulfoxide (DMSO).
14. 100 μM activity-based probe MV201 or MV202 in DMSO (*see* **Note 1**).
15. 10 mM E-64 protease inhibitor in DMSO.
16. Acetone (optional).
17. Heat block.
18. Polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
19. 12% Polyacrylamide gel.
20. Protein gel loading dye: 4 M Tris–HCl pH 6.8, 6% (w/v) SDS, 0.15% (w/v) Bromophenol blue, 60% (v/v) glycerol.
21. 1× SDS running buffer: 25 mM Tris–HCl pH 8.3, 192 mM glycine, 4 mM ultrapure SDS.

Fig. 1 (continued) inhibitor is present in E+ apoplastic fluid, E− and E+ are combined in a 1:1 ratio before labeling (BL) with a fluorescent activity-based probe (ABP). As control E− and E+ are also combined after labeling (AL). (**b**) Readout of a convolution ABPP. Proteins are separated via SDS-PAGE and signals derived from active PLCPs labeled with fluorescent MV202 are detected. In principle, the signal derived from AL represents the average signal intensities of E− and E+. Any reduction in signal intensity of BL compared to AL indicates an excess of PLCP inhibitor present in the E+ sample able to suppress PLCP activity in the E− sample

22. Container for washing and SyproRuby staining of polyacrylamide gel.
23. Platform shaker.
24. 4 °C Tabletop centrifuge (optional).

2.4 Detection

1. Chemi-Doc MP System (or equivalent imaging system), with filter sets suitable for rhodamine (Ex. 532 nm, Em. 580 nm) and SyproRuby (Ex. 450 nm, Em. 610 nm) detection.

2.5 Protein Loading Control

1. SyproRuby.
2. Fixing solution: 50% MeOH, 7% acetic acid.
3. Gel wash solution: 10% MeOH, 7% acetic acid.

2.6 Quantification of Protease Activity

1. Image analysis software compatible with imaging system used (e.g., Image Lab Software for the Chemi-Doc system, Bio-Rad).

3 Method

3.1 Apoplastic Fluid Isolation

1. Cut *Lolium perenne* leaves 2 cm above soil to ensure the shoot apical meristem remains intact and collect leaves. Do so for *Epichloë festucae* infected (E+) and uninfected (E−) plants.
2. Place leaves in a beaker and fill the beaker with MilliQ water. Ensure that all leaves are fully submerged.
3. Place beakers in a desiccator and vacuum infiltrate the leaves with MilliQ water three times for 10 min at 60 mbar with a 2 min interval of atmospheric pressure. Ensure that the leaves remain submerged during vacuum infiltration (*see* **Note 2**).
4. Remove the leaves from the beaker and thoroughly dry the outer surface to prevent diluting your apoplastic fluid.
5. Place the leaves in centrifugation tubes with sieve and removable bottom and centrifuge for 20 min at 2000 × *g* and 4 °C to collect the apoplastic fluid (*see* **Note 3**).
6. Keep the extracted apoplastic fluid on ice. If it is not used immediately, freeze at −20 °C (*see* **Note 4**).

3.2 Preparation of 12% Polyacrylamide Gels

1. Combine components of 12% resolving gel (Table 1; *see* **Note 5**) and pour into gel cassette leaving ca. 2 cm room for the 6% stacking gel. Add a few drops of isopropanol to remove any air bubbles.
2. Once the 12% resolving gel is polymerized, remove isopropanol.

Table 1
Pipetting scheme for one 12% polyacrylamide gel

6% stacking gel		12% resolving gel	
H_2O	2	H_2O	2.3
30% acrylamide	0.6	30% acrylamide	2.8
1 M Tris–HCl, pH 6.8	0.378	1.5 M Tris–HCl, pH 8.8	1.75
10% SDS	0.03	10% SDS	0.07
10% APS	0.03	10% APS	0.07
TEMED	0.003	TEMED	0.0028
Total volume	3	Total volume	7

All entries are in milliliter (mL)

3. Combine components of 6% stacking gel (Table 1; *see* **Note 5**) and fill the remaining space in the gel cassette. Insert comb and leave to polymerize.

3.3 Convolution Activity-Based Protein Profiling

1. Combine 1X volume of apoplastic fluid of *E. festucae* infected (E+) plants with 1X volume of apoplastic fluid of uninfected (E-) plants and incubate at room temperature (RT) for 45 min. This sample will be hereafter referred to as before labeling (BL) sample (*see* Table 2, **Note 6**).
2. Meanwhile, aliquot 3X the volume of E− apoplastic fluid used in the BL sample. This sample will be referred to as E− sample (*see* Table 2). For the E-64 control, aliquot 2x the volume of E− apoplastic fluid used in the BL sample. This sample will be referred to as E− (E-64) sample. Do the same for the E+ apoplastic fluid. For the no probe control (NPC) sample combine E− and E+ apoplastic fluid (1X volume each). Keep all samples on ice.
3. After the 45 min incubation add 50 mM NaAcO and 10 mM DTT to all samples (see Table 2).
4. Add 10 μM E-64 to the E− (E-64) and E+ (E-64) samples and an equivalent amount of DMSO to all other samples (*see* Table 2, **Note 7**).
5. Incubate at RT for 15 min.
6. Add 0.2 μM of fluorescent MV202 to all samples but NPC, here add an equivalent amount of DMSO (*see* Table 2).
7. Incubate for 2 h in darkness at RT (*see* **Note 8**).
8. Take 1/3 of the E− sample and 1/3 of the E+ sample (50 μL each) and combine them in a new tube. This sample will be hereafter referred to as after labeling (AL) sample.

Table 2
Convolution ABPP pipetting scheme

	E−	E+	BL	E− (E-64)	E+ (E-64)	NPC
Apoplastic fluid (E−)	141.9	–	47.3	94.6	–	47.3
Apoplastic fluid (E+)	–	141.9	47.3	–	94.6	47.3
1 M NaOAc pH 6	7.5	7.5	5	5	5	5
1 M DTT	0.15	0.15	0.1	0.1	0.1	0.1
DMSO	0.15	0.15	0.1	–	–	0.1
10 mM E-64	–	–	–	0.1	0.1	–
100 μM MV202	0.3	0.3	0.2	0.2	0.2	–
DMSO	–	–	–	–	–	0.2
Total	150	150	100	100	100	100

All entries are in microliter (μL)
E− uninfected apoplastic fluid, *E+ E. festucae* infected apoplastic fluid, *BL* before labeling sample, *NPC* no probe control

9. (optional) Stop reaction by adding 500 μL cold acetone and precipitate sample for at least 1 h at −20 °C (*see* **Note 9**).
10. (optional) Centrifuge samples for 30 min at 4 °C and maximum speed.
11. (optional) Fully remove acetone.
12. (optional) Resuspend pellet in MilliQ water.
13. Add 1X Protein gel loading dye and boil samples for 5 min at 95 °C.
14. Load samples onto 12% polyacrylamide gel and run SDS-PAGE in darkness.

3.4 Detection

1. Carefully cut the wells and remaining loading dye from the rest of the polyacrylamide gel. Ensure that the gel does not dry out.
2. Thoroughly rinse the polyacrylamide gel with water to remove any leftover SDS running buffer.
3. Place the polyacrylamide gel into fluorescence imaging system (e.g., Chemi-Doc MP System, Bio-Rad). Ensure the surface is clean and free of dust or any other type of dirt.
4. Use a filter suited for rhodamine imaging (Ex. 532 nm, Em. 580 nm) to detect the signals derived from active PLCPs labeled with the fluorescent activity-based probe MV202.
5. Save image (for the Chemi-Doc system: as Image Lab Image Document .scn).

3.5 Protein Loading Control

1. Place the polyacrylamide gel into fixing solution for 30 min while gently shaking.

2. Remove fixation solution and add SyproRuby. Stain overnight or according to the manufacturer's instruction.
3. Wash the polyacrylamide gel twice for 15 min in gel wash solution.
4. Remove gel wash solution and rinse with water.
5. Place the polyacrylamide gel into the imaging system. Ensure the surface is clean and free of dust or any other type of dirt.
6. Detect proteins with the SyproRuby settings (Ex. 450 nm, Em. 610 nm).
7. Save image (for Chemi-Doc system: as Image Lab Image Document .scn).

3.6 Quantification of Protease Activity

1. Open fluorescent ABPP image saved in Chemi-Doc .scn format with Image Lab Software (Bio-Rad) (*see* **Note 10**).
2. Go to "Volume Tools."
3. Use the "Rectangle" function and draw a rectangle around the perceived signal in the E− sample.
4. Copy the rectangle and place the new rectangles to the corresponding positions in all other samples (Fig. 2a).
5. Repeat for each signal detected in E−.
6. Go to "Analysis Table."
7. Select "Volume Table" and export the results in an excel file.
8. Open SyproRuby image with Image Lab Software.
9. Go to "Lane and Bands."
10. Select "Automatic." If necessary, adjust lane number and size manually with options available in "Lane and Bands" (Fig. 2b).
11. Go to "Analysis Table."
12. Select "Lane Statistics" and export the results in an excel file.
13. Use function displayed in Fig. 2c to calculate protease activity for each sample. Divide the Adjusted Volume (Int) of the PLCP signal (Fig. 2a) by the Adjusted Total Lane Volume (Int; Fig. 2b) to normalize against the protein loading control.
14. To eliminate signals not deriving from PLCPs and obtain the specific PLCP labeling intensities, subtract the corresponding E-64 sample. For AL and BL use the average of the E− (E-64) and E+ (E-64) samples.
15. To obtain the protease activity [%] relative to the E− sample, set the E− labeling intensities to 100% and calculate the percentage of activity for other samples using the rule of three.
16. Repeat the entire assay (3.1–3.5) at least twice to obtain enough data for statistical analyses and graphical presentation (Fig. 2d).

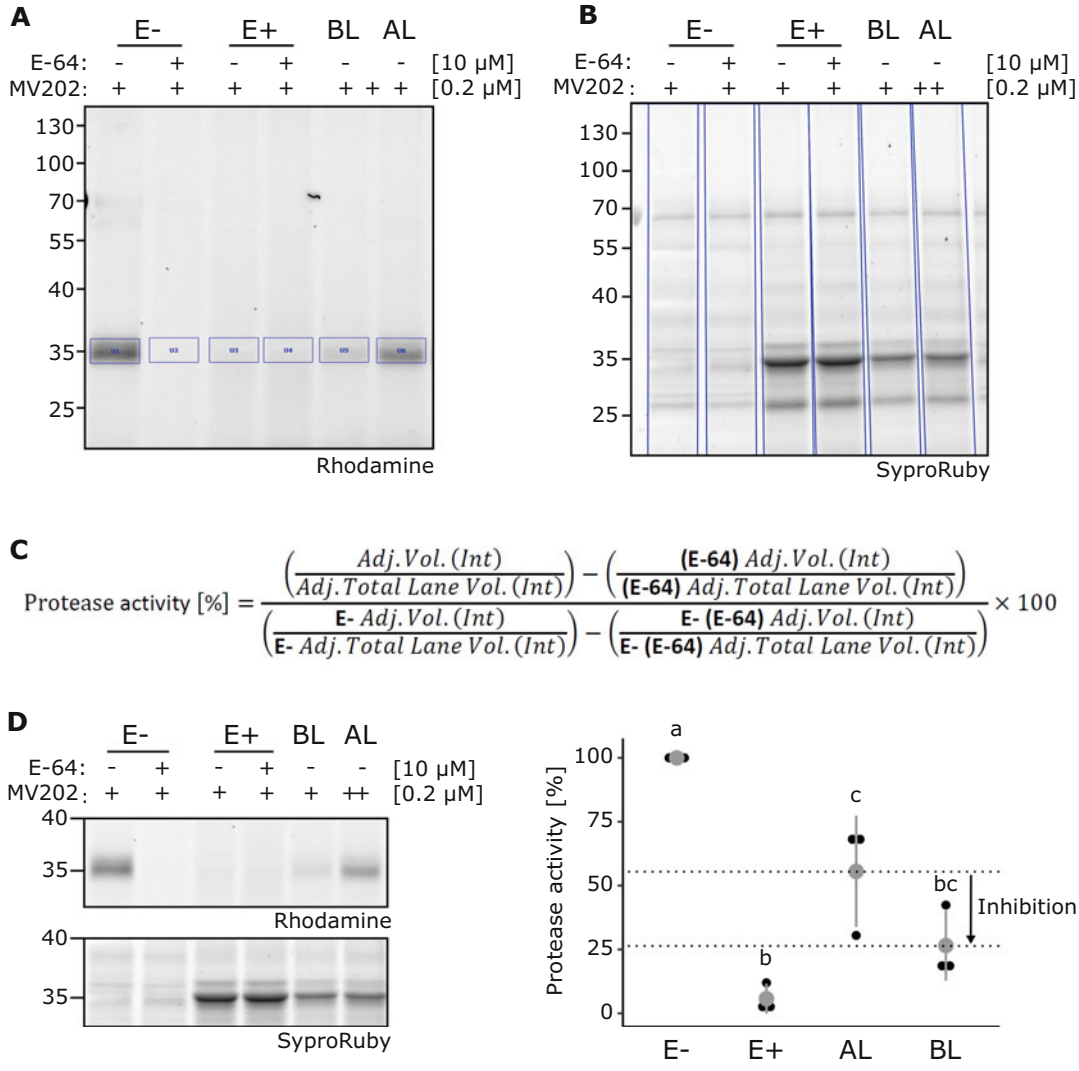

Fig. 2 Signal quantification of convolution experiment. (**a**) Selection of signals from fluorescent ABPP image. (**b**) Selection of lanes for calculation of protein loading control from SyproRuby image. (**c**) Function to calculate protease activity in percentage relative to the mock (E−) sample. (**d**) Final output with statistical analysis. (Figure adapted from [5])

4 Notes

1. Instead of MV202, the fluorescent probe MV201 can be used for PLCP detection. MV201 lacks the biotin tag present in MV202. Probes can be provided by Prof. Hermen Overkleeft (Leiden University) upon request.

2. After vacuum infiltration, leaves should appear greener and firmer to the touch. If this is not the case, most likely the vacuum infiltration did not work properly.
3. Alternatively, 50 mL reaction tube (Falcons) combined with 20 mL needleless syringes can be used. Place the leaves in the barrel of 20 mL needleless syringes. Then place the barrels in 50 mL falcon tube and centrifuge.
4. Since the apoplastic fluid contains many proteolytic enzymes that remain active even at −20 °C, storing the apoplastic fluid longer than a few days is not recommended.
5. Add the polymerization components 10% APS and TEMED last to avoid premature polymerization.
6. The volumes of apoplastic fluid used can be upscaled, but the ratios must be kept.
7. If PLCP signal did not fully disappear in the E-64 control sample during detection, the E-64 concentration can be increased. Generally, a 1:50 or 1:100 probe:inhibitor ratio is used.
8. Fluorescent probes such as MV202 are light sensitive. Therefore, any light exposure should be kept to a minimum to prevent the probes from losing fluorescence.
9. Recommendation for weak signals: protein precipitation and resuspension in a smaller amount of MilliQ water or buffer concentrates the apoplastic proteins; thus more can be loaded onto the polyacrylamide gel.
10. The Chemi-Doc and Image Lab software are used as examples due to availability. Other imaging systems and software may be used in similar fashion.

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Chapter 9

Cysteine Reactivity Profiling to Unveil Redox Regulation in Phytopathogens

Kyoko Morimoto, Monika Stegmann, Farnusch Kaschani, Shabaz Mohammed, and Renier A. L. van der Hoorn

Abstract

Reactivity-based chemical proteomics is a powerful technology based on the use of tagged chemicals that covalently react with surface-exposed residues on proteins in native proteomes. Reactivity profiling involves the purification, identification, and quantification of labeled peptides by LC-MS/MS. Here, we have detailed a protocol for reactivity profiling of Cys residues using iodoacetamide probes, displaying >1000 reactive Cys residues in the proteome of phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto*DC3000). Comparative reactivity profiling of *Pto*DC3000 treated with or without hydrogen peroxide (H_2O_2) identified ~200 H_2O_2-sensitive Cys residues in antioxidant enzymes, metabolic enzymes, and transcription regulators. Interestingly, half of these H_2O_2-sensitive Cys residues are more reactive in response to H_2O_2 and several proteins have multiple Cys residues with opposite reactivities in response to H_2O_2 exposure.

Key words Reactivity profiling, Biotinylated iodoacetamide, BIAM, LC-MS/MS, Redox-sensitive Cys residues, Hydrogen peroxide, *Pseudomonas syringae* pv. *tomato* DC3000

1 Introduction

Reactivity profiling is an emerging chemical proteomics method, primarily developed in pharmaceutical research [1, 2]. Reactivity profiling is used in two ways: competitive profiling and comparative profiling [3–6]. Competitive profiling is used to screen chemical libraries to discover new drug targets, whereas comparative profiling identifies differentially reactive residues in biological samples generated from two different conditions.

Reactivity profiling is based on the use of reactivity probes that consist of a reactive group and an affinity tag or chemical tag.

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Reactivity probes have a simple structure to minimize steric hindrance when compared to the more selective activity-based probes that contain an additional binding group. The reactivity probes used here carry an iodoacetamide (IAM) reactive group to alkylate cysteine (Cys) residues that are exposed on proteins in native proteomes.

Cys reactivity profiling with IAM-based probes has been established with different probes and approaches [2, 4, 7–9]. The method typically includes 4–5 steps: (1) labeling with reactivity probes (in vitro or in vivo); (2) coupling to biotin by click-chemistry; (3) digesting the labeled proteome with, e.g., trypsin; (4) enrichment of labeled peptides on various avidin beads; (5) identification and quantification of the labeled peptides by LC-MS/MS.

In this chapter, we describe the method that we developed using the commercially available biotinylated IAM (BIAM; Fig. 1a). By using BIAM, we omit the click-chemistry reaction (**step 2**) (Fig. 1b). We also employ label-free quantification (LFQ) instead of isotopic labeling, which is often used for Cys reactivity profiling [10]. LFQ simplifies the procedure and

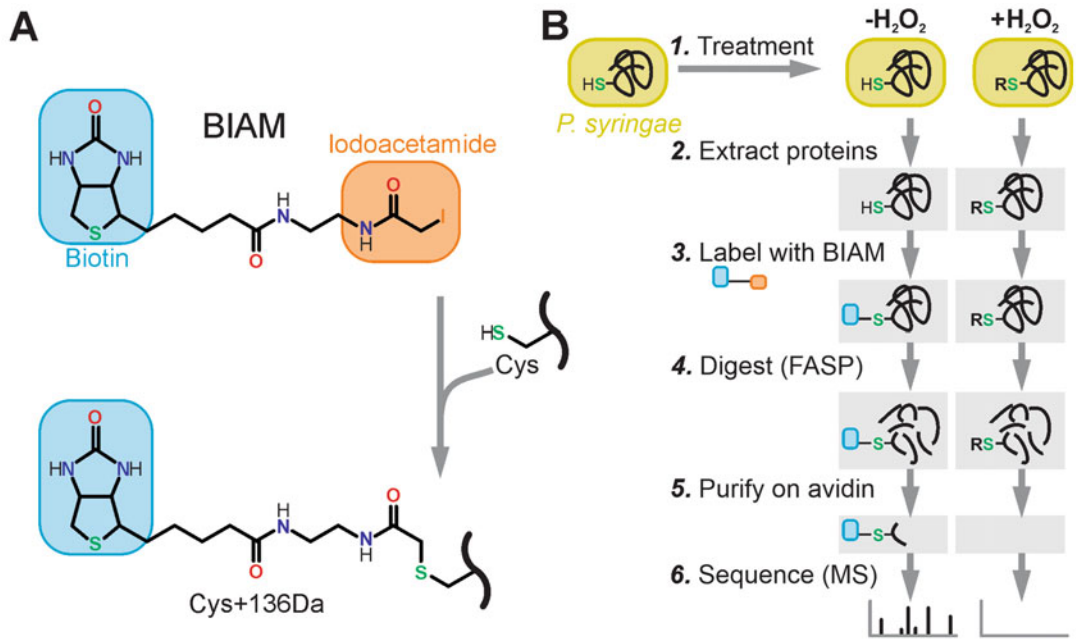

Fig. 1 Concept of Cys reactivity profiling with BIAM (**a**) Alkylation of Cys residues with BIAM. BIAM contains iodoacetamide as a reactive group (organe) and biotin as affinity tag (blue). Labeled peptides have 136 Da adducts on Cys residues. (**b**) Comparative Cys reactivity profiling of *P. syringae* during oxidative stress consists of six steps: (1) Mock treatment and hydrogen peroxide (H_2O_2) treatment of two identical *P. syringae* cultures; (2) Protein extraction under non-denaturing conditions; (3) Labeling with BIAM; (4) Peptide preparation using FASP; (5) Purification of BIAM labeled peptide on monomeric avidin beads; and (6) Sequence labeled peptides by LC-MS/MS

mitigates the risks of sample loss. This method allows us to detect ~1000 labeled peptides from only 200 μg proteins.

To provide a step-by-step protocol, we show comparative profiling of the bacterial plant pathogen *Pseudomonas syringae* exposed to hydrogen peroxide (H_2O_2) as an example. *P. syringae* bacteria are exposed to H_2O_2 during early stages of infection, generated by plant respiratory burst oxidase homologs (RBOHs [11, 12]). This experiment aims to identify redox-sensitive Cys residues in pathogen proteins by mimicking the host-delivered oxidative burst through exposure to 10 mM H_2O_2.

Following this procedure, we identified ~1000 labeled peptides assigned to ~760 unique Cys residues and ~200 redox-sensitive Cys residues in antioxidant enzymes, metabolic enzymes, and transcription regulators (Fig. 2a and **Supplemental Table S1**). Interestingly, half of the 192 Cys residues that show differential reactivity upon H_2O_2 treatment are more reactive in H_2O_2-treated samples (Fig. 2a). These results indicate that this method uncovers not only Cys residues that become oxidized, but also Cys residues becoming more reactive during oxidative stress. Moreover, several proteins have multiple sites with opposite reactivities in response to H_2O_2 treatment. For instance, two glutaraldehyde 3-phosphate dehydrogenases (GAPDHs), Gap1 and Gap2, show a complete oxidation of the active site residue, consistent with the literature [13], while the other Cys residues have unaltered reactivity or even an increased reactivity upon H_2O_2 treatment (Fig. 2b). Taken together, this experiment provides a good example of intriguing observations to be made using Cys reactivity profiling following the protocol detailed below.

2 Materials

2.1 H_2O_2 Treatment of Pseudomonas Syringe Cultures

1. *Pseudomonas syringae* pv. *tomato* DC3000 frozen stock, stored at −80 °C.
2. Rifampicin (25 mg/mL): dissolve 125 mg of rifampicin in 5 ml DMSO, vortex well, and store at −20 ° C in 500 μL aliquots.
3. Liquid NYG medium: 5 g/L of Bacto™ peptone, 3 g/L of Bacto™ yeast extract, 20 g/L of glycerol; autoclave at 121 °C and 100 kPa for 15 min.
4. Solid NYG medium: NYG medium supplemented with 1.5% Agar.
5. Minimal (mannitol-glutamate (MG)) medium: 10 g/L of mannitol, 2 g/L of L-glutamic acid, 0.5 g/L of KH_2PO_4, 0.2 g/L of NaCl; adjust pH to 7.0 with 1 N KOH and adjust pH to 7.4 and the final volume to 900 mL with distilled water, autoclave at 121 °C and 100 kPa for 15 min and add sterile-

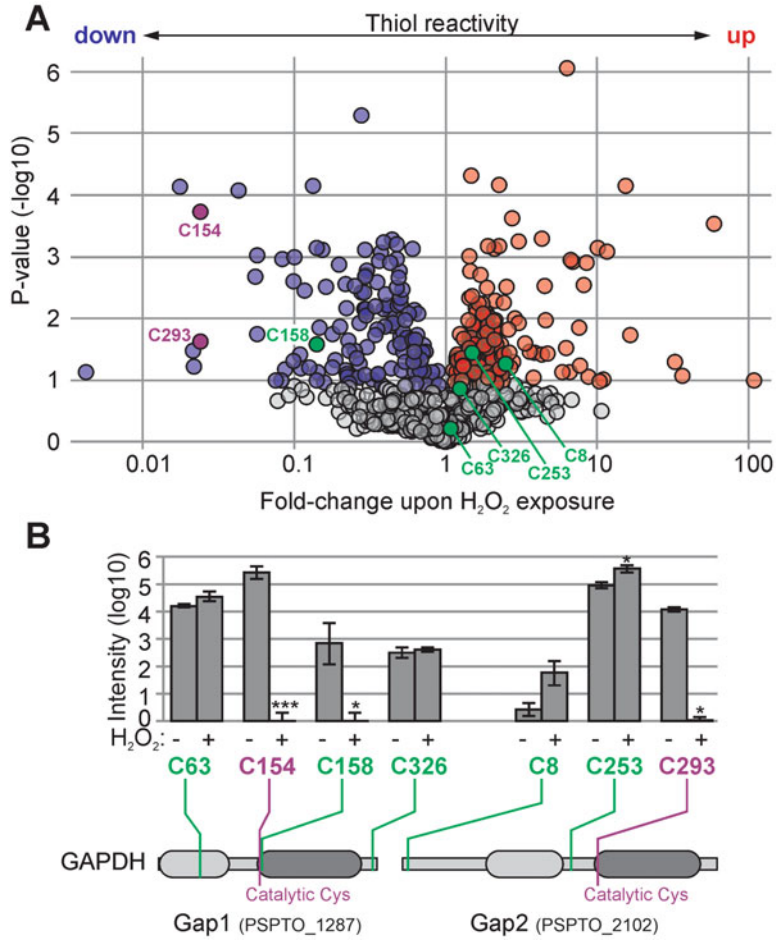

Fig. 2 Comparative Cys reactivity profiling in *Pseudomonas syringae* (**a**) Comparative Cys reactivity profiling on *P. syringae* exposed to H_2O_2 displays significantly altered Cys reactivities. Volcano plot showing all BIAM-labeled peptides identified at least three times, plotted against statistical significance (*P*-value, y-axis) and fold-change of intensities of labeled peptides in mock-treated versus H_2O_2 treated proteome (x-axis). The peptides with significantly decreased or increased mass spectra intensities upon H_2O_2 treatment were highlighted in blue and red, respectively. Gap1/Gap2-derived peptides are indicated. (**b**) GAPDH proteins Gap1 and Gap2 carry multiple Cys residues with opposite reactivities upon H_2O_2 treatment. The graph shows the fold change of MS intensity of each labeled peptide of Gap1 and Gap2. Cartoons depict the PFAM domain structures with labeled Cys residues. Highlighted are catalytic Cys residues (purple) and remaining Cys (green)

filtered (0.22 μm membrane filter) $MgSO_4$ to a final concentration of 0.8 mM (0.2 g/L).

6. 30% (w/w) Hydrogen peroxide solution in H_2O (Sigma Aldrich). Please beware that the stock can expire when not properly stored.

7. 0.22 μM Syringe filter units (Millipore).
8. Incubator with shaker for incubating bacterial cultures at 28 °C, 220 rpm.
9. Spectrophotometer and UV cuvettes for measuring optical density (OD) at 600 nM.

2.2 Protein Isolation and BIAM Labeling

1. Protein extraction buffer: 1× PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4); filter sterilized using 0.22 μm membrane filter.
2. Protein LoBind tubes, 1.5 mL (Eppendorf).
3. Ultrasonic disintegrator/Probe sonicator (Sanyo MSE Soniprep 150).
4. BOECO Tube Rotator RS-24 (BOECO, BOE 8024000) or equivalent.
5. DC Protein Assay (Bio-Rad, 5000116).

2.3 BIAM Labeling

1. BIAM (N-(Biotinoyl)-N′-(Iodoacetyl)Ethylenediamine (Invitrogen™).
2. Dimethyl sulfoxide (DMSO).

2.4 Peptide Preparation by FASP

Please make all solutions for FASP fresh on the day of use.

1. Acetonitrile (ACN) for UHPLC and LC-MS (Sigma-Aldrich).
2. Trifluoroacetic acid (TFA), LC-MS Grade.
3. 1.0 M TEAB (triethylammonium bicarbonate), pH 8.5 ± 0.1 (Sigma-Aldrich).
4. Urea Reagent Plus®, ≥99.5%, pellets (Sigma-Aldrich).
5. Tris-(2-carboxyethyl)-phosphine-hydrochloride (TCEP, Sigma-Aldrich).
6. 2-Chloroacetamide (Sigma-Aldrich).
7. Lysyl Endopeptidase® (Lys-C), Mass Spectrometry Grade (Wako).
8. 50 mM Tris–HCl, pH 8.5; filter sterilized through 0.22 μm membrane filter.
9. Trypsin Gold, Mass Spectrometry Grade (Promega).
10. 100% Acetic acid (HAc).
11. Vivacon 500 centrifugal concentrators (10,000 MWCO) (Sartorius).
12. Refrigerated centrifuge (Eppendorf, 5427 R).
13. Digital Heating Shaking Drybath (Thermo Scientific) or equivalent.
14. Vacuum centrifuge with cold trap for volatile organics (Thermo Scientific).

2.5 Purification of Biotinylated Peptides

Please make all solutions for purification fresh on the day of use.

1. Acetonitrile (ACN) for UHPLC and LC-MS (Sigma-Aldrich).
2. Formic acid (FA, Merck).
3. Monomeric Avidin (Invitrogen).
4. Ammonium bicarbonate (NH_5CO_3).
5. Gilson Diamond Filter Tips Sterile DFL10ST, 0.1–10 µL (Gilson).
6. Visiprep™ SPE Vacuum Manifold DL (Supelco).
7. Vacuum pump (Welch Vacuum Piston Pump 2522C).

2.6 Sequencing Labeled Peptides

1. Trap column: 5 mm, PepMap RSLC, C18, 300 µm ID particle size 2 µm (Thermo Fisher).
2. C18 reversed-phase capillary column: 50 cm, PepMap RSLC, EASY-Spray column, C18, 75 µm ID particle size 2 µm (Thermo Fisher).
3. Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher) with a Dionex UltiMate™ 3000 HPLC (Thermo Fisher).
4. V-bottom 96-well plate.
5. Loading buffer: 5% formic acid, 5% DMSO. Make fresh before use.
6. Mobile phase A: 0.1% formic acid, 5% DMSO. Make fresh before use.
7. Mobile phase B: 100% acetonitrile, 0.1% formic acid, 5% DMSO. Make fresh before use.

2.7 Data Acquisition and Interpretation

1. Computer with operating system: Windows (64-bit) with .NET Framework, 4.7.2 or higher.
2. MaxQuant1.5.3.30 (https://maxquant.org/ [14, 15]).
3. MSFileReader 16.0 (Thermo Fisher).
4. Perseus 1.5.5.3 data analysis software (https://maxquant.org/perseus/).
5. Microsoft Excel (www.microsoft.com).

3 Methods

3.1 H_2O_2 Treatment of Pseudomonas syringae Cultures

How you prepare the proteome defines the potential coverage of labeling (*see* **Note 1**).

1. Revive *Pseudomonas syringae* p.v. *tomato* DC3000 from the glycerol stock by streaking on LB agar medium containing 25 µg/mL rifampicin and incubate the plate for 48 h at 28 °C.

2. Pick a single bacterial colony from the plate and inoculate 10 mL liquid NYG medium containing 25 μg/mL rifampicin and grow the bacteria overnight, shaking at 28 °C.
3. Centrifugate the overnight grown bacterial culture at 2000 × *g* for 10 min at room temperature (RT).
4. Remove the supernatant and suspend the pellet in 10 mL minimal medium.
5. Measure the optical density at 600 nm (OD_{600}) and dilute the bacterial suspensions with minimal medium to $OD_{600} = 0.5$.
6. Dilute the bacterial cultures 1:100 in 50 mL minimal medium to $OD_{600} = 0.005$ and incubate them for ~8 h until they reach $OD_{600} = 0.3–0.5$.
7. Centrifugate the bacterial cultures at 2000 × *g* for 10 min at RT; remove the supernatant and suspend the pellet in 40 mL minimal medium.
8. Split the bacterial suspensions into two to make two identical 20 mL bacterial suspensions in 50 mL falcon tubes.
9. Treat one of the 20 mL bacterial suspensions with 20.4 μL of 9.8 M H_2O_2 (10 mM final concentration) and another with the same amount of water as a mock treatment for 20 min at 28 °C.
10. Centrifugate the bacterial cultures at 2000 × *g* for 10 min at RT, remove the supernatant, and suspend the pellet in 1 mL 1× PBS.
11. Transfer the bacterial suspensions to 1.5 mL tubes and centrifugate them at 2000 × *g* for 2 min at RT.
12. Remove the supernatant and snap-freeze the pellet in liquid nitrogen.

3.2 Protein Isolation

Important: you need to minimize protein oxidation during protein isolation (*see* **Note 2**).

1. Add 100 μL 1x PBS per one 1 mL bacterial cultures with $OD_{600} = 1.0$.
2. Lyse the bacterial cells by sonication on ice, three times for 10 s each.
3. Centrifugate the lysate at 15,000 × *g* for 10 min at 4 °C.
4. Transfer the supernatant into a 1.5 mL tube.
5. Determine the protein concentration using a suitable assay.
6. Dilute extract to 1 mg protein/mL with 1x PBS.

3.3 Labeling with BIAM

Important: labeling conditions are dictated by used probes (*see* **Note 3**).

1. Dispense 200 μL of the lysate into 1.5 mL tubes.
2. Add 2 μL of 1 mM BIAM (10 μM final concentration) or DMSO as a no-probe control.
3. Incubate the labeling mixtures for 1 h at RT while tumbling on a tube rotator.

3.4 Peptide Generation by FASP

In this step, proteins will be digested with trypsin/Lys-C using Filter Aided Sample Preparation (FASP), originally described in [16] (*see* **Note 4**).

1. Assemble the Vivacon 500 filter units and wash them with 200 μL of 0.1% TFA in 50% ACN by centrifugation at 15,000 × *g* for 15 min at 20 °C. There should be thin layer of liquid. If not, the filters might be leaking.
2. Prewash the filter with 200 μL of 8 M urea in 100 mM TEAB by centrifugation at 15,000 × *g* for 15 min at 20 °C.
3. Load the labeling mixtures on the filter and centrifugate at 15,000 × *g* for 20 min at 20 °C. This step removes excess probe.
4. Wash the filter with 200 μL of 8 M urea in 0.1 M TEAB by centrifugation at 15,000 × *g* for 20 min at 20 °C.
5. Repeat **step 4** a further four times. Detergent-containing samples need to be washed intensively until the flow-through no longer forms bubbles.
6. Reduce the proteins by adding 4 μL of 500 mM TCEP in 200 μL of 8 M urea in 0.1 M TEAB (final concentration 10 mM) on the filter and incubate them for 30 min at room temperature.
7. Alkylate the proteins by adding 20 μL of 500 mM 2-chloroacetamide in water (final concentration 50 mM) on the filter and incubate them for 30 min at room temperature in the dark.
8. Centrifugate the samples at 15,000 × *g* for 20 min at 20 °C.
9. Wash the filter with 200 μL of 6 M urea in 50 mM TEAB and centrifugate them at 15,000 × *g* for 20 min at 20 °C.
10. Repeat **step 9** once.
11. Change to a fresh collection tube for peptide collection.
12. Prepare the 1 μg/μL Lys-C stock solution by dissolving 20 μg Lys-C in 20 μL 50 mM Tris–HCL (pH 8.5).
13. Dilute 5 μL of 1 μg/μL Lys-C stock solution with 95 μL of 1 M urea in 50 mM TEAB. Load 1 μg Lyc-C per 40 μg protein on

the filter with a total volume of 100 μL and incubate for 4 h at 37 °C.

14. Prepare the 1 μg/μL trypsin stock solution by dissolving 100 μg of Lys-C in 100 μL 50 mM acetic acid (2.875 mL acetic acid solution in 1 μL MS water).
15. Dilute 5 μL of 1 μg/μL trypsin stock solution with 95 μL of 50 mM TEAB. Load 1 μg trypsin per 40 μg protein on the filter with a total volume of 100 μL and incubate for 16–20 h at 37 °C.
16. Seal the lid with Parafilm to prevent evaporation.
17. Collect the digested peptides by centrifugation at 15,000 × *g* for 20 min at 20 °C.
18. Transfer the peptide-containing flow-through in 1.5 mL tubes.
19. Wash the remaining peptides off the filter with 150 μL 0.1% TFA by centrifugation at 15,000 × *g* for 15 min at 20 °C.
20. Wash the remaining peptides off the filter with 150 μL 0.1% TFA in 50% ACN by centrifugation at 15,000 × *g* for 15 min at 20 °C.
21. Pool the flow-through collected from **steps 16–19** in 1.5 mL tubes.
22. Dry the peptides by vacuum centrifugation.

3.5 Purification of Biotinylated Peptides

In this step, biotinylated peptides are purified using SPE (solid-phase extraction) operated through a vacuum manifold to increase the sample processing capacity (*see* **Note 5**).

1. Dissolve the 200 μg peptides in 200 μL of 25 mM ammonium bicarbonate (1 μg/μL final concentration) using sonication in a water bath, if necessary.
2. Assemble the Visiprep™ SPE Vacuum Manifold DL, place the Gilson Diamond Filter Tips on the disposable liner, and fasten the valves.
3. Wash the filter tips with 400 μL of 0.1% formic acid (FA) 50% acetonitrile (ACN) by applying the vacuum.
4. Repeat **step 3** once.
5. Wash the filter tips with 400 μL of 25 mM ammonium bicarbonate by applying the vacuum.
6. Repeat **step 5** twice.
7. Load 150 μL monomeric avidin beads on the filter tips.
8. Wash the filter tips with 200 μL of 25 mM ammonium bicarbonate by applying the vacuum.
9. Repeat **step 8** once.

10. Place the new collection tube and slowly pass the peptides solutions through the filter tip by applying the vacuum.
11. Reload the flow-through on the filter tips.
12. Repeat **step 11** once.
13. Wash the filter tips with 200 μL of 25 mM ammonium bicarbonate by applying the vacuum.
14. Repeat **step 12** once.
15. Close the valves and place the new collection tube.
16. Load 200 μL of 0.4% TFA in 30% acetonitrile and incubate for 30 min.
17. Elute the peptides by applying the vacuum in the collection tube.
18. Load another 200 μL of 0.4% TFA in 30% acetonitrile straightaway.
19. Dry down the biotinylated peptides by vacuum centrifugation.

3.6 Sequencing Labeled Peptides

LFQ requires individual MS runs of all samples sequentially (*see* **Note 6**).

1. Reconstitute the peptides in 10–20 μL of 5% DMSO, 5% formic acid solution and add the sample to a V-bottom 96-well plate.
2. Place the plate in the Dionex UltiMate™ 3000 UHPLC system.
3. Trap the peptides on a C18 PepMap100 pre-column (300 μm i.d. × 5 mm, 100 Å, Thermo Fisher) using solvent A (0.1% formic acid in water) at a flow rate of 10 μL/min.
4. Separate peptides on an in-house packed analytical column (75 μm i.d. packed with ReproSil-Pur 120 C18-AQ, 1.9 μm, 120 Å, Dr. Maisch GmbH).
5. Acquire data in data-dependent acquisition (DDA) mode.
6. Acquire full scan MS spectra in the Orbitrap (scan range 350–1500 m/z, resolution 70,000, AGC target 3e6, maximum injection time 50 ms).
7. Select the 10 most intense peaks for HCD fragmentation at 30% of normalized collision energy at resolution 17,500, AGC target 5e4, maximum injection time 120 ms with first fixed mass at 180 m/z.
8. Select charge exclusion for unassigned and 1+ ions.

3.7 Data Acquisition and Interpretation

1. Download the Uniprot reference proteome for *Pseudomonas syringae* pv. *tomato* (strain ATCC BAA-871/DC3000), Proteome ID; UP000002515.

2. Submit the RAW spectra to Andromeda [17].
3. Annotate the spectra using MaxQuant (1.5.3.30) with default settings [18].
4. Select oxidation of methionine residues (16 Da) and acetylation of the protein N-terminus (42 Da), modification of cysteine by alkylation with iodoacetamide (57 Da) or with BIAM ($C_{14}H_{22}O_3N_4S$, 326.1413 Da) as the "Variable Modifications." Set the "Enzyme specificity" was set to "Trypsin/P" with two missed cleavages allowed. Switch on "Label-free protein quantification." Retention times are recalibrated based on the in-built nonlinear time-rescaling algorithm. Switch on the "match between runs" option with the maximal match time window set to 0.7 min and the alignment time window set to 20 min. The quantification is based on the "value at maximum" of the extracted ion current. At least two quantitation events are required for a quantifiable protein.
5. For further analysis and filtering of the results, upload the text file for modification sites (e.g., BIAMSites.txt) to Perseus v1.5.5.3 [16]. MaxQuant generates several text files. The file with modification sites appears with the name you set in the MaxQuant configuration. Use the modification sites of your interest.
6. Load the columns named "Intensity (sample name)_ (1,2,3...)" as main column. The rest of the columns are default. One sample can have multiple columns depending on how many modifications per peptides are allowed in MaxQuant.
7. To have only one column per sample and the appropriate number of rows per site, process the data by using "Expand site table."
8. Log2-transform the MS intensities.
9. Create categorical groups by selecting categorical annotation rows (e.g., Control, H_2O_2).
10. Optionally, import protein annotations (e.g., added annotations: mainAnnot.pseudomonas_syringae_pv._tomato_ (strain_dc3000).txt.gz).
11. Filter the data, e.g., detected at least three times in at least one group.
12. Impute the missing values from normal distribution using default settings.
13. Normalize the values by subtracting from the median of each column.
14. Visualize the data with a volcano plot using default settings (Fig. 2a).

4 Notes

1. It is critical to select the appropriate conditions that create the proteome containing the proteins of your interest. We chose a minimal medium because the virulence gene expression (e.g., type III secretion system (T3SS) genes) of *P. syringae* are suppressed in rich media [19]. The transient exposure of *P. syringae* to H_2O_2 is aimed to mimic the oxidative burst which *P. syringae* experiences in the plant apoplast during the infection [12].
2. The lysis method and buffer should be carefully considered to prevent alteration of the redox balance before labeling. For instance, high concentrations of reducing agents (e.g., DTT and TCEP) will change the redox status of the lysates. Conversely, too much exposure to air may cause oxidation of extracts. Also catalases, peroxidases, and other redox enzymes will change the redox balance.
3. There are several probes for reactive thiols [20]. The probe concentration and incubation time depend on the potency of the used probe and the proteome and should be adjusted accordingly.
4. The FASP protocol takes advantage of MW filter microfuge tubes and includes all steps from removal of excess probes, buffer exchange, protein denaturation, disulfide-bond reduction, alkylation to digestion on one filter column, whilst minimizing sample loss. Also, an appropriate molecular weight (MW) cutoff column efficiently filters out the contaminants such as intact trypsin and Lys-C that have MWs more than 20 kDa from the flow-through that is your peptide pool at the final step.
5. The vacuum manifold can handle 12 samples simultaneously but it is easier to handle less than 6 samples. When assembling, please be aware of inserting the disposable liner inside the collection tubes and make sure that the unused ports are tightly closed and do not apply more vacuum than 20 Hg (0.6772778 bar).
6. LFQ analysis requires the MS analysis of all samples, intermitted by MS standards. If included, the no-probe controls should be analyzed first. A prerun with a probe-labeled sample is useful to adjust the loading, if needed.

Acknowledgments

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Chapter 10

Determination of Caspase-Like Activities in Roots by the Use of Fluorogenic Substrates

Katarina Šoln and Marina Klemenčič

Abstract

Activity of proteases in tissues can be influenced by various intrinsic and extrinsic factors. One of the activities that is regularly monitored in organisms ranging from prokaryotes to metazoans is the -aspase-like activity: activity of proteases, which cleave their substrates after the negatively charged amino acid residues, especially the aspartic acid. This activity is also known as the caspase-like activity, since the caspases, metazoan cysteine proteases, are one of the best characterized proteases with Asp-directed activities. Plants do not contain caspases; however, various plant proteases have been shown to exhibit caspase-like activity including saspases, phytaspases, and legumains (VPEs). The activity of these proteases can change in plants in response to stress. Here we present a simple method for monitoring of the caspase-like protease activity in roots, which have been treated with allelopathic extracts, using a set of commercially available caspase substrates. We show that activity towards some, but not all, caspase substrates is upregulated in treated but not control samples. The protocol can be used also for other plant tissues as well as for other stressors.

Key words VPE, Phytaspase, Saspase, Caspase-like activity, Allelopathy, PCD

1 Introduction

Caspases, the cysteine dependent aspartate directed proteases, are among the best characterized metazoan proteases, crucial for execution of the programmed cell death (PCD) in animals [1]. In humans, 12 different caspases are present and this number varies among other animals [2]. Their cleavage specificities in the P1–P4 positions of the substrate vary and this property can be used for monitoring of their distinct activities.

For this reason, tetrapeptides harboring an AFC (7-Amino-4-trifluoromethylcoumarin) fluorescent compound covalently bound to the P1 residue can be used as substrates. Only after cleavage, the fluorescent group is released, which is then detected using 400 nm excitation and 505 nm emission wavelength light. For detection of caspase activities in animal cells or tissues, the following substrates are commonly used (the fluorophore is in this case AFC although

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others, such as AMC (7-Amino-4-methylcoumarin) are often employed as well): Ac-YVAD-AFC (caspase-1 substrate), Ac-VDVAD-AFC (caspase-2 substrate), Ac-DEVD-AFC (caspase-3 substrate), Ac-LEVD-AFC (caspase-4 substrate), Ac-WEHD-AFC (caspase-5 substrate), Ac-VEID-AFC (caspase-6 substrate), Ac-IETD-AFC (caspase-8 substrate), Ac-LEHD-AFC (caspase-9 substrate), and Ac-AEVD-AFC (caspase-10 substrate).

Despite the fact that no caspases were yet identified in any non-metazoan organism, these organisms were observed to undergo cell death in a manner strongly reminiscent of the metazoan apoptosis. Examples of unicellular eukaryotes include the parasitic *Leishmania donovani* [3], *Plasmodium falciparum* [4] and *Trypanosoma cruzi* [5], yeast [6–8] and algae [9–14]. The cell death phenotypes of these cells share several features with apoptosis including DNA fragmentation, increased caspase-like activity, changes in mitochondrial membrane potential, and enlargement of organelles. Also in plants, caspase-like proteolytic activities accompany hypersensitive response and PCD. The enzymes, responsible for the observed activities, were found to be the vacuolar processing enzymes (VPEs, also called legumains), which exhibit caspase-1-like (YVADase) activity [15, 16], saspases with predominant caspase-8-like activity [17], and phytaspases with predominant caspase-3-like activity [18, 19]. A very good overview of caspase-like activities in plants is reviewed by Bonneau et al. [20].

Here, we present a simple protocol of measurement of caspase-like activities in roots of the radish (*Raphanus sativus*), which have been watered with or without an allelopathic extract from rhizomes of an invasive Japanese knotweed plant (*Fallopia japonica*). This protocol can be used for other plants as well as other plant tissues, where relative differences in caspase-like activities are to be known.

2 Materials

Prepare all solutions using deionized miliQ distilled water (18.2 MΩ-cm at 22 °C). Prepare and store all reagents at room temperature unless indicated otherwise.

2.1 Treatment of Raphanus sativus Seedlings Using Knotweed Extract and Extract Preparation

1. *Raphanus sativus* seeds, stored at 4 °C (*see* **Note 1**).
2. Rhizomes of Japanese knotweed (*Fallopia japonica*).
3. Lyophilizer.
4. Cutting mill (cut to 1 mm pieces or smaller, such as Cutting mill SM 200, Retsch).
5. Orbital shaker, which can be used at room temperature.
6. Filter paper, such as Grade 520A.

7. Sterile round plastic Petri dishes (diameter 14 cm, one for each treatment replicate).
8. Scalpel.
9. Liquid nitrogen in a Dewar flask.
10. Tabletop vortex.
11. Ice.
12. Growth chamber for plants in which temperature and light regimes can be changed.
13. Vacuum pump.
14. Mortar and pestle with liquid nitrogen.

2.2 Reagents and Chemicals for Fluorescence Determination

1. 2-(N-morpholino)ethanesulfonic acid (MES) buffer: 20 mM MES hydrate, 150 mM NaCl, pH 5.5 (*see* **Note 2**).
2. DTT: Prepare 1 M 1,4-dithiothreitol (DTT) in deionized miliQ water. Store at 4 °C before the use. DTT should always be prepared fresh.
3. EDTA: Prepare 0.5 M EDTA in dH_2O, pH 8.0 (adjusted with concentrated NaOH).

2.3 Caspase Substrates

The fluorogenic substrates can be either bought as lyophilized powder or already in DMSO solution. If bought as powder, they should be resuspended in DMSO to the concentration of 1 mM. Vortex well and spin down shortly before use. Stock solutions of substrates must be stored at −20 °C and kept on ice during the analyses. The following substrates can be used to monitor caspase-like activities:

1. Ac-YVAD-AFC.
2. Ac-DEVD-AFC.
3. Ac-VDVAD-AFC.
4. Ac-WEHD-AFC.
5. Ac-LEHD-AFC.
6. Ac-LEVD-AFC.
7. Ac-IETD-AFC.
8. Ac-VEID-AFC.
9. Ac-AEVD-AFC.

2.4 Determination of Total Protein Concentration

1. 5 × Bradford reagent: dissolve 100 mg of Coomassie Brilliant Blue G-250 (not R-250) in 50 mL of 95% ethanol (mix for 5–10 min using a magnetic stirrer). Add 100 mL of 85% (w/v) orthophosphoric acid and 50 mL of distilled water. Filter the solution through filter paper and store at −20 °C.
2. Deionized miliQ distilled water.

2.5 Equipment for Measurement of Absorbance and Fluorescence

1. Microtiter plate, 96-well, transparent.
2. Plastic cuvettes for measurement of absorbance in the visible spectra.
3. Fluorescence microtiter plate reader.
4. UV-Vis spectrophotometer.
5. Computer with numerical data analysis software (such as Excel).

3 Methods

3.1 Treatment of Raphanus sativus Seedlings with Knotweed Extract

Schematic representation of the whole experiment is shown in Fig. 1.

1. Collect rhizomes of approximately 50 Japanese knotweed (*Fallopia japonica*) plants from nature, wash to remove soil, lyophilize (app. 5 days, −98 °C, 0.003 mbar), and cut with cutting mill to get rhizome powder (or pieces up to 1 mm in length).
2. Extract preparation: add 10 g of rhizome material to 100 mL distilled water and incubate on a shaker (175 rpm) for 24 h at room temperature (RT). Vacuum filter through filter paper and store the extract at 4 °C before use. Please be aware that extracts must be used within 3 days.
3. Germination test: place 50 *Raphanus sativus* seeds on a filter paper in a plastic Petri dish. Use one Petri dish for one replicate of each treatment. Water with 10 mL of extracts. Control seeds are watered with 10 mL of distilled water. Allow the seeds to grow for 3 days under laboratory conditions (22 ± 2 °C, 15 h light/9 h dark). Petri dishes must be covered with a lid to prevent evaporation.

3.2 Preparation of Root Cell Lysate

1. Cut the root tips of *Raphanus sativus* seedlings of the treated and non-treated plants, app. 1 cm from the root apex. Use the scalpel. You will need app. 30 root tips (*see* **Note 3**).
2. Weigh 100 mg of fresh root tissue for treated and non-treated samples.
3. Homogenize 100 mg of fresh root tissue with a mortar and pestle in liquid nitrogen (*see* **Note 4**). Add 1 mL MES buffer and continue grinding.
4. Transfer 1 mL of the grinded cell lysate to a precooled 1.5 mL tube and vortex for approximately 15 s. The samples must be kept on ice during the procedure (*see* **Note 5**).

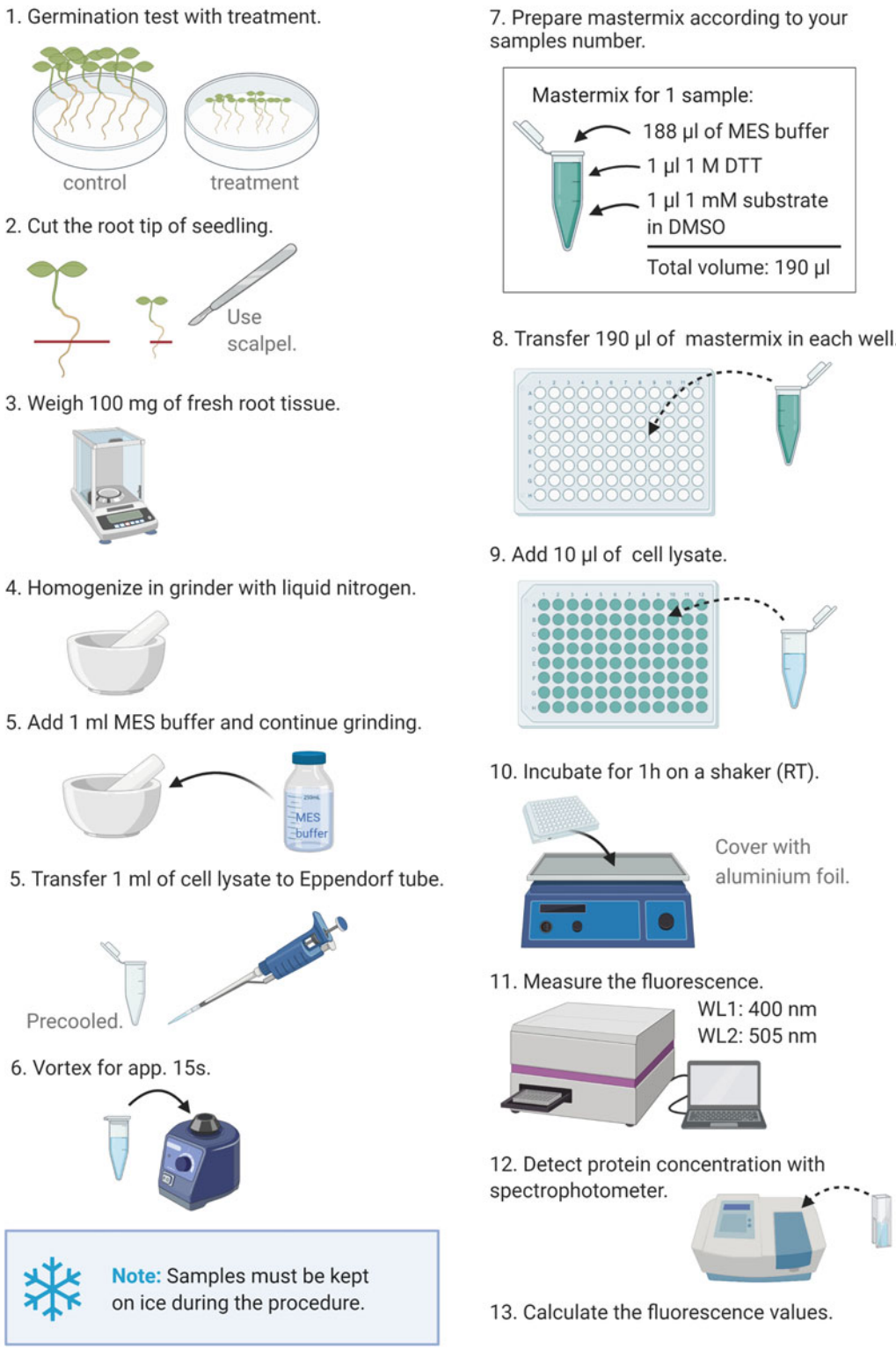

Fig. 1 Schematic representation of the experiment

3.3 Preparation of the Protease Reaction Mixture

For each of the substrates, a mastermix can be prepared consisting of the buffer, the substrate, and DTT. This mastermix can be stored at −20 °C up to a month; however, frequent freeze-thawing or longer storage times are discouraged. Below, a mixture for one substrate and one biological sample in one technical replicate is described. The volumes should be multiplied by the number of biological and technical replications.

1. Prepare the reaction mixture by mixing of 188 μL of MES buffer, 1 μL 1 M DTT, and 1 μL 1 mM substrate in DMSO. The total volume of the reaction mixture for one sample is therefore 190 μL. Keep the mixture on ice. For every substrate at least 3 technical replicates should be prepared (*see* **Note 6**).
2. Pipette 190 μL of this mastermix per well on the microtiter plate. When finished pipetting, cover the microtiter plate with aluminum foil (*see* **Note 7**) and leave on ice before adding the cell lysate. Plates prepared like this can be kept in dark at +4 °C overnight.

3.4 Fluorometric Determination of Caspase-Like Activities

1. Add 10 μL of the prepared cell lysate per one well containing 190 μL of the reaction mixture and mix gently by pipetting. For the negative control use 10 μL of the MES buffer. Cover the microtiter plate with aluminum foil (*see* **Note 7**) and incubate for 1 h on a shaker (50–100 rpm) at room temperature.
2. Measure the fluorescence on the microtiter plate reader using 400 nm excitation and 505 nm emission wavelengths. Measurements can also be repeated after 12 h (*see* **Notes 8** and **9**).

3.5 Determination of Protein Concentration

In order to determine the correct intensities of fluorescence activities, fluorescence values must be normalized to the protein content in each of the lysates. For this reason, the concentration of proteins in each of the cell lysates is determined by Bradford method.

1. Mix 790 μL deionized miliQ water, 200 μL Bradford (5×) reagent, and 10 μL cell lysate in one 1.5 mL tube. Mix carefully with pipetting or inverting several times. Prepare at least 3 technical replicates per one lysate sample.
2. Prepare also the blank sample by mixing 800 μL deionized miliQ water and 200 μL Bradford reagent.
3. Measure the absorbance of each of the samples at 595 nm using the spectrometer and write down the values.

3.6 Determination of Fluorescence Values

For determination of caspase-like activities, fluorimetric data has to be analyzed first by removal of the baseline and by normalization in regard to the total protein content in a lysate as calculated in Subheading 3.4.

1. Deduct the values of the blank samples (containing only the buffer, DTT and the substrate) from the samples for each of the substrates used.
2. Divide these values by the absorbance values of the respective cell lysate used. The higher the protein content, the higher is the absorbance.
3. Calculate the average, standard error, and other statistical characteristics for every fluorescence data point.

4 Notes

1. In this experimental setup, we used three biological replicates (3 Petri dishes) for treated and three biological replicates (3 Petri dishes) for the non-treated samples. In the treated sample, fewer biomass is expected, which is needed for further experiments. For this reason, additional plates can be used. Since approximately 50 seeds are used for germination for each biological replicate, 6 plates and thus approximately 300 seeds are needed.
2. All up to now characterized enzymes with caspase-like activities have acidic pH optima. Nevertheless, buffers with other pH values lower or higher than 5.5 can also be used.
3. Approximately 20–25 non-treated root tips suffice for 100 mg. In the treated sample, fewer biomass is expected. However, we here suggest pooling the root tips from all three treated plates as one sample and the root tips from all three non-treated plates as one sample as well to obtain a 100 mg.
4. Fresh root material is required. Be quick, root samples must be homogenized immediately after the cutting.
5. If cell lysate is needed for other analyses, 1 μL of 0.5 M EDTA can be added to each sample prior to freezing at −20 °C.
6. Prepare also blank samples in which 10 μL of the lysate is substituted with 10 μL of the buffer. Blank samples must be prepared for each individual substrate. Optionally, blank samples without the cell lysate and substrate can also be prepared.
7. Reagents in the microtiter plate must be stored in the dark before the analysis/fluorometric measurement.
8. Prepare in advance a detailed plan for pipetting in the microtiter plate. Do not forget the blank samples and all the technical replicates (we suggest 3). For this, a 96-well template can be used.
9. If absorbance is measured after 12 h, cover your microtiter plate with aluminum foil and incubate it on the shaker until fluorometric analysis.

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Chapter 11

Detection of Damage-Activated Metacaspase Activity by Western Blot in Plants

Simon Stael, Luke P. Miller, Álvaro D. Fernández-Fernández, and Frank Van Breusegem

Abstract

Metacaspases are cysteine proteases that are present in plants, protists, fungi, and bacteria. Previously, we found that physical damage, e.g., pinching with forceps or grinding on liquid nitrogen of plant tissues, activates *Arabidopsis thaliana* METACASPASE 4 (AtMCA4). AtMCA4 subsequently cleaves PROPEP1, the precursor pro-protein of the plant elicitor peptide 1 (Pep1). Here, we describe a protein extraction method to detect activation of AtMCA4 by Western blot with antibodies against endogenous AtMCA4 and a PROPEP1-YFP fusion protein. It is important to (1) keep plant tissues at all times on liquid nitrogen prior to protein extraction, and (2) denature the protein lysate as fast as possible, as metacaspase activation ensues quasi immediately because of tissue damage inherent to protein extraction. In theory, this method can serve to detect damage-induced alterations of any protein-of-interest in any organism for which antibodies or fusion proteins are available, and hence, will greatly aid the study of rapid damage-activated proteolysis in the future.

Key words Metacaspase, PROPEP1, Physical damage, Protease activity, *Arabidopsis thaliana*, Western blot quantification

1 Introduction

Metacaspases are a family of cysteine proteases originally identified by homology search to animal caspases [1]. Metacaspases and seven other families of cysteine endopeptidases are allocated in the CD clan, according to the MEROPS database (http://www.ebi.ac.uk/merops) [2]. Within the CD clan, metacaspases, caspases, and paracaspases are grouped in the C14 family, with a further subdivision of caspases in the subfamily C14A and metacaspases and paracaspases in the subfamily C14B. Despite sharing the cysteine-histidine catalytic dyad and a similar protein structure, metacaspases' functions, substrate specificity, and their mode of action clearly differ from those of caspases [3–5]. Caspases, as

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reflected in their name (Cysteine-dependent ASPartate specific proteASE), cleave their substrates after aspartic acid [6], whereas metacaspases cleave after arginine or lysine residues and cannot process synthetic capase-3 substrates [7–9]. In fact, screening of a synthetic substrate library for metacaspases resulted in an optimal minimal substrate corresponding to VRPR↓X, representing a double basic amino acid motif at the positions P1 and P3 (*see* **Note 1**). Metacaspases are classified as type-I, type-II, and type-III subclasses, depending on the structural arrangement of their large (p20) and small domains (p10). In type-I and type-II metacaspases the large subunit precedes the small subunit, while the order is inverted in type-III metacaspases [10, 11]. Type-I metacaspases bear a distinctive N-terminal prodomain with a proline-rich repeat region, a Zinc finger like motif (in plants), and a relatively shorter linker between their domains compared to type-II metacaspases. An additional sub-classification among type-II metacaspases can be made based on the requirements for enzymatic activity, whether it is availability of free calcium ions (Ca^{2+}) or low pH [7]. The activation of most metacaspases requires Ca^{2+} in vitro, such as *Arabidopsis thaliana* AtMCA4, with the exception of the pH-dependent type-II metacaspases, such as AtMCA9 [3, 7]. Whereas most metacaspases have a pH optimum of 7–7.5, AtMCA9 activity is highest around pH 5.5 and does not require Ca^{2+}.

Plant metacaspases are known to have functions in cell death [8, 12, 13] and are involved in responses to pathogens [14–16]. Previously, we have extended the range of metacaspase functions to include plant wound response [17, 18]. We found that AtMCA4 is activated by physical damage and subsequently cleaves and releases the plant elicitor peptide 1 (Pep1) from its precursor pro-protein PROPEP1 [17]. Peps are important molecular players in the plant wound response and induce immune-like responses by binding the cognate transmembrane PEP Receptor kinases (PEPRs) [19–25]. We found that PROPEP1 is a bona fide substrate of AtMCA4 that is cleaved both in vitro and in vivo to release the mature Pep1 small signaling peptide [17]. Cleavage of PROPEP1 by AtMCA4 was independently confirmed by Shen et al., who further showed that all Arabidopsis type II metacaspases can cleave other *Arabidopsis* PROPEP family members [26]. AtMCA4 activation is likely due to damage-induced membrane permeabilization and accumulation of high amounts of Ca^{2+} in the cytosol. Accordingly, PROPEP1 processing did not occur when metacaspase activity was depleted by treatment with specific inhibitors or Ca^{2+} chelating agents [17]. A recently solved X-ray crystal structure confirms the critical importance of Ca^{2+} binding to AtMCA4 [27]. Ca^{2+} binds to aspartic rich motifs in the linker region resulting in subsequent autocatalytic cleavage and disengagement of the p20 domain from the p10 part. Autocatalytic cleavage of a p20* fragment (p20 plus an additional AtMCA4 protein sequence) is

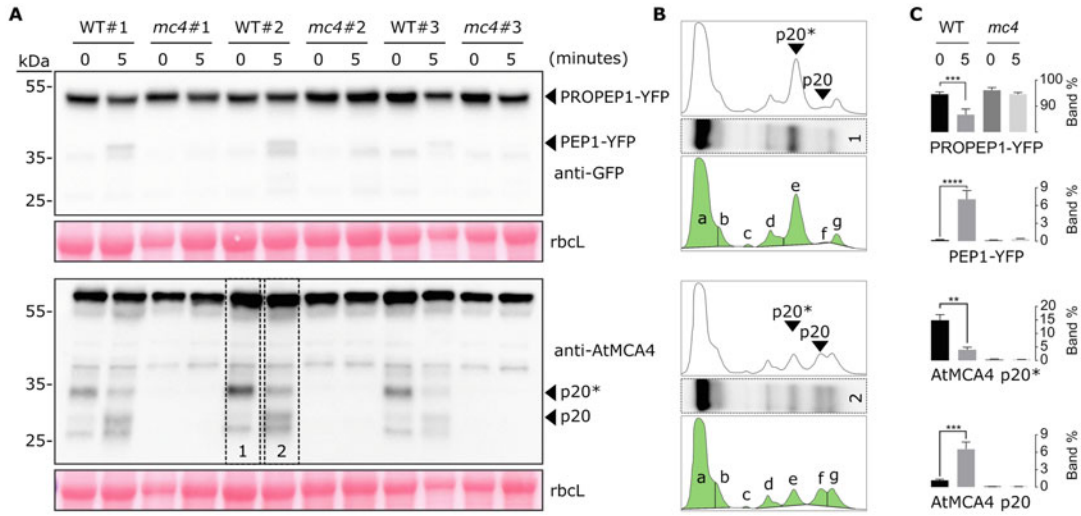

Fig. 1 Western blot of AtMCA4 and a PROPEP1-YFP fusion protein in Arabidopsis seedlings damaged with forceps. (**a**) Western blot of Arabidopsis wild type (WT) and *atmca4* mutant (*mc4*) seedlings expressing a PROPEP1-YFP fusion protein were damaged with forceps and incubated for 5 min at room temperature (three biological replicates labeled #1, #2 and #3). *rbcL* Ponceau S stained rubisco large subunit as total protein loading control. (**b**) As an example, plot profiles were generated with ImageJ for two lanes highlighted in the anti-AtMCA4 Western blot in (**a**). Peaks corresponding to protein bands, labeled **a–g**, were delimited manually and the peak densities (area under the curve indicated in green) were calculated by ImageJ. (**c**) The quantification process was repeated for all lanes and bands in (**a**) and the highlighted bands (with a triangle) were plotted as the percentage of overall density in the corresponding lane (band %; two-way ANOVA test, *P* value). For example, AtMCA4 p20* band % is the area under the curve of "peak e" divided by the sum of the areas under all peaks in lane 1

indicative of induction of AtMCA4 activity in vivo (Fig. 1a). Upon high Ca^{2+} availability, AtMCA4 activity rapidly ensues within seconds and maximal cleavage of substrates, e.g., a PROPEP1-YFP fusion protein, in vivo within 5 min after damage [17]. PROPEP1-YFP cleavage is significantly reduced in mutant *atmca4 Arabidopsis* seedlings [17]. Moreover, PROPEP cleavage was shown to be inhibited when the catalytic cysteine of metacaspases performing the nucleophilic attack was mutated as well as when the arginine at position P1 preceding the cleavage site in PROPEP1 was replaced by a different amino acid [17, 26]. Together, AtMCA4 autocatalytic cleavage and cleavage of the substrate PROPEP1-YFP form a reliable read-out of damage-activated AtMCA4 activity.

Here, we describe a method to process samples in order to detect damage-activated AtMCA4 activity, taking into account the potential problems that arise from rapid in vivo activation of AtMCA4 during physical damage. Plant tissues are frozen and ground to a powder with a mortar and pestle under constant supply of liquid nitrogen to ensure that all enzymatic activity, including metacaspase derived proteolysis, is inhibited (*see* **Note 2**). Subsequently, the protein lysates are denatured as fast as possible, again to inhibit unwanted damage-activated metacaspase activity during

sample preparation. AtMCA4 activity is evaluated on Western blots by the detection of autocatalytic cleavage (reduction of the p20* band and accumulation of the p20 band) and cleavage of the bona fide substrate PROPEP1-YFP (accumulation of a Pep1-YFP band; Fig. 1a). Subsequent quantification of the bands on the Western blot [28] (Fig. 1b) allows for a statistical comparison between different treatments or plant genotypes (e.g., wild type versus *atmca4* mutant seedlings; Fig. 1c).

2 Materials

1. Wild type and PROPEP1-YFP transgenic *Arabidopsis thaliana* seeds [17, 19].
2. Small forceps for handling of seedlings.
3. Serrated forceps for damage treatments of seedlings.
4. Liquid nitrogen.
5. Extruded polystyrene foam box and sample tube holder.
6. 1.5 mL Tubes.
7. Small lab spoon or spatula that fits inside a 1.5 mL tube.
8. Mortar and pestle.
9. Two-times (2x) Laemmli buffer: 125 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 0.005% bromophenol blue [29]. Store at room temperature. Before use, add 5% β-mercaptoethanol.
10. Semi-micro lab balance.
11. Dry block incubator.
12. Western blot appliances.
13. Anti-AtMCA4 and anti-GFP antibody.
14. ½ MS medium (solid): Dissolve 2.2 g of Murashige & Skoog (MS), 0.1 g of myo-Inositol, 0.5 g of MES monohydrate, 10 g of sucrose in ddH2O, 7.5 g of agar. Adjust the pH to 5.7 with 1 M KOH, and sterilize at 121 °C for 20 min.
15. ImageJ release 1.45 or higher [30]. ImageJ is an open-source image processing program that can be freely downloaded: https://imagej.nih.gov/ij/.

3 Methods

3.1 Sowing of Arabidopsis Seeds on Plates

1. Sow two lines above each other of sterilized seeds on ½ MS solid medium plates, with 15–18 seeds for each line.
2. Stratify the seeds for 2 days at 4 °C.
3. Transfer the plates to grow for 10–14 days at 25 °C, 50 μmol photons m^{-2} s^{-1}, 16 h/8 h light/dark.

3.2 Damaging Arabidopsis Seedlings with Forceps

Sample extractions are performed on liquid nitrogen. Take the necessary precautions to handle liquid nitrogen, such as protective gloves and protective eye wear.

1. Before collecting 10- to 14-day-old seedlings, pre-cool the mortar(s) and pestle(s) on liquid nitrogen. Also, label the required number of 1.5 mL tubes and weigh them on a semi-micro lab balance (*see* **Note 3**).
2. Collect the seedlings gently with small forceps to minimize unwanted damage and place them together on a clean surface (*see* **Note 4**).
3. Damage the seedlings by pinching with serrated forceps (*see* **Note 5**). Keep the damaged seedlings at room temperature for the required amount of time (*see* **Note 6**).
4. Transfer the damaged seedlings to the pre-cooled mortar and grind under a constant supply of liquid nitrogen with a pestle to a fine powder.
5. Transfer the freeze-ground tissue powder to a pre-cooled 1.5 mL tube placed on a polystyrene foam sample tube holder floating on liquid nitrogen in a polystyrene foam box with the help of a pre-cooled small lab spoon.
6. Keep the samples on liquid nitrogen or store at −70 °C for later use.

3.3 Damage by Thawing of Freeze-Grinded Arabidopsis Seedlings

As alternative to damage with forceps, *Arabidopsis* seedlings are ground on liquid nitrogen, and the resulting tissue powder thawed at room temperature for a required amount of time, to mimic severe damage (*see* **Note 7**).

1. Collect the seedlings gently with small forceps to minimize unwanted damage in a pre-cooled mortar and grind under a constant supply of liquid nitrogen to a fine powder.
2. Transfer the freeze-ground tissue powder to a pre-cooled 1.5 mL tube (as in Subheading 2.5 **step 3**).
3. Weigh the amount of freeze-grinded tissue powder on a semi-micro lab balance. One at a time, the tubes can be removed from the liquid nitrogen and placed on the balance shortly without thawing (enough to note down the weight of the tube plus sample). Quickly place the tubes back on liquid nitrogen. The weight of the sample is obtained by subtracting the weight of the tube (*see* **Note 3**).
4. Transfer the tubes to room temperature and open and close the lids to prevent potential build-up of nitrogen gas (*see* **Note 8**).
5. Thaw the tubes (*see* **Note 9**) and keep them at room temperature for the required amount of time (*see* **Note 6**).

3.4 Termination of Damage-Induced Metacaspase Proteolysis and Protein Extraction

Sample tubes coming from **steps 3.2** and **3.3** are processed in the same way in the following steps.

1. Heat up a dry block incubator and a required volume of 2x Laemmli buffer in 1.5 mL tubes to 70 °C. Work in a fume hood, as the 2x Laemmli buffer contains β-mercaptoethanol.
2. Place the sample tubes, coming from liquid nitrogen, on a rack at room temperature (*see* **Note 8**) and add three volumes (v/w ratio) of pre-heated (70 °C) 2x Laemmli buffer (*see* **Note 10**).
3. Mix the samples with the use of a vortex or by slight tapping with the hand and incubate at 70 °C for 10 min (*see* **Note 11**).
4. Centrifuge the sample tubes for 5 min at 16,000 × *g* at room temperature, to pellet cellular debris, and transfer the supernatant to a new tube.

3.5 Western Blot and Protein Band Quantification

1. Load an equal volume of the supernatants (protein extracts) on an SDS polyacrylamide gel and perform a Western blot with the anti-GFP and anti-AtMCA4 antibodies (*see* **Note 12**; Fig. 1a).
2. Open the Western blot image file using *File* > *Open* in ImageJ. Only use non-overexposed images for the quantitative analysis (*see* **Note 13**). Convert the image to grayscale: *Image* > *Type* > *8-bit*.
3. Select the Rectangular Selections tool from the ImageJ toolbar and draw a rectangle around the first lane, covering all bands in that lane (*see* **Note 14**).
4. After drawing the rectangle over your first lane, press the 1 key or go to *Analyze* > *Gels* > *Select First Lane* to set the rectangle in place. The first lane will now be highlighted and have a 1 in the middle of it.
5. Use your mouse to click and hold in the middle of the rectangle on the first lane and drag it over to the next lane (*see* **Note 15**). You can also use the arrow keys to move the rectangle, though this is slower.
6. Press the 2 key or go to *Analyze* > *Gels* > *Select Next Lane* to set the rectangle in place over the second lane. A 2 will appear in the lane.
7. Repeat **steps 5** and **6** for each subsequent lane on the gel, pressing 2 each time to set the rectangle in place.
8. After you have set the rectangle in place on the last lane (by pressing 2), press 3, or go to *Analyze* > *Gels* > *Plot Lanes* to draw a profile plot of each lane (*see* **Note 16**).
9. Select the Straight Line selection tool from the ImageJ toolbar and for each peak in the profile plot, draw a line across the base of the peak to enclose the peak (Fig. 1b; *see* **Note 17**).

10. When each peak has been closed off at the base with the Straight Line selection tool in a given lane (*see* **Note 18**), select the Wand tool from the ImageJ toolbar. Click inside each peak in the plot profile of the lane. For each peak that you highlight, measurements should pop up in the Results window that appears.
11. When all of the peaks have been highlighted in a given lane, go to *Analyze* > *Gels* > *Label Peaks*. This labels each peak with its size, expressed as a percentage of the total size of all of the highlighted peaks.
12. Repeat **steps 10** and **11** for the plot profile of each lane.
13. Paste the values into a spreadsheet. The values from the Results window can be moved to a spreadsheet program by selecting *Edit* > *Copy All* in the Results window. Data visualization and statistics can be performed in a program of choice.

4 Notes

1. Substrate cleavage site nomenclature according to Schechter and Berger, where the downward arrow indicates the position of the peptide bond that is cleaved and N-terminal and C-terminal amino acids are designated as follows: P4 P3 P2 P1↓P1′P2′P3′P4′ [31].
2. We have occasionally witnessed that the use of automated homogenizers and handling of plant tissues on room temperature led to thawing and detection of a Pep1-YFP cleavage band by Western blot. To reduce interference from the inherent damage caused by sample homogenization for protein extraction on readout of AtMCA4 activity, we keep samples at all times on liquid nitrogen or stored at −70 °C.
3. Tubes are weighed before and after addition of freeze-grinded material to estimate the approximate amount of each sample. An appropriate volume of Laemmli buffer is added afterward to balance protein concentration in the sample extraction. Quantification of the protein bands on Western blot as a percentage of total band intensity in one lane (Fig. 1b and c) alleviates the absolute need for equal sample load or comparison to a reference band or loading control.
4. Alternatively, shoot and root parts of the seedling can be divided to test the damage response separately. To obtain sufficient root tissue, seedlings are grown longer (14 rather than 10 days). We routinely cut roots from seedlings with clean scissors at the base of the hypocotyl, which minimally interferes with subsequent AtMCA4 activation.

5. This treatment is inherently prone to variation between samples. Leaf tissue will become visibly "wetted" and darker green when pinched with forceps. Try to achieve the same level of damage judged by the degree of this leaf wetting. In one sample, multiple (minimally three, but the amount depends on your downstream requirements) seedlings are pressed together. Generally, we suggest to perform multiple biological repeats and quantification of the Western blots to obtain more significant results, especially when comparing the degree of PROPEP1-YFP or AtMCA4 cleavage between conditions (e.g., inhibitor treatments, or genotypes).
6. Room temperature is in our case 22 °C. We routinely sample timepoints at 1, 5, 10, 15, 30, 45, and 60 min after damage. An undamaged sample (0 min) is prepared by collecting and grinding seedlings directly on liquid nitrogen.
7. Whereas forceps-damaged seedlings contain a heterogenous mixture of damaged and intact cells or tissues, freeze-grinded seedlings are more homogenously damaged.
8. When thawing 1.5 mL tubes coming from liquid nitrogen, always open up the tubes first to vent rapidly expanding nitrogen gas, which potentially got trapped in the tube during preparation, to prevent the tubes from exploding. Partial localized heating of the lids, coming from the warmth of your hands, will prevent breaking of the frozen tubes.
9. We warm the tubes by hand until the tissue powder becomes visibly thawed ("wetted and darker green").
10. The volumes are calculated according to the sample weights that were recorded earlier in the protocol. We routinely add three volumes to obtain a good balance between heating of the sample and protein extraction on the one side and protein concentration and visualization of proteins on Western blot on the other side. Of course, the w/v ratio can be changed to fit your experimental requirements.
11. Addition of preheated 2x Laemmli buffer to the tissue powder will ensure that proteins will be denatured, and damage-activated metacaspase activity inhibited, as fast as possible. Minimize the time between transferring the samples at room temperature and the addition of 2x Laemmli buffer as much as possible, for example, by transferring only two to three samples at a time.
12. For best separation results, we use 10% SDS polyacrylamide gels for visualization of PROPEP1-YFP and 4–20% gradient SDS polyacrylamide gels for AtMCA4 [17]. Subsequently, we routinely perform semi-dry Western blots on polyvinylidene difluoride membranes. A detailed step-by-step explanation of the Western blot conditions is subject to the specific antibodies

that are used to visualize PROPEP1-YFP and AtMCA4 [17], or any other antibody that might be used, and therefore, is out of the scope of this protocol.

13. Overexposure of bands is detected and highlighted by most digital camera systems (e.g., Bio-Rad ChemiDoc Imaging System). On film, assess overexposure by making a time series of exposures and choosing the Western blot with a minimum of visible signal for the protein cleavage bands (more subjective).
14. ImageJ assumes that your lanes run vertically (so individual bands are horizontal), so your rectangle should be tall and narrow to enclose a single lane. If you draw a rectangle that is short and wide, ImageJ will switch to assuming the lanes run horizontally (individual bands are vertical), leading to much confusion.
15. Center the rectangle over the lane left-to-right, but do not worry about lining it up perfectly at the same vertical position because ImageJ will automatically align the rectangle's vertical position with the first rectangle in the next step.
16. The profile plot represents the relative density (i.e., darkness) of the contents of the rectangle over each lane. The rectangles/lanes are arranged top to bottom on the profile plot, so that the top profile plot represents the lane designated "1" in the earlier steps. Higher peaks represent darker bands with more protein. Wider peaks represent bands that cover a wider size range on the Western blot. Western blots will always have some background signal, so the peaks do not reach down to the baseline of the profile plot, so the peak appears to float above the baseline of the profile plot (Fig. 1b). It is necessary to close off the peak, so that we can measure its size.
17. This step requires some subjective judgment on your part to decide where the peak ends and the background noise begins. For a close-up look at the profile plot, ImageJ lets you zoom easily with the + and − keys. Bands that are close together in a lane can appear as double (merged) peaks. In this case you can draw a vertical line down to the x-axis of the profile plot (holding the shift key at the same time to obtain a vertical line). Stay consistent with enclosing the peaks in the profile plots that represent different biological repeats of the same timepoint/genotype. We recommend that you duplicate the plot profile image (*right click with your mouse > duplicate*) before drawing on it, in case you want to alter the closed peaks, and to avoid going through **steps 2–8** again.
18. Note that if you have many lanes highlighted, the later lanes will be hidden at the bottom of the profile plot window. To see these lanes, press and hold the space bar, and use the mouse to click and drag the profile plot upward.

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Chapter 12

Sensitive Plant N-Terminome Profiling with HUNTER

Fatih Demir, Andreas Perrar, Melissa Mantz, and Pitter F. Huesgen

Abstract

Protein N-termini provide unique and distinguishing information on proteolytically processed or N-terminally modified proteoforms. Also splicing, use of alternative translation initiation sites, and a variety of co- and post-translational N-terminal modifications generate distinct proteoforms that are unambiguously identified by their N-termini. However, N-terminal peptides are only a small fraction among all peptides generated in a shotgun proteome digest, are often of low stoichiometric abundance, and therefore require enrichment. Various protocols for enrichment of N-terminal peptides have been established and successfully been used for protease substrate discovery and profiling of N-terminal modification, but often require large amounts of proteome. We have recently established the High-efficiency Undecanal-based N-Termini EnRichment (HUNTER) as a fast and sensitive method to enable enrichment of protein N-termini from limited sample sources with as little as a few microgram proteome. Here we present our current HUNTER protocol for sensitive plant N-terminome profiling, including sample preparation, enrichment of N-terminal peptides, and mass spectrometry data analysis.

Key words N-termini, N-terminomics, Proteolysis, Proteomics, Positional proteomics, N-terminal protein modifications, HUNTER

1 Introduction

Protein termini provide crucial information on the presence of different sequence variants arising from a protein-coding gene, termed proteoforms that reflect the functional state of the proteome [1]. Many genes are alternative spliced or use alternative translation initiation sites [2] and the resulting proteins are further co- and post-translationally modified at the N-terminus by acetylation [3], myristoylation [4], N-terminal methionine removal, or proteolytic cleavage of pro-, signal-, and transit-peptides [1]. In addition, regulatory processing by site-specific proteolysis generates new proteoforms that can show altered activity, location, and function. Each of these proteoforms is distinguished by a unique N-terminus that provides a unique peptide sequence not found in the precursor protein. Global profiling of protein N-termini thus

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provides insights into protein maturation [5–7], protein stability determinants [8, 9] and proteolytic regulation [10] as well as the function of specific proteases of interest [11, 12].

Unbiased identification of protein N-termini can be achieved by mass spectrometry-based proteomics (survey degradomics) [13]. In standard proteomics workflows, proteins are digested into peptides to enable mass spectrometric analysis [14], mainly using trypsin. However, among the large number of peptides generated by tryptic digest only a low number of peptides are derived from N-termini. In addition, neo-N-terminal peptides indicative of endoproteolytic processing are often of low abundance. As for other post-translationally modifications, a large variety of dedicated enrichment procedures have been developed to achieve unbiased, proteome-wide N-termini identification as reviewed in [1, 15]. Recently, we have established High-efficiency Undecanal-based N-Termini EnRichment (HUNTER) as a robust, fast, sensitive, and scalable method that requires only common commercially available reagents [16] (Fig. 1). As in other protocols for negative selection of N-termini, free primary amines including protein N-termini are chemically modified on the protein level. Amine-reactive stable isotope labeling reagents may be used at this point to facilitate comparison across multiple conditions. The modified proteins are then digested with a specific protease, typically trypsin. In the resulting peptide mixture, all internal peptides and C-terminal peptides contain a free primary amine that is further modified with undecanal as a hydrophobic tag, while all peptides derived from protein N-termini do not contain a primary amine and remain inert in the tagging reaction. In the final step, the peptide mixture is passed through a C_{18} reverse-phase column or cartridge, where undecanal-tagged peptides are retained while N-termini-derived peptides selectively remain in the flow through.

Enriched N-terminal peptides can be analyses by LC-MS/MS and identified from fragment ion spectra using standard proteomics software packages such as MaxQuant [17], OpenMS [18], or TransProteomicPipeline [19], but search settings have to be modified. Proteolytically generated neo-N-termini will (mostly) not be delimited by the cleavage specificity of the protease used for proteome digest (Arg or Lys when trypsin is used). Therefore, a more open, "semi-specific" database search has to be performed that considers peptides with variable N-terminal sequence, but only C-terminally adhering to the specificity of the proteome digestion enzyme.

Here we present a step-by-step protocol for application of the HUNTER procedure to plant samples with *Arabidopsis thaliana* leave proteome as a test case (Fig. 2). This includes proteome isolation, labeling, enrichment of N-terminal peptides by negative selection, optional fractionation, clean-up, and guidelines for mass spectrometry data analysis.

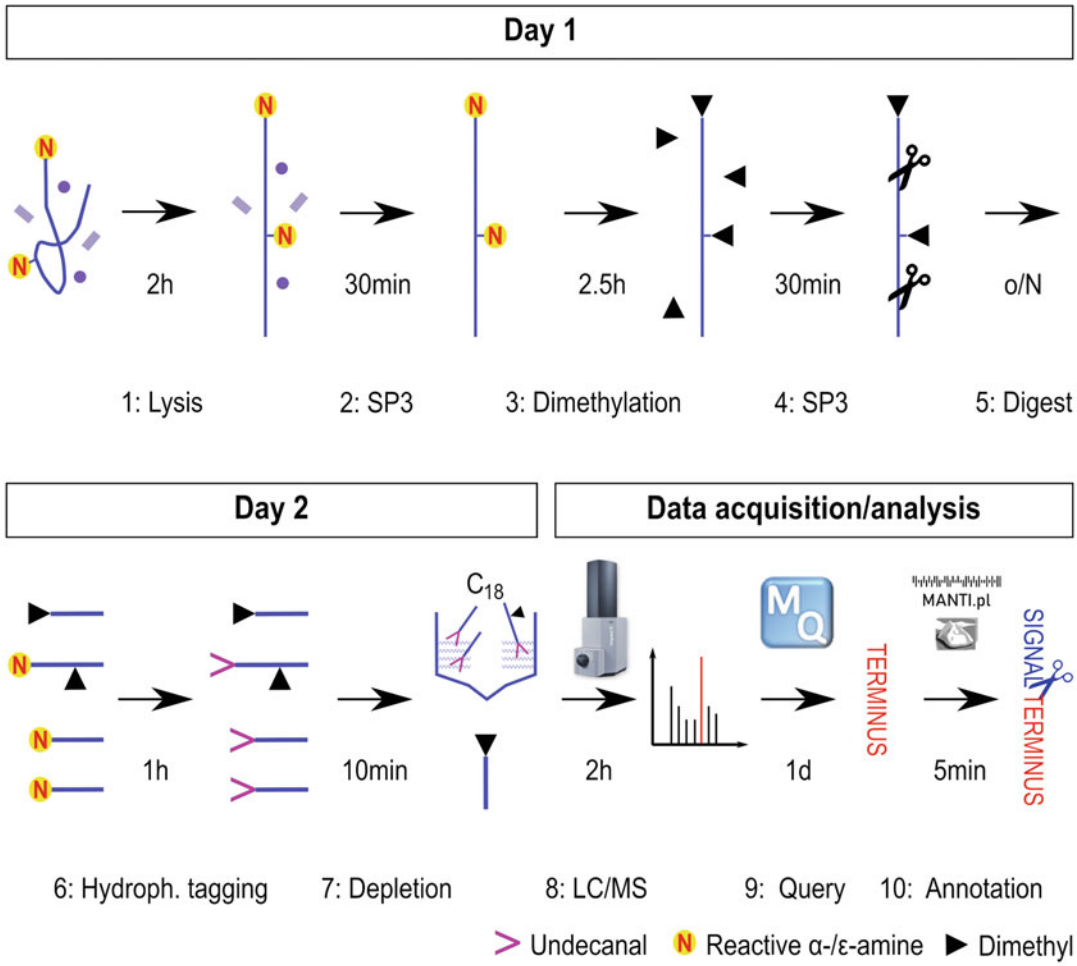

Fig. 1 Workflow for High-efficiency Undecanal-based N Termini EnRichment (HUNTER). The initial step comprises the lysis of the sample in a denaturing buffer containing guanidine hydrochloride or SDS, followed by carbamidomethylation (**step 1**). Subsequently, the sample is purified using SP3 beads and resuspended in an amine-free buffer with 6 M guanidine hydrochloride (**step 2**). All naturally free primary amines (yellow circles) are labeled with an amine-reactive reagent, here formaldehyde (black triangles) (**step 3**). Note that this step offers a convenient opportunity to introduce stable isotope labels, as described for formaldehyde-based reductive dimethylation in this protocol. Subsequently, all protein N-termini are blocked by dimethylation and the sample is purified with SP3 beads to remove remaining guanidine hydrochloride (**step 4**). The digestion with a specific endoprotease of choice, typically trypsin, follows in an overnight (o/N) reaction (**step 5**). During digestion, proteins are cleaved into smaller peptides. Internal and C-terminal peptides featuring a "new" N-terminal primary amine, resulting from digestion, are hydrophobically tagged with undecanal in 40% ethanol (**step 6**). After hydrophobic tagging, all peptide N-termini generated by the digestion protease in **step 5** are thus modified with undecanal (pink symbol), resulting in much higher hydrophobicity. In contrast, protein N-termini that were either endogenously modified or chemically modified by reductive dimethylation in **step 3** do not contain reactive primary amines and remain inert in this reaction. Undecanal-labeled peptides are depleted by C_{18} reverse phase chromatography: The hydrophobic undecanal-modified peptides are retained on the C_{18} material whereas the protein N-termini are directly eluted (**step 7**). Subsequently, the protein N-terminal peptides remaining in the flow-through need to be desalted and purified or fractionated before analysis by nano- LC-MS/MS systems (**step 8**). The resulting mass spectra are queried using MaxQuant with a semi-specific search strategy (**step 9**, see also Table 3) yielding peptide identifications. With additional annotation tools like MANTI, the identified N-terminal peptides can be classified into different known, canonical functional categories like signal or transit peptide processing or non-canonical, putatively protease-generated neo-N termini (**step 10**)

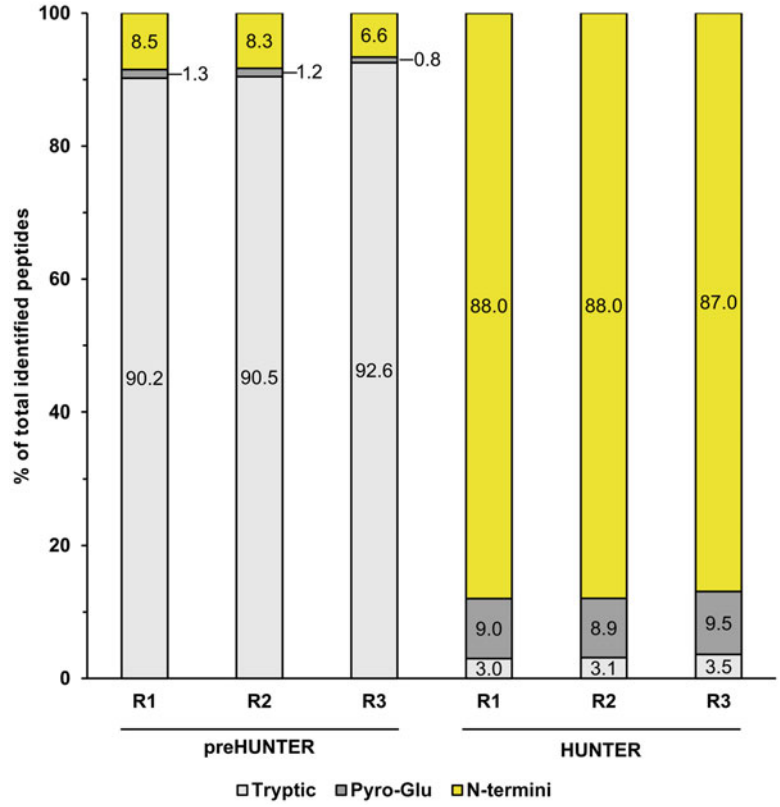

Fig. 2 Amount of digest-generated peptides in different samples from the HUNTER workflow. Relative amounts of digest-generated peptides (in gray) in *Arabidopsis* whole leaf proteome (preHUNTER) and N-termini (HUNTER) samples in each of the three biological replicates (data from [16], ProteomeXchange identifier: PXD012821, *Arabidopsis* whole leaf HUNTER evaluations). Typically, $<$10% of the peptides in a classical proteomics experiment comprise protein N-termini whereas $\geq$87% of the peptides in a HUNTER workflow represent true protein N-termini. The peptides were evaluated by semi-specific searches with variable dimethylated N-termini modification in MaxQuant to equally search for protein N-termini as well as normal tryptic peptides

2 Materials

1. Use fresh, high-quality reagents to avoid contaminations. All solvents should be HPLC-grade and reagents should be proteomics/mass spectrometry-grade if not mentioned otherwise. Formaldehyde and cyanoborohydride are toxic; please handle carefully in the fume hood and wear appropriate personal protection equipment (lab goggles, lab coat, gloves).

2.1 Proteome Preparation and Labeling

1. 90% Acetonitrile (ACN).
2. Ethanol (EtOH), absolute.
3. 1 M Dithiothreitol (DTT) in water, store frozen at −20 °C.
4. 1 M Sodium cyanoborohydride ($NaBH_3CN$) dissolved in 0.2 M NaOH. For comparative experiments with three samples, provide additionally 1 M sodium cyanoborodeuteride ($NaBD_3CN$) dissolved in 0.2 M NaOH (*see* **Note 1**).
5. 2 M Formaldehyde solution (CH_2O) in water (toxic, handle in fume hood).

   For comparative stable isotope labeling of two samples or determination of endogenous N-terminal dimethylation, provide additionally 2 M carbon 13-labeled deuterated formaldehyde ($^{13}CD_2O$) (in D_2O). For stable isotope labeling of three samples, provide additionally 2 M deuterated formaldehyde ($^{12}CD_2O$) (in D_2O).
6. 500 mM Chloroacetamide (CAA) in water, aliquot in amber tubes, and store frozen at −20 °C.
7. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES): 1 M, adjusted to pH 7.5.
8. Benchtop centrifuge.
9. Benzonase.
10. Bicinchoninic acid (BCA) protein assay.
11. Dispersing device (e.g., Kinematica Polytron or IKA Turrax).
12. Filter cloth (e.g., Merck Miracloth).
13. Guanidine protein extraction buffer: 6 M guanidine hydrochloride, 100 mM HEPES pH 7.5, 5 mM EDTA; store at 4 °C.

    Alternatively, SDS protein extraction buffer: 4% SDS, 100 mM HEPES, 5 mM EDTA, pH 7.5; store at room temperature (RT).
14. Liquid nitrogen (N_2).
15. Magnet rack for 1.5 mL reaction tubes (e.g., Invitrogen DynaMag).
16. Mortar and pestle.
17. pH test strips.
18. Protease inhibitor cocktail (e.g., Thermo Halt or Roche cOmplete Protease Inhibitor Cocktail).
19. Protease for proteome digestion, e.g., trypsin or other specific endoproteases such as LysargiNase [20], Legumain [21], GluC, or others [22].
20. Proteome digestion buffer. For trypsin: 100 mM HEPES, 2.5 mM $CaCl_2$, pH 7.5; for legumain: 100 mM MES, 2 mM DTT, pH 6.0.

21. Reaction tubes 1.5 mL and 2.0 mL (*see* **Note 2**).
22. Single-pot solid-phase (SP3) beads (Sera-Mag Carboxylate Speed Beads, hydrophobic Cat. #45152105050250 and hydrophilic Cat. #65152105050250).
23. Temperature-controlled incubation shaker with a lid heater.
24. Vacuum concentrator.

2.2 Enrichment of N-Terminal Peptides (Day 2)

1. 100% Ethanol (EtOH).
2. 100% Formic acid (FA).
3. HR-X spin columns for fractionation (e.g., Macherey Nagel, HR-X spin columns, catalog number 730525, Chromabond HR-X 45 μm, 20 mg, SPE Spin tubes).
4. Undecanal, 97% (4.7 M) (e.g., Sigma, Catalog number U2202).
5. Undecanal stock solution: a 6:4 undecanal solution in 100% EtOH (prepared freshly) yielding a final 40% EtOH solution with 0.5 mg/μL (2.9 M) undecanal, store at RT, protected from light.

2.3 Peptide Fractionation by High pH Reverse Phase Chromatography

1. Ammonium hydroxide (AmOH).
2. Solutions for elution with increasing ACN concentration: 5%, 10%, 20%, and 50% ACN in 10 mM ammonium hydroxide (*see* **Note 3**), prepared freshly.

2.4 Desalting of N-termini Peptides for MS Analysis

1. 100% ACN for activation of C_{18} membranes.
2. 50% ACN, 0.1% FA.
3. 0.1% FA (can be stored at 4 °C).

2.5 Mass Spectrometry and Data Analysis

1. High-resolution tandem mass spectrometry system on-line coupled to a nano-high-performance liquid chromatography system via an electrospray ionization source (nano-HPLC-ESI-MS/MS).
2. Personal computer with a software suite for peptide identification and quantification of MS/MS data (e.g., MaxQuant, OpenMS, or TransProteomicPipeline).

3 Method

3.1 Proteome Preparation and Labeling (Day 1)

1. Add a protease inhibitor cocktail to the protein extraction buffer according to the manufacturer's instructions directly before use. For plant samples, 5–10 mL homogenization buffer is recommended per g fresh weight, e.g., up to 12 leaves of 6-week-old *Arabidopsis thaliana* plants. For tissue culture,

10–15 mL homogenization buffer per g fresh weight is a good starting point.

2. Determine the fresh weight and homogenize the material by mechanical disruption, using, e.g.:
   (a) A mortar and pestle: Pre-chill the mortar by applying small amounts of liquid N_2. Quickly add the leaves and instantly supply further liquid N_2. Grind the leaves to a fine powder while supplying further liquid N_2 as necessary to keep the leaves frozen. Transfer powder to a tube with a suitable amount of guanidine hydrochloride protein homogenization buffer supplemented with protease inhibitors and mix by pipetting (*see* **Note 4**).
   (b) A mechanic disperser (e.g., Kinematica Polytron or IKA Turrax): Pre-chill a small beaker with homogenization buffer in an ice bath. Add leaves to the beaker and homogenize at full speed for 30 s. Inspect the sample for homogenization. Always keep your beaker in the ice bath to keep your sample chilled until the sample is fully homogenized, but do not perform too prolonged homogenization steps.
3. Filter homogenate through two layers of filter cloth into a new tube and squeeze gently with your hands to obtain all liquids.
4. Centrifuge at 500 × *g* for 5 min at 4 °C to pellet cell debris and transfer the supernatant into a new tube—this is the total lysate.
5. Determine the protein concentration using the BCA protein assay (*see* **Note 5**).
6. Proceed with 100 μg of proteome per condition at a protein concentration between 0.5 and 2 mg/mL (*see* **Note 6**). Use equal amounts for each sample condition in comparative experiments (*see* **Note 7**).
7. Confirm that the pH of the sample is between 6.5 and 8.0 (*see* **Note 8**).
8. Treat with at least 1 μL of Benzonase (~ 26 U/μL) at 37 °C for 30 min to degrade DNA and RNA, especially if the sample is rich in nucleic acids (e.g., total cell extracts, cell culture experiments).
9. Reduce the proteins by adding 10 mM DTT (final) and incubate the sample at 56 °C for 30 min (*see* **Note 9**).
10. Let the sample cool down to 21 °C.
11. Alkylate the sample by adding 50 mM CAA (final concentration) and incubate at 21 °C in the dark for 30 min.
12. Quench the reaction by adding further 50 mM DTT (final concentration of 60 mM) and incubate at 21 °C for 20 min.

This and all subsequent reactions do not have to be performed in the dark.

13. Prepare the SP3 bead stock by combining hydrophilic and hydrophobic SP3 beads in a 1:1 ratio. For 100 µL combined beads: Wash 2x with 400 µL dH_2O and reconstitute in 100 µL dH_2O (*see* **Note 10**).
14. To bind protein to SP3 beads: Add 4x sample volume of 100% EtOH to the sample (to a final concentration of 80% EtOH), add 5 µL of the bead stock to each 100 µg protein sample, mix well by short sonication in a water bath for 15 s (*see* **Note 11**), and incubate for 15 min at 21 °C.
15. Place your sample tubes into the magnetic stand to separate the beads from the liquid; allow 30 s for full separation.
16. Remove and discard liquid, wash the beads with 400 µL 90% ACN, and place the sample tubes in the magnetic stand for separation.
17. Repeat **step 16**.
18. After removing the wash liquid, spin down the beads shortly (10 s at max. 2000 × *g*), place tubes again in the magnetic stand, and remove the remaining liquid.
19. Evaporate any residual organic liquids under the hood for 2 min, but do not dry the beads (*see* **Note 12**).
20. Re-suspend the beads in 50 µL of 6 M guanidine hydrochloride, 100 mM HEPES, pH 7.5 for each 100 µg proteome to yield a protein concentration of 2 µg/µL (*see* **Note 13**). Resuspend the beads by brief sonication.
21. To label free primary amines including free N-termini, add 30 mM sodium cyanoborohydride (final concentration) and 30 mM formaldehyde (final concentration (*see* **Note 1**). Choose appropriate combinations of stable isotope-labeled formaldehyde and sodium cyanoborohydride for each experimental condition in comparative studies (*see* **Note 7**, Table 1).

Table 1
Dimethyl labeling combinations and resulting monoisotopic mass shifts

Multiplicity	Formaldehyde	Cyanoborohydride	Mass shift
Single—heavy label	$^{13}CD_2O$	$NaBH_3CN$	+34.0631
Duplex—light label	$^{12}CH_2O$	$NaBH_3CN$	+28.0313
Duplex—heavy label	$^{13}CD_2O$	$NaBH_3CN$	+34.0631
Triplex—light label	$^{12}CH_2O$	$NaBH_3CN$	+28.0313
Triplex—medium label	$^{12}CD_2O$	$NaBH_3CN$	+32.0564
Triplex—heavy label	$^{13}CD_2O$	$NaB\mathbf{D}_3CN$	+36.0756

22. Mix by short sonication and incubate for 1 h at 37 °C.
23. To ensure complete labeling, add the same amount of the same fresh cyanoborohydride and formaldehyde isotopes as in the previous step and incubate at 37 °C for a further 1 h. Take care not to mix the labels in comparative experiments.
24. Quench the labeling reaction by addition of 500 mM Tris–HCl, pH 6.8 (final concentration). Incubate for 30 min at 37 °C.
25. Bind the proteins to the SP3 beads again by addition of 4 reaction volumes of 100% EtOH (final concentration of 80%) and incubate for 15 min at 21 °C.
26. Place the sample tubes into the magnetic stand to separate the beads from the liquid (~ 30 s).
27. Remove the liquid and wash the beads with 400 µL 90% ACN. For comparative experiments, combine the differentially labeled sample pairs (*see* **Note 14**) before placing the reaction tubes back on the magnetic stand. In case of a comparative labeling approach (*see* Table 1), mix in equal ratio based on estimated protein amount.
28. Place the combined samples back into the magnetic stand and let the beads separate from the liquid. Remove the liquid and rinse once more with 400 µL 90% ACN to complete the washing procedure.
29. Let the beads separate again from the liquid by placing the samples into the magnetic stand and remove all liquids by pipetting.
30. Shortly spin down the beads at low speed (10 s at max. 2000 × *g*), place them back into the magnetic stand, and remove all remaining liquids.
31. Let any remaining liquids evaporate under the hood for max. 2 min, but do not dry the beads!
32. Re-suspend the beads in 50 µL for each 100 µg proteome (e.g., 100 µL for 200 µg combined proteomes), to an estimated protein concentration of 2 mg/mL, of the appropriate digestion buffer for the selected digestion protease (*see* **Note 15**).
33. Add digestion protease of choice in the corresponding ratio, e.g., 1 µg Trypsin per 100 µg of Proteome (*see* **Note 16**).
34. Mix the samples by shortly placing them into the ultra-sonic bath, spin down, and incubate overnight at 37 °C in an oven or an incubator with a heated lid (*see* **Note 17**).
35. Spin the samples shortly down and let the beads separate from the liquid. Take a small aliquot of the supernatant (~10% or max. 20 µg) of the digested sample. This is the "pre-HUNTER" sample and can be used to determine the

dimethylation efficiency and sample quality. Furthermore, this can provide information on protein abundance and facilitate isoform assignment.

3.2 Enrichment of N-Terminal Peptides (Day 2)

1. Add 100% EtOH to a final concentration of 40% EtOH in the reaction mixture, which still contains the beads.
2. Add 20-fold excess of undecanal stock solution (e.g., 2 mg undecanal = 3.3 μL 2.9 M undecanal stock solution for each 100 μg of proteome in the sample) and thoroughly mix the samples by vortexing and ultrasonic bath treatment**.**
3. Add 30 mM sodium cyanoborohydride (final concentration), mix thoroughly by pipetting, and incubate at 37 °C for 30 min.
4. Sonicate the tubes briefly, collect the samples by spin centrifugation, and bind the SP3 beads to the magnet by placing the sample tubes into the magnetic stand.
5. Repeat the addition of 30 mM sodium cyanoborohydride, mix by pipette, and incubate at 37 °C for another 30 min.
6. Top up with 40% EtOH to a volume of 400 μL and mix the beads by pipetting and brief ultrasonication for 15 s. The total concentration of EtOH in the supernatant should now be 40% (*see* **Note 18**).
7. Let beads settle on the magnetic stand and prepare the depletion spin columns or cartridges according to the manufacturer's instruction in the meantime (*see* **Note 19**). For samples with up to 400 μg of total protein we use Macherey-Nagel HR-X spin columns:
   (a) Activate the HR-X spin columns with 2 subsequent washes of 400 μL MeOH at 50 × *g* for 30 s at 21 °C, discarding the flow-through.
   (b) Wash HR-X spin columns with 2 subsequent washes of 400 μL 40% EtOH at 50 × *g* for 30 s at 21 °C, discarding the flow-through.
   (c) Directly continue to load the sample.
8. Load the well-separated SP3 bead supernatant, which contains the digested peptides, from the reaction tubes onto the depletion column. Take care to avoid transfer of beads. Spin at 50 × *g* and collect the flow-through. Important: The flow-through contains the desired N-terminal peptides. Wash the remaining beads with 400 μL of 40% EtOH and use this to elute any remaining N-terminal peptides from the depletion column used for the same sample (*see* **Note 20)**. Discard the spin columns.
9. Combine both eluates (total volume of 800 μL), evaporate the organic solvent, and reduce the sample volume to 100 μL in a

vacuum concentrator to enable desalting and/or fractionation by C_{18} solid-phase extraction cartridges or C_{18} STAGE Tips.

3.3 Peptide Fractionation by High pH Reverse Phase Chromatography

1. Fractionation can improve N-terminome coverage (*see* **Note 21**).
2. Choose an appropriately sized C_{18} solid phase extraction column depending on the amount of N-terminal peptides in the HUNTER sample (double layered STAGE tip for <20 μg, HR-X Spin Column for <400 μg, HR-X Cartridge for >400 μg).
3. Activate the HR-X Spin column with 2 subsequent washes of 400 μL MeOH at 50 × *g* for 1 min at 21 °C, discarding the flow-through.
4. Wash the HR-X spin column with 2 subsequent washes of 400 μL 0.1% FA at 50 × *g* for 1 min at 21 °C to equilibrate to acidic pH.
5. Acidify the N-terminal peptide samples using 0.5–2.5% FA (final) to obtain a pH between 2 and 3 (*see* **Note 8**).
6. Load the acidified sample onto the equilibrated HR-X Spin column at 50 × *g* for 1 min at 21 °C.
7. Reload the flow-through once more and repeat **step 5** to ensure complete peptide binding to the column. Discard the flow-through.
8. Wash the loaded peptides with 400 μL of 0.1% FA at 50 × *g* for 1 min, discarding the flow-through.
9. Wash the loaded peptides with 400 μL of 10 mM AmOH to alter the pH, discarding the flow-through.
10. Subsequently, elute the peptides from the column using a series of elution buffers each containing 10 mM AmOH and an increasing percentage of ACN (e.g., 5, 10, 15, 20, and 50%), collect the flow-through after each elution step in an individual tube.
11. Evaporate the organic solvents using a vacuum concentrator device and proceed to mass spectrometry.

3.4 Desalting of N-Terminal Peptides for MS Analysis

1. Acidify the sample to a pH between 2 and 3 by adding FA to a final concentration of 0.5–2.5% (*see* **Note 8**).
2. Craft a double-layer STAGE tip by excising two pieces of C_{18} membrane using a Hamilton plunger and inserting them into a 200 μL pipette tip. Alternatively, commercially available C_{18} STAGE tips can also be used.
3. Activate the STAGE tip by adding 20 μL of 100% ACN and pressing the liquid through. Avoid drying out the membrane

by not completely pressing through the liquid! Discard the flow-through.

4. Equilibrate the STAGE tip by adding 50 μL 0.1% FA in H_2O and pressing the liquid through. Avoid drying of the membrane! Discard the flow-through.
5. Load the acidified sample. Avoid drying of the membrane! Discard the flow-through.
6. Wash the bound peptides by adding 50–100 μL of 0.1% FA and pressing the liquid through. This time the whole liquid can be pressed through. Membrane may be dried at this point and stored for later processing. Discard the flow-through.
7. Elute desalted peptides by adding 20 μL of 50% ACN, 0.1% FA and completely pressing through. Collect the eluate which contains the desired peptides in a new tube.
8. Evaporate the organic solvent in a vacuum concentrator device.

3.5 Identification and Bioinformatics Analysis of N-Terminal Peptides

1. Analyze the HUNTER and (optionally) preHUNTER samples by LC-ESI-MS/MS. Details of the mass spectrometry analysis are instrument-specific and should be discussed with the operator. Typically, settings used for shotgun proteome analysis are sufficient, at least when using trypsin as a digestion protease.
2. Specific search settings vary depending on the algorithm and the instrument used for data acquisition; full coverage of all combinations is well beyond the scope of the protocol (*see* **Note 22**). However, the following HUNTER-specific considerations apply:
   (a) Trypsin is virtually inactive toward dimethylated Lys residues. Hence, protease specificity should be defined as semi-specific ArgC (variable peptide N-terminus) with up to two missed cleavages for trypsin-digested samples (*see* **Note 23**).
   (b) For simple termini profiling, dimethylation of Lys residues may be set as a fixed modification (for exact mass of modification, *see* Table 1). Variable modifications should include N-terminal dimethylation with the chosen formaldehyde variant used in Subheading 3.3, **step 18** (for mass differences, *see* Table 1), common endogenous and artificial modifications such as N-terminal acetylation (+42.010565), N-terminal pyro-Glu from Gln (−17.026549) or Glu (−18.010565) (*see* **Note 24**), along with other N-terminal modifications of interest. For characterization of endogenous N-terminal monomethylation, include modification with a mixed label consisting of one (endogenous) isotopically light methyl

Table 2
Exemplary MaxQuant search settings for analysis of "preHUNTER" samples (trypsin digested sample, duplex labeling with $^{12}CH_2O/NaBH_3CN$ and $^{13}CD_2O/NaBH_3CN$). Settings can be adapted for simplex or triplex labeling by including the relevant dimethyl modifications. Note that variable dimethyl modifications are not standard modifications listed in the MaxQuant releases but must be defined by the user

Settings for	Label	Var. Modifications	Specificity
QC dimethylation and digest efficiency (duplex)	–	Oxidation (M) Acetyl (Protein N-term) Var_DimethLys0 Var_DimethLys6 (^{13}C)	Specific, Trypsin/P, 4 missed cleavages
Shotgun proteome (duplex)	DimethLys0 DimethLys6 (^{13}C)	Oxidation (M) Acetyl (Protein N-term)	Specific, ArgC

group and one added isotopically heavy methyl group (+31.0472).

(c) For quantitation in comparative experiments (*see* **Note 25**), include searches for all employed dimethylation variants at Lys side chains and peptide N-termini (Table 1) and perform MS1-based quantitation with quantitation modules such as XPRESS [23], FeatureFinderMultiplex [24], or MaxQuant (*see* **Note 26**).

(d) To assess the efficiency of the reductive dimethylation reaction, perform a database search of the pre-HUNTER sample using specific digestion enzyme settings with up to four missed cleavages and Lys dimethylation (modification mass *see* Table 1) set as variable modification. The ratio of modified Lys/total number of Lys residues identified in all peptides indicates the labeling efficiency. A high number of missed cleavages at Arg indicates incomplete proteome digest. The preHUNTER sample can also be used to obtain information on protein abundance (Table 2).

3. Positional annotation is the key for classifying and understanding protein N-termini (*see* **Note 27**). N-termini mapping to position 1 of their gene models represent (canonical) genome-encoded termini and those matching to position 2 indicate co-translational processing by Met aminopeptidase. Alternative splicing, alternative translation initiation, or post-translational proteolytic processing results in N-termini matching within the protein model. These can be further interpreted with the help

of databases such as UniProt [25] that allow assignment of known processing events such as signal-, transit-, or propeptide cleavages and other position-specific features. For human and murine data, the TopFIND database enables additional annotation of proteases known to cut at or near the identified termini and the activity of proteolytic cascades [10].

4 Notes

1. Handle in a fume hood as both $NaBH_3CN$ and $NaBD_3CN$ release highly toxic cyanic acid upon decomposition. To minimize decomposition, stock solutions of $NaBH_3CN$ may be prepared in 5 M NaOH. Always prepare fresh working stock solutions of 2 M $NaBH_3CN$ and $NaBD_3CN$ and dispose them as hazardous chemical waste after the HUNTER N-termini enrichment.
2. We strongly recommend high-quality plastic ware with low protein absorption to avoid contamination with plasticizers and loss of sample. We also recommend contacting the person in charge of the mass spectrometry system for their preferred brand/reference.
3. Fractionation with ammonium hydroxide yields a slightly different elution pattern/behavior as peptides tend to elute earlier than usually, thus the stronger bias on the low organic content solutions for elution.
4. SDS protein extraction buffer is not recommended if you homogenize by mortaring under liquid N_2 as the SDS will immediately precipitate in the mortar or in contact with the frozen material on transfer to the test tube, which may result in sample loss.
5. Protein concentration measurements by microplate BCA assay are prone to overestimation, particularly in the presence of reduction agents such as DTT. We recommend assaying at least two dilutions of each sample and including a buffer control for evaluation of potential background absorbance of the buffer components. Further, the BCA assay tolerates up to 3 M guanidine hydrochloride and calibration standards ranging from 25 to 2000 μg/mL. Therefore, dilute your total lysates at least 1:2 or higher to ensure no interferences of the test and appropriate protein concentrations within the calibrated range.
6. The protein amount can be scaled according to sample availability. We have successfully performed HUNTER from as little as 2 μg material up to mg scale for subsequent peptide-level fractionation. For quantities >500 μg we use chloroform/methanol precipitation for protein clean-up [16], as scaling to

large quantities can be challenging with SP3 beads. In our hands, protein concentrations in the range of 0.5–2 mg/mL yield efficient reactions and binding. Higher protein concentrations tend to cause protein aggregation, while low concentrations can result in poor sample recovery.

7. For single profiling experiments or "label-free" quantitation, we recommend using deuterated $^{13}CD_2O$ as amine-modifying blocking reagent to distinguish between protein termini with free amines that are dimethylated in vitro, and protein termini that are endogenously modified by methylation or dimethylation. For comparison of two or three experimental conditions or genotypes, we recommend using stable isotope labeling with appropriate combinations of formaldehyde and cyanoborohydride isotopes (Table 1). Also isobaric stable isotope labeling reagents, which allow comparison of up to 10 conditions in a single experiment, have been used as described elsewhere [26].
8. Small-scale pH estimation, apply 0.5 μL of sample directly to the interface of two sections on a pH test strip. If required, adjust the pH by addition of 5 μL 1 M NaOH or 1 M HCl, mix, test the pH again, and repeat until the desired pH is reached.
9. Prepare the DTT stock (1 M in water), which can be stored at −20 °C, without prolonged storage at ambient temperature. During the reduction (**step 7**) and quenching (**step 10**) steps, the DTT should be kept chilled on ice. The reaction works at room temperature, but is more effective if the sample is heated up to 56 °C.
10. The prepared SP3 bead stock can be stored at 4 °C for weeks. Do not freeze SP3 bead stocks!
11. Brief sonication in a water bath is the preferred method to resuspend SP3 beads, as pipetting often results in loss of beads and sample. For larger sample amounts (>300 μg) traditional clean-up protocols such as chloroform/methanol precipitation should be considered [27].
12. Dry carefully but avoid over-drying. If the beads get too dry, they clump and are not easily dispersed in the next buffer. If too much organic is left, proteins may not fully be released from the beads.
13. The volume should be at least 30 μL to cover the SP3 beads, but with a maximum volume of 300 μL to allow for the addition of 4 volumes of organic to bind the protein to the SP3 beads again.
14. Flush the sample tube if necessary with an additional amount of 90% ACN to ensure complete combination of sample pairs.
15. A variety of proteases can be used for proteome digestion, each with individual requirements. Most buffers can be

accommodated, but avoid the use of amine-containing buffers such as ammonium bicarbonate as these would interfere with subsequent undecanal modification and depletion. If amine-containing buffers must be used during proteome digestion, perform an additional peptide purification with the SP3 beads by addition of >95% acetonitrile (final volume) after completion of digest and before continuing with the protocol.

16. Parallel digest with different proteases increases protein sequence and proteome coverage [22]. This is even more important for N-terminome profiling, where individual N termini may be too short, too long or contain an unfavorable combination of amino acids for ionization and reliable mass spectrometry-based identification [28]. Each parallel digest with an enzyme with different specificity provides an additional chance to identify termini missed in the tryptic digest, at the expense of multiplied experiments. We often use Legumain [21], which cleavages after Asn and Asp residues, or GluC, which cleaves specifically after Glu and Asp residues.
17. Temperature gradients in the tube must be avoided when working with small volumes as used during the SP3 procedure. If the digest is performed overnight at 37 °C in a lid-less incubator, the sample will display condensation on the lid and the digest will not be complete. Alternatively, the sample may be placed in a clean oven or bacteria incubator set to 37 °C (or other temperature appropriate for the protease used for digestion).
18. The ethanol concentration used for the undecanal tagging must be between 35 and 40% ethanol. Lower concentrations can result in loss of N-terminal peptides binding to the column. Too high concentrations will lead to the elution of undecanal-tagged peptides and/or carry-over of undecanal which will pose problems for the LC/MS equipment.
19. Choosing a C_{18} column with sufficient capacity to completely retain the added undecanal is critical to avoid carryover. The recommended HR-X spin column works well for up to 8 mg undecanal (total) load.
20. Both eluates should not display the typical scent of undecanal anymore since the undecanal-tagged peptides are trapped inside the depletion column.
21. Samples may be analyzed directly as a single sample. However, peptide-level fractionation of the HUNTER and preHUNTER samples by strong cation exchange (SCX) or high pH reverse-phase chromatography will increase peptide coverage.
22. Many different software tools for the identification of peptide sequences by matching acquired MS/MS spectra to proteome databases have been established [29]. Analysis of HUNTER

data with multiple of these search engines is recommended as this can significantly increase the number of identified N-termini [28].

23. Due to the greater search space for database queries in semi-specific searches, the number of peptides identified in semi-specific searches will be low compared to standard proteomics workflows.
24. Formation of pyro-Glu is a common side reaction under the digestion conditions. These peptides should not be considered as true N-termini present in vivo if the preceding amino acid matches the specificity of the employed enzyme (Arg in the case of trypsin). However, these peptides, along with any peptides with free primary amines that may have been carried over by incomplete undecanal tagging and peptides identified in preHUNTER samples, provide valuable additional shotgun proteomics-type information on proteins present in the sample and enable better discrimination between different isoforms [30].
25. Note that HUNTER is a peptide-centric technique. Differential proteolysis affects the cleaved internal peptide and results in newly generated N-termini, but may not affect stability or quantity of any other peptide in the protein. Hence, quantitation must be evaluated on the peptide and not on the protein level.
26. MaxQuant [17] is currently one of the most popular and convenient tools for MS1-based quantitation. Note that MaxQuant does not designate labels as modifications; hence all non-Met-oxidized peptides will be classified as "unmodified" in the output files. To validate the specificity of the HUNTER workflow N-termini enrichment, two independent MaxQuant database searches should be performed as outlined in Table 3. The search window for both protein N-termini searches should be set to 35 (Sequences tab in MaxQuant, "Max. peptide length for unspecific search"). The "Var_" variable modifications can be set easily in the "Configuration" tab in MaxQuant by duplicating the corresponding label (DimethNter0) and setting them as standard modifications instead of labels. Both labeling efficiency in the preHUNTER sample and pullout efficiency in the HUNTER sample should be >90%.
27. For MaxQuant searches, the software package MaxQuant Advanced of N-termini Interpreter (MANTI) offers a convenient solution for semi-automated N-termini dataset validation, annotation, and classification [31] (https://sourceforge.net/projects/manti/). See also a separate chapter, describing the MANTI workflow, in this issue.

Table 3
MaxQuant search settings for HUNTER workflow validation (trypsin-digested sample, duplex labeling with CH2O/NaBH3CN and 13CD2O/NaBH3CN). Detailed instructions for the database search parameters are available in Note 26

Search settings	Label	Var. Modifications	Specificity
N-terminal peptide identification	DimethLys0 DimethNter0 DimethLys6 (^{13}C) DimethNter6 (^{13}C)	Oxidation (M) Acetyl (N-term) Gln->pyro-Glu Glu->pyro-Glu	Semi-specific free N-terminus, ArgC
Pullout efficiency	DimethLys0 DimethLys6 (^{13}C)	Oxidation (M) Acetyl (N-term) Gln->pyro-Glu Glu->pyro-Glu Var_DimethNter0 Var_DimethNter6 (^{13}C)	Semi-specific free N-terminus, ArgC

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Chapter 13

Profiling Sequence Specificity of Proteolytic Activities Using Proteome-Derived Peptide Libraries

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Abstract

Substrate sequence specificity is a fundamental characteristic of proteolytic enzymes. Hundreds of proteases are encoded in plant genomes, but the vast majority of them have not been characterized and their distinct specificity remains largely unknown. Here we present our current protocol for profiling sequence specificity of plant proteases using Proteomic Identification of Cleavage Sites (PICS). This simple, cost-effective protocol is suited for detailed, time-resolved specificity profiling of purified or enriched proteases. The isolated active protease or fraction with enriched protease activity together with a suitable control are incubated with split aliquots of proteome-derived peptide libraries, followed by identification of specifically cleaved peptides using quantitative mass spectrometry. Detailed specificity profiles are obtained by alignment of many individual cleavage sites. The chapter covers preparation of complementary peptide libraries from heterologous sources, the cleavage assay itself, as well as mass spectrometry data analysis.

Key words Protease specificity, Proteolysis, Proteomics, Proteome-derived peptide library, Proteomic identification of protease cleavage sites

1 Introduction

Proteases regulate virtually all aspects of plant life and death [1–3]. However, the vast majority of the hundreds of proteases encoded by plant genomes remain uncharacterized. A fundamental property of proteolytic enzymes is their sequence specificity, which determines their substrates and consequently function and thus allows development of specific and selective substrates, inhibitors, and activity-based probes [4]. A large variety of biological and chemical approaches have been developed to determine the sequence specificity of proteases in detail (reviewed in [4]). Among these, proteomic identification of protease cleavage sites (PICS) has found wide application due to its unique ability to comprehensively identify the amino acid preferences of the protease

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on both sides of the cleavage site simultaneously in a single mass spectrometry experiment [5].

PICS uses specific proteases with well-known and characterized cleavage specificity such as trypsin, GluC, chymotrypsin [5, 6], or legumain [7] to digest proteomes into peptides with predictable sequences (Fig. 1). These "proteome-derived peptide libraries" are then used as test substrates for proteolytic activity of the protease of interest. After incubation for a chosen time, the cleaved peptides are identified by mass spectrometry (Fig. 2). Due to the non-random origin of the peptides, the full sequence of each cleaved peptide substrate can be inferred by matching the identified fragment peptide to the corresponding proteome databases (Fig. 3) [9, 10]. In a typical PICS experiment, hundreds of cleaved peptides are identified and aligned to provide a detailed specificity profile (Fig. 3a). Notably, for most proteases the source proteome does not have a strong impact on the obtained specificity profile [6].

In plant sciences, PICS has been successfully applied to profile proteases with both very strict [8] and rather loose sequence specificity [11], including aspartic [12], cysteine [8, 13, 14], serine [15–18], and metalloproteases [11]. PICS experiments can determine differences in sequence specificity among closely related enzymes (e.g., [11, 12, 17]), reveal kinetic preferences when suitable time points are collected [8] (Fig. 3b), and reveal the specificity of the dominating proteolytic activity in crudely enriched fractions despite the presence of multiple proteolytic enzymes [19]. In this chapter, we describe a step-by-step protocol to generate complementary proteome-derived peptide libraries using different digestion enzymes, the specificity assay itself, and provide detailed instructions for mass spectrometry data analysis using a freely available software.

2 Materials

Use fresh high-quality reagents to avoid contaminations. All solvents should be HPLC-grade and reagents should be proteomics/mass spectrometry-grade if not mentioned otherwise. Formaldehyde and cyanoborohydride/cyanoborodeuteride are toxic; the latter also release toxic hydrogen cyanide gas on acidification. Always handle in the fume hood and wear appropriate personal protection equipment (lab goggles, lab coat, gloves).

2.1 *Preparation of* E. coli *Proteome-Derived Peptide Libraries*

1. 1 M 2-(N-Morpholino)ethanesulfonic acid buffer (MES buffer) pH 6.0.
2. Acetonitrile (ACN).
3. Benchtop centrifuge.

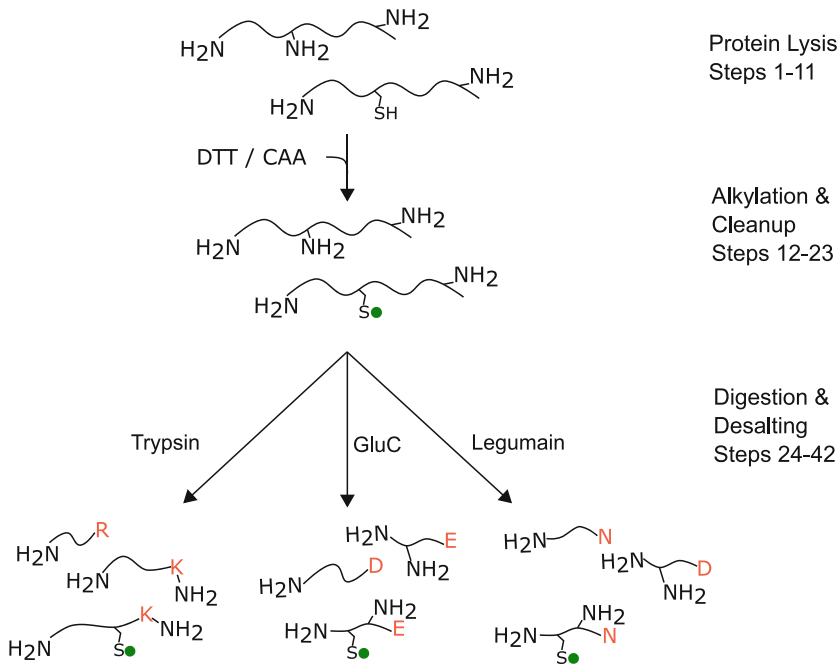

Fig. 1 Preparation of proteome-derived peptide libraries. Proteins are extracted from lysed *E. coli* cells or any other a suitable or convenient source. Proteins are denatured, reduced, and alkylated before parallel digestion with endoprotease that show complementary sequence specificity, for example, trypsin (cleaves after Arg, Lys), GluC (cleaves after Glu and Asp), or legumain (cleaves after Asn and Asp). The final products are purified proteome-derived peptide libraries

4. Bicinchoninic acid (BCA) assay for protein and peptide. (Protein/peptide BCA kit).
5. C-18 reversed phase solid phase extraction cartridges.
6. Chloroform ($CHCl_3$).
7. 500 mM Chloroacetamide (CAA) (freshly prepared).
8. 1 M Dithiothreitol (DTT) (freshly prepared in dH_2O).
9. Escherichia coli (*E. coli*) DH5a or any other source proteome (*see* **Note 1**).
10. Formic acid (FA).
11. Luria-Bertani (LB) broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl dissolved in 1 L of distilled water, pH adjusted with NaOH to pH 7.0 and autoclaved at 120 °C for 25 min.
12. 100× Protease Inhibitor cocktail (or commercial equivalent): 100 mM AEBSF-HCl, 80 μM Aprotinin, 5 mM Bestatin, 1.5 mM E-64, 2 mM Leupeptin, 1 mM Pepstatin A dissolved in DMSO.
13. Lysis Buffer: 4% SDS, 100 mM HEPES-NaOH, 5 mM EDTA, 1× protease inhibitor cocktail.

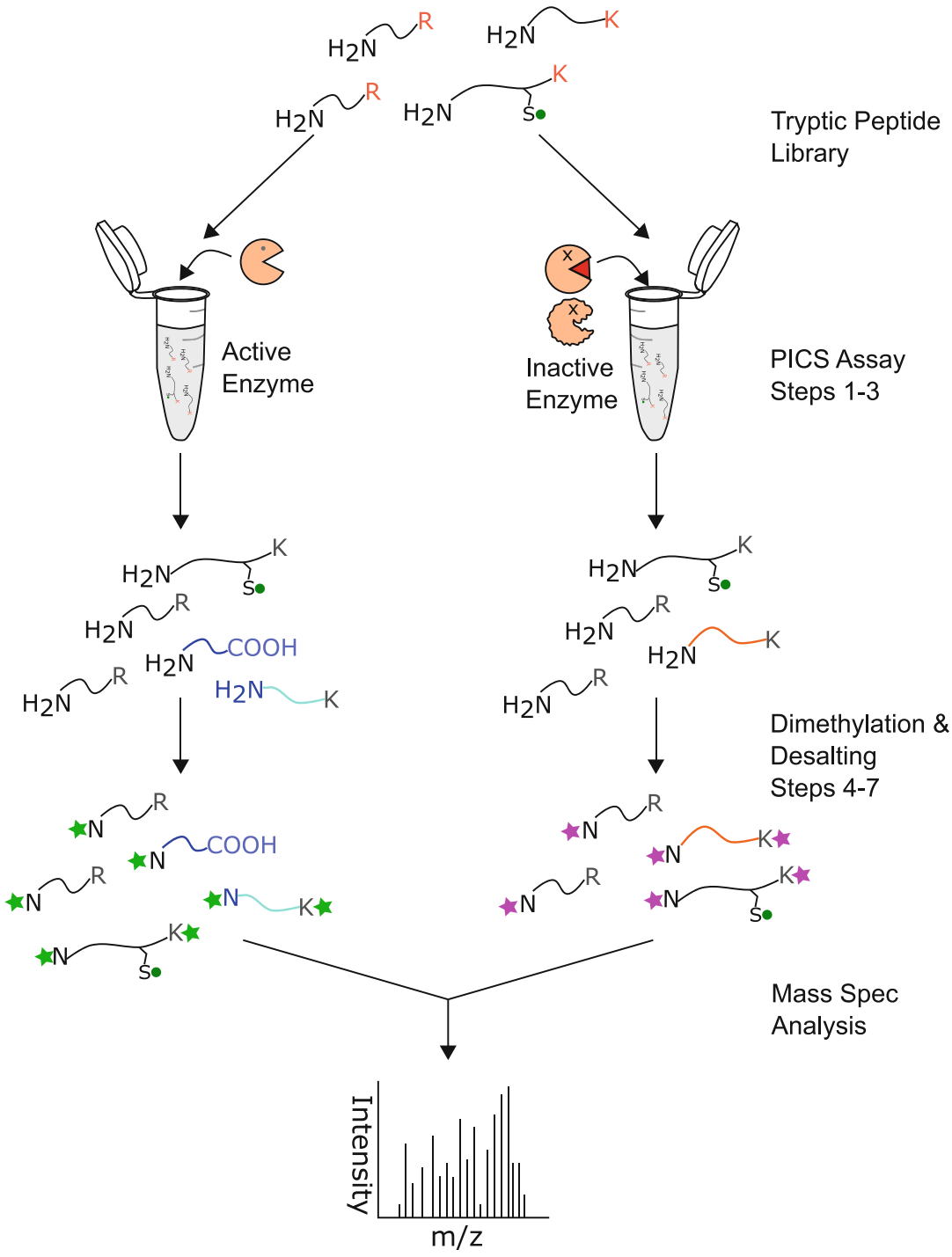

Fig. 2 Proteomic Identification of Cleavage Sites (PICS) assay. Proteome-derived peptide libraries, shown here for a tryptic library, are then split in two or more aliquots and incubated with active enzyme (purified protease or partially enriched, proteolytically active fraction) or suitable control (point-mutated protease, proenzyme, inhibitor or heat inactivated enzyme/enriched fraction). The active protease will cleave peptide substrates that

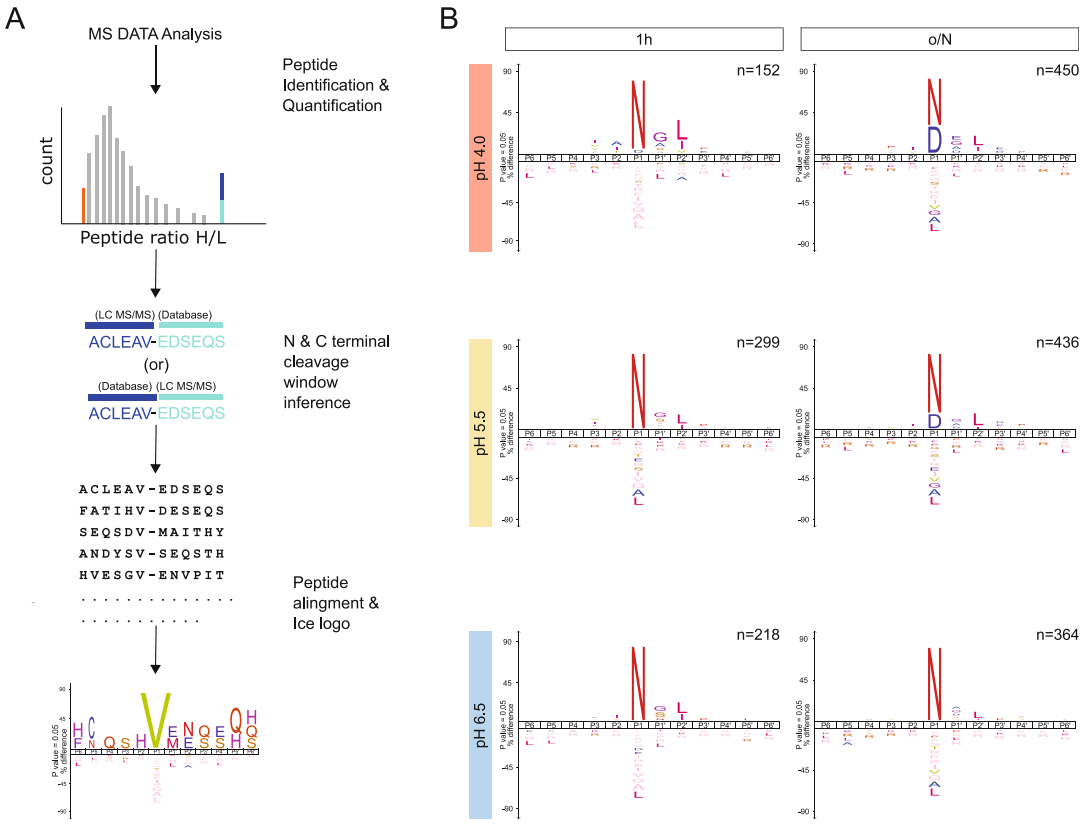

Fig. 3 PICS data analysis. (**a**) workflow. Peptide sequences are identified by matching the acquired mass spectra to a source proteome database. Peptides are then quantified based on the dimethyl label, with uncleaved peptides forming a distribution centered on 1:1, while cleaved substrate peptides will show a high ratio in the protease-treated condition. Cleaved peptide termini that are not derived from the protease used for library digestion protease, e.g., C-termini that are not ending with K or R and N-termini that are not preceded by K or R in the case of a trypsin-generated library, are considered to be derived from the test protease. For these peptide termini, the complete cleaved sequence is reconstructed by matching the peptides to the protein database and extending the sequence in the appropriate direction. Finally, unique cleaved sequences, also termed cleavage windows, are aligned and used to calculate positional frequencies and generate sequence logos. (**b**) Exemplary application of PICS. Arabidopsis VPEbeta was incubated with a tryptic library for different times and at different pH. Comparison of the individual results reveals a strong kinetic preference for cleavage with Asn at P1, with a secondary preference for Asp at P1 under acidic conditions. (Figure panel is based on the dataset published in [8])

Fig. 2 (continued) contain a suitable cleavage site, releasing N-terminal fragments that reflect non-prime-side specificity and C-terminal fragments that reflect prime-side specificity. Samples may be withdrawn at multiple time points, the reaction is stopped and protease- and control-treated peptides differentially stable isotope labeled by reductive dimethylation. The reaction is quenched, peptides combined, desalted and analyzed by tandem mass spectrometry

14. Methanol (MeOH), chilled at −20 °C.
15. 1 M N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)-NaOH (HEPES-NaOH) buffer, pH 7.5.
16. 10 mM Sodium Hydroxide (NaOH, ice cold).
17. MS-grade trypsin or other specific digestion enzymes such as Legumain [20], GluC, or other enzymes [21].
18. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis apparatus, Coomassie staining solution, 12% SDS-PAGE gel, 4× loading dye, Protein molecular weight ladder.
19. Temperature-controlled incubation shaker with a lid heater.
20. Temperature-controlled incubator for bacterial culture.
21. Trifluoroacetic acid (TFA).
22. 1 M Tris (hydroxymethyl) aminomethane hydrochloride pH 6.8.
23. Proteome digestion buffer: 100 mM HEPES, 2.5 mM $CaCl_2$, pH 7.5 for trypsin or 100 mM MES, 2 mM DTT, pH 6.0 for legumain (*see* **Note 2**).
24. 1.5 mL and 2.0 mL reaction tubes (*see* **Note 3**).
25. UV spectrophotometer.
26. Vacuum concentrator.
27. Wash buffer: 1× Phosphate Buffered Saline (PBS) pH 7.4: 137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl.

2.2 Protease Specificity Assay

1. 2 M Formaldehyde solution (CH_2O) in water (toxic, handle in fume hood).
2. 2 M ^{13}C-labeled deuterated formaldehyde ($^{13}CD_2O$) in D_2O.
3. For stable isotope labeling of three samples additionally 2 M deuterated formaldehyde ($^{12}CD_2O$) in D_2O and 1 M sodium cyanoborodeuteride ($NaBD_3CN$) in D_2O.
4. Protease incubation buffer suited for test protease (*see* **Note 4**).
5. Isolated test protease or its enriched fraction as well as control, ideally using a catalytically inactive version. If no inactive form is available, then chemical inhibitors or heat inactivation can be used as controls.
6. 1 M Sodium cyanoborohydride solution ($NaBH_3CN$) (*see* **Note 5**).
7. 1 M Tris–HCl, adjusted to pH 6.8.

2.3 Desalting of Peptides

1. C18 reversed phase solid phase extraction membranes.
2. Hamilton Plunger.

2.4 Peptide Identification and Data Analysis

1. High-resolution tandem mass spectrometer with coupled chromatography system.
2. Personal computer with a software suite for peptide identification and quantification of MS/MS data, e.g., MaxQuant [22], OpenMS [23], or TransProteomicPipeline [24].
3. PINCIS (Pics N-/C-terminal Inferred Substrates) Perl script (available at https://pincis.sourceforge.io).

3 Method

3.1 Preparation of E. coli Proteome-Derived Peptide Libraries (Day 1)

1. Grow *E. coli* starter culture by inoculating the glycerol stock in 5 mL of LB media and incubating overnight at 37 °C with mild agitation at 200 rpm (*see* **Note 1**).
2. Inoculate 5 mL of the overnight culture in 100 mL of LB media and monitor the growth of the bacteria using a UV spectrophotometer until it reaches exponential growth phase, i.e., $OD_{600} = 0.8$.
3. Prepare 50 mL tubes for harvest; note the weight of the empty tubes.
4. Once the cells reach the desired OD, divide the 100 mL cell culture into two 50 mL tubes and centrifuge at 400 × *g* at 4 °C for 15 min and discard the supernatant.
5. Wash the pelleted cells with 20 mL of ice-cold PBS, combine the cell pellets from both the tubes, and collect by centrifugation at 400 × *g* at 4 °C for 15 min.
6. Repeat the wash step with 5 mL of PBS and discard the supernatant after centrifugation.
7. Determine the fresh weight of the cell pellet. The cells can be frozen at −80 °C for later use.
8. To resuspend the cell pellet in lysis buffer add 1 mL of the lysis buffer containing protease inhibitor per 50 mg fresh weight of the cells and transfer the lysate to a 5 mL tube.
9. Heat the cell lysate at 95 °C for 5 min, cool it down on ice, and reheat again at 95 °C for 5 more min to lyse the cells.
10. Centrifuge for 30 min at maximum speed at 4 °C and transfer the supernatant to a new tube.
11. Determine the protein concentration of supernatant by Bradford or BCA assay. The experiment can be stopped here by freezing the proteome in liquid N_2 and stored at −20 (for weeks) or −80 °C (for months).
12. Alkylate proteins by adding 10 mM DTT (final concentration) to the lysate and incubating for 30 min at 37 °C (*see* **Note 6**) and let it cool down shortly.

13. Add 50 mM iodoacetamide (final concentration) and incubate for 30 min at room temperature (RT) in the dark.
14. Quench unreacted iodoacetamide by adding another 50 mM DTT and incubate at RT for 20 min.
15. Purify proteome by chloroform/methanol precipitation (*see* **Note 7**) [25]: Add 4× starting volume of ice-cold MeOH (chilled at −20 °C or in ice bath) and mix.
16. Add 2× starting volume ice-cold $CHCl_3$ (chilled at −20 °C or in ice bath) and mix.
17. Add 3× starting volume chilled water and mix. A fluffy, perturbed solution should be visible indicating the presence of protein precipitate.
18. Centrifuge for 5 min at 4000 × *g* (for 15 or 50 mL reaction tubes) using a swing bucket rotor or at >10,000 g (for 1.5 mL or 2.0 mL reaction tubes) at 4 °C. A white or gray precipitate should be visible at the interphase (*see* **Note 8**).
19. Add 3× starting volume ice-cold MeOH (chilled at −20 °C or in ice bath) and vortex.
20. Centrifuge for 5 min at 4000 × *g* (for 15 or 50 mL reaction tubes) or at >10,000 g (for 1.5 mL or 2.0 mL reaction tubes) at 4 °C, discard the supernatant (*see* **Note 9**).
21. Wash the precipitated pellet 2–3× with 1 mlL cold MeOH (chilled to −20 °C) by vortexing and centrifuging again at maximum speed for 5 min.
22. Briefly air-dry the pellet in a fume hood after the second wash step, but do not over-dry or heat.
23. Overlay pellet with ice-cold 10 mM NaOH. Use sufficient volume to reach an assumed 2.0 mg/mL protein concentration on the basis of the total protein amount determined previously by Bradford/BCA assay.
24. Add 100 mM (final concentration) of appropriate buffer for digestion with selected specific library protease (*see* **Note 2**):
    (a) For trypsin (cleavage after K,R): Add 100 mM HEPES, pH 7.5 (final concentration), 2.5 mM $CaCl_2$ (final concentration).
    (b) For GluC (cleavage after D,E): Add 100 mM HEPES, pH 7.5 (final concentration).
    (c) For Legumain (cleavage after N,D): Add 100 mM MES pH 6.0 (final concentration), 2 mM DTT (final concentration).
25. Centrifuge at 20,000 × *g* for 10 min or at 8500 × *g* for 30 min at 4 °C and collect the supernatant (*see* **Note 10**).

26. Determine the protein concentration and total protein amount using the BCA assay method, use 1:10 and 1:15 dilutions.
27. Digest with Trypsin, GluC, or legumain. Use a protease-to-proteome ratio of 1:100 (w/w) for trypsin, 1:50 (w/w) for GluC or chymotrypsin or legumain and incubate at 37 °C for 16 h (O/N).
28. Analyze a small aliquot of the digest (approx.10 μg) by 12% SDS-PAGE to assess the completion of the proteome digest (*see* **Note 11**).
29. If the proteome is not completely digested, add more of the protease, incubate for an additional 3–16 h at 37 °C, and repeat SDS-PAGE analysis.
30. To desalt the peptides, acidify to 0.5% (v/v) TFA and degas by applying mild vacuum (5 min in speed vac). Final pH should be <3.
31. Clean the C18 cartridges by applying 1 mL of 100% methanol followed by 1 mL of 100% acetonitrile, do not let the column dry during any step.
32. Equilibrate the cartridge with 1 mL of 0.1% FA in H_2O.
33. Load the sample on to your column and collect the flow-through, consider the maximal capacity of the column to avoid sample loss by overloading.
34. Load the flow-through from the previous step once again.
35. Wash the peptides with 2–3 mL of 0.1% FA in H_2O.
36. Elute the peptides with 2 mL of 80 or 90% ACN (without FA).
37. Speed vac the samples until all the organics have evaporated or until dry.
38. Add about 300–500 μL H_2O without any acid.
39. Measure the actual peptide concentration using peptide BCA (use 1:5 and 1:10 dilutions of the peptide sample for the peptide BCA).
40. After measuring the peptide concentration, adjust the volume of H_2O so that the final concentration is 2 μg/μL.
41. Optional: Withdraw an aliquot of 10 μg and analyze by mass spectrometry for quality control (*see* **Note 12**).
42. Store peptide libraries in aliquots of 100 to 200 μg at −80 °C (longer term for months) or −20 °C (short term for a few weeks).

3.2 Protease Specificity Assay

1. Thaw 100 μg of the peptide library and divide into two tubes of 50 μg each (*see* **Note 13**).
2. Adjust the buffer conditions appropriately for the protease of choice. This includes the buffer system, pH, reducing agents, cofactors, and inhibitors (to inactivate the protease).

3. Add the active protease and inactive protease/control in one of the two separate tubes each and incubate at suitable temperature (e.g., at 30 °C for a plant protease) at a time series or overnight (*see* **Note 14**).
4. The next day stable isotope label the digested samples using heavy formaldehyde ($^{13}CD_2O$) for active sample and light formaldehyde ($^{12}CH_2O$) for inactive sample: Add 30 mM $NaCNBH_3$ and 30 mM formaldehyde (add the $NaCNBH_3$ first). Incubate at 37 °C for 1 h in a shaker incubator.
5. Again add 30 mM $NaCNBH_3$ and 30 mM formaldehyde and incubate at 37 °C for 1 more hour.
6. Quench the remaining formaldehyde by adding Tris–HCl pH 6.8 to a final concentration of 100 mM and incubating at 37 °C for 1 h.
7. Mix the heavy and light dimethylated aliquots in a 1:1 ratio.

3.3 Desalting of Peptides

1. Acidify 10 μg of the peptides with FA to pH 2–3 (final concentration between 0.5 and 2.5% FA).
2. Manufacture a self-packed, double-layered C18 stage tip by excising C18 membrane with a Hamilton plunger and inserting the membrane stack into a 200 μL pipette tip.
3. Activate the C18 membrane inside the stage tip by pushing through 40 μL of 100% ACN by applying the liquid with a syringe or by centrifuging the stage tip in an 1.5 mL reaction tube with an adapter at 1400 × *g* for 1 min. Avoid drying out the membrane by stopping before all liquid has passed. Discard the flow-through.
4. Equilibrate stage tip for loading acidified sample by pushing through 50 μL of 0.1% FA in H_2O. Again, avoid drying of membrane by stopping prematurely. Discard the flow-through.
5. Load the acidified sample. Avoid drying of membrane by stopping before all liquid has passed. Discard flow-through.
6. Desalt bound peptides by completely pushing through 50–100 μL of 0.1% FA in H_2O. This time, push the complete washing liquid through the membrane stack. Discard flow-through. Dried stage tip with desalted, bound peptides can be stored for several weeks for later processing or convenient transport.
7. Elute desalted peptides by pushing 20 μL of 50% ACN, 0.1% FA through the membrane stack. Collect eluate in a fresh tube as it contains the desired peptides.
8. Evaporate the organic solvents in a vacuum concentrator device.
9. Resuspend in 0.1% FA and proceed to mass spectrometric analysis (*see* **Note 15**).

3.4 Peptide Identification and Data Analysis

1. Load acquired mass spectrometry data in the latest version of MaxQuant (*see* **Note 15**).
2. For a tryptic peptide library, after loading the measured files, use standard search settings defined for your instrument but adjust the following parameters:
   (a) Enzyme—Trypsin/P, Digestion node—"Semi-specific"
   (b) Multiplicity—2, Label 0—N-terminal light dimethyl and lysine-light dimethyl, Label 1—N-terminal heavy dimethyl and lysine-heavy dimethyl.
   (c) "Re-quantify"—off
   (d) Identification—"Match between runs"—off (*see* **Note 17**).
3. Load the appropriate FASTA proteome database downloaded from UniProt [26].
4. Open the "peptides.txt" file from MaxQuant search result "txt" folder in Microsoft excel and remove peptides flagged as "contaminants" and "reverse identifications".
5. Filter for peptides that appear as singletons in the active enzyme channel (i.e., intensity in the inactive/light-labeled channel = 0 and intensity in the heavy labeled channel with active protease >0). Copy and paste the sequences of the peptides in a new excel file.
6. Then remove the filter for intensities and apply another filter for peptides with ratio H/L normalized >4. Copy the sequences of these peptides to the same new excel file and save it in .csv format.
7. Install a Perl interpreter and download the pincis.pl script on https://pincis.sourceforge.io (*see* **Note 18**).
8. Copy the .csv file (e.g., PICS_list.csv) and the appropriate FASTA file to the location of the PINCIS script (the E-coli.fasta file is already supplied with the downloaded PINCIS package). Run PINCIS using PowerShell or other command line tools. For instance, open up a PowerShell instance under Windows by right clicking on the Explorer window with the corresponding directory while holding the shift key and selecting the "Open PowerShell window here."
9. Define the necessary arguments for PINCIS on the command-line—the available options are:
   (a) --fasta: FASTA file (default FASTA "E-coli.fasta" supplied with PINCIS)
   (b) --enzyme: Digestion enzyme used for generation of the proteome-derived library: available enzymes are: [**T**]rypsin (def.), [**G**]luC, [**L**]egumain or [**C**]hymotrypsin

(c) --output-dir: Output directory (def. "PINCIS_Output_DATE_TIME")

(d) --peptides: PICS peptide list input (file has to be in CSV file format)

(e) --replace: Replace all cutting chars for the digestion enzymes with "X" in the output

(f) --help: Display a short help message on the command line

10. Enter the following commands for PINCIS and press enter:

(a) perl pincis.pl --fasta E-coli.fasta --peptides PICS_list.csv --enzyme Trypsin

Alternatively, enter in short notation:

(b) perl pincis.pl -f E-coli.fasta -p PICS_list.csv -e T

11. Evaluate the PINCIS script output by inspecting the "Inferred_Peptides.tab" file, which gives information about the N- and C-terminally inferred peptide cleavage windows and the corresponding peptides as well as a short summary about the inferred peptides at the end of the file.

12. Open the "Specific_Cleavage_Windows.tab file" in the output directory in any text editor or in Microsoft Excel. Copy all the cleavage windows from the file and make an iceLogo using https://iomics.ugent.be/icelogoserver/create online tool.

13. In the iceLogo online tool [27], select "Use precompiled Swiss-Prot composition" and choose the corresponding organism ("E.coli K12" for *E. coli* peptide library) in the Swiss-Prot composition field. Choose percentage scoring system and enter the start position as −6 (PINCIS generates by default 12 amino acid long cleavage windows representing P6 to P6'), leave the P-value cutoff at 0.05 and click generate.

14. Save the generated iceLogo as SVG and PNG files.

4 Notes

1. Proteome-derived peptide libraries can be prepared from any organism or tissue, but proteomes with large dynamic range such as mammalian blood plasma or plant leaves may introduce a bias toward low-efficient cleavage sites in a few high-abundance proteins. We typically use *E. coli* DH5a or similar lab strain as a convenient source of proteome.

2. A variety of proteases can be used to generate proteome-derived peptide libraries. Use of libraries generated with complementary enzymes with distinct specificity is strongly recommended to obtain information on all amino acids.

3. We strongly recommend high-quality plastic ware with low protein absorption to avoid contamination with plasticizers and loss of sample. We also recommend contacting the person in charge of the mass spectrometry system for their preferred brand/reference.
4. Conditions where the test protease is active should be known beforehand. PICS can then be used to assess cleavage specificity at different conditions.
5. Handle in fume hood as $NaBH_3CN$ releases highly toxic cyanic acid on decomposition after acidification. To minimize decomposition, stock solutions of $NaBH_3CN$ may be prepared in 1 M NaOH. Always prepare fresh working stock solutions of 1 M $NaBH_3CN$ and dispose them as hazardous chemical waste after the experiment.
6. 37 °C may be exceeded in suitable high-quality plastic ware (e.g., Eppendorf brand) at least for the incubation times indicated in this protocol.
7. The final volume of the precipitation will be 10× the starting volume, so starting volume should be maximal 200 µL for each 2 mL tube or 5 mL for each 50 mL tube.
8. Often the lower phase can be poured out as well while slightly twisting the tube, which causes the protein pellet to stick to the side wall.
9. If the pellet fails to precipitate at 4000 × *g*, transfer suspended pellet to 2 mL tubes and centrifugation at higher speed.
10. Sometimes proteins start to precipitate quickly. Alternative to the "clean" centrifugation: Add protease to allow clearance of the aggregates by digestion. In this case, centrifuge after incubation, but before SDS-PAGE analysis, to remove remaining aggregates.
11. We recommend silver staining for visualization to conserve protein. PICS assumes use of flexible peptides, not folded proteins. Therefore, except for the protease used for digestion, no major bands >10 kDa should be observed at this stage.
12. Analysis of this aliquot should identify the number of peptides expected for standard shotgun proteome analysis of the chosen tissue under the chosen mass spectrometry settings. Assess cleavage efficiency, for trypsin <10% missed cleavage sites are expected.
13. To test proteases with unknown specificity, use more than one peptide library generated with distinct proteases to obtain complementary results. Avoid using of peptide libraries generated with a digestion protease that at least partially matches the substrate specificity of the test enzyme. For example, peptide libraries generated by GluC or legumain are suboptimal for

profiling caspase activity due to the overlapping preferred cleavage after Asp.

14. A typical application uses PICS as an end-point assay at long incubation times such as overnight incubation, where every suitable peptide substrates are expected to be cleaved. However, withdrawing aliquots at various time points can indicate kinetically preferred cleavage sites [8].
15. The amount of peptides injected into the mass spectrometry system varies depending on the instrument setup. Please consult with your mass spectrometry operator or core facility. We typically inject 500 ng to 1 μg for nano-LC-MS/MS with a 2 h total runtime gradient for separation. Peptides can be fractionated to achieve higher coverage.
16. Many different software tools for the identification of peptide sequences by matching acquired MS/MS spectra to proteome databases have been established and can be used to identify peptides in PICS experiments. Here we describe the use of MaxQuant [22], which is currently one of the most popular and convenient tools for MS1-based quantitation, for seamless compatibility with our data analysis script PINCIS. Search data from each peptide library separately.
17. Note that PICS is a peptide-centric technique. Proteolytic activity results in cleavage of library peptides and results in new, shorter peptides that are delimited by the specificity of the enzyme used for library generation only one side. Therefore, "semi-specific" search settings are mandatory. Adjust the enzyme setting according to the enzyme used for proteome digestion during the library generation.
18. Perl interpreters come pre-installed on many Linux systems; for users on Windows systems, there are several Perl distributions, e.g., Strawberry Perl (https://www.strawberryperl.com) or ActiveState Perl (https://www.activestate.com/products/perl/downloads/), the latter being also available for MacOS and other operating systems. The current version 12 of PINCIS can be downloaded from https://pincis.sourceforge.io and is released under the Perl Artistic License v2.0 (https://www.perlfoundation.org/artistic-license-20.html), so free to use and modify.

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Chapter 14

Monitoring Cell Death Via Ion Leakage and PAM Fluorometry

Nick Dunken, Lisa Mahdi, Rainer E. Häusler, and Alga Zuccaro

Abstract

Cell death in plants plays a major role during development as well as in response to certain biotic and abiotic stresses. For example, plant cell death can be triggered in a tightly regulated way during the hypersensitive response (HR) in defense against pathogens or be elicited by pathogenic toxin deployment. Monitoring cell death and its impact on plant health can aid in the quantification of plant disease symptoms and help to identify the underlying molecular pathways. Here, we describe our current protocol for monitoring plant cell death via ion leakage and Pulse-Amplitude-Modulation (PAM) fluorometry. We further provide a detailed protocol for the sample preparation, the measurement, and the data evaluation and discuss the complementary nature of ion leakage and PAM fluorometry as well as the potential of PAM fluorometry for high-throughput screenings.

Key words Plant cell death, ION leakage, Pulse-Amplitude-Modulation (PAM) fluorometry, Pathogen treatment, Chemical treatment, Large-scale screening

1 Introduction

Plant cell death is essential for plant development [1] as well as in response to biotic and abiotic stresses [2, 3]. Forms of regulated cell death (RCD) such as apoptosis-like cell death, necroptosis, and the hypersensitive response (HR) are induced in response to different stimuli and exhibit distinct cell death characteristics. As a drastic and irreversible transition, RCD is tightly regulated by the plant [4]. However, RCD can also be induced or suppressed by plant-associated microorganisms and pathogens can trigger host cell death independently of the plant cell death machinery via the secretion of toxins and cell-wall degrading enzymes.

Monitoring cell death allows to quantify disease as well as stress symptoms of the plant and helps to unravel the underlying molecular mechanisms.

Plant cell death results in a loss of cell membrane integrity that ultimately leads to the leakage of cellular electrolytes into the surrounding medium. Measuring the corresponding increase in

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electric conductivity in the surrounding liquid is a commonly used, direct proxy to quantify plant cell death in response to abiotic and biotic stresses [5]. However, ion leakage measurements are time-consuming as each sample has to be measured individually. Thus, they are less suitable for large-scale screenings.

The photosynthetic activity reflects the health status of a plant [6, 7]. The maximum potential quantum yield of photosystem II electron transport is defined as the variable fluorescence (F_V) divided by the maximum fluorescence (F_M) (F_V/F_M), where $F_V = F_M\text{-}F_0$ (F_0. ground fluorescence). Healthy and non-stressed plants obtain a F_V/F_M value of approximately 0.8 [8, 9] whereas biotic and abiotic stresses lead to a decrease in the F_V/F_M ratio up to a total loss of photosynthetic activity upon cell death. Thus, while PAM fluorometry is an indirect proxy for cell death, it provides additional information about the overall health status of the plant. Due to its simple implementation and time-effectiveness, PAM measurements are especially useful for large-scale screenings. Furthermore, PAM measurements do not require a liquid-based system and can also be used for plants on solid medium or soil. Both ion leakage and PAM fluorometry are noninvasive and thus suitable to measure cell death progression over time on the same samples.

Here, we show the function and specificities of cell death monitoring via a combination of ion leakage and PAM fluorometry, exemplified by a chemically induced, developmental cell death as well as a biotic stress induced cell death.

Representing a chemical induced cell death, we treated *Arabidopsis thaliana* seedlings with methyl jasmonate (MeJA), a bioactive derivate of jasmonic acid (JA) that induces leaf senescence [10]. Treatment with MeJA leads to increased electric conductivity as well as to a constant, uniform decrease in the F_V/F_M ratio (Fig. 1).

Representative of a biotic interaction inducing cell death, we took advantage of a previously described tripartite system consisting of the detrimental fungal pathogen *Bipolaris sorokiniana* and the beneficial fungal root endophyte *Serendipita vermifera* in barley [11]. While *B. sorokiniana* colonization results in host cell death, co-colonization with *S. vermifera* counteracts these detrimental effects [11]. We here applied this tripartite system in *Arabidopsis thaliana* (Fig. 1). We inoculated Arabidopsis roots with *S. vermifera*, *B. sorokiniana,* or a combination of both fungi (Fig. 3). As observed in barley, *B. sorokiniana* was able to colonize Arabidopsis roots. Upon colonization, *B. sorokiniana* caused typical disease symptoms on Arabidopsis roots and shoots including root browning. Consistently, *B. sorokiniana* colonization results in a constant increase in electric conductivity. This increase is diminished in plants when the roots are additionally inoculated with *S. vermifera*, reflecting the protective function of *S. vermifera*.

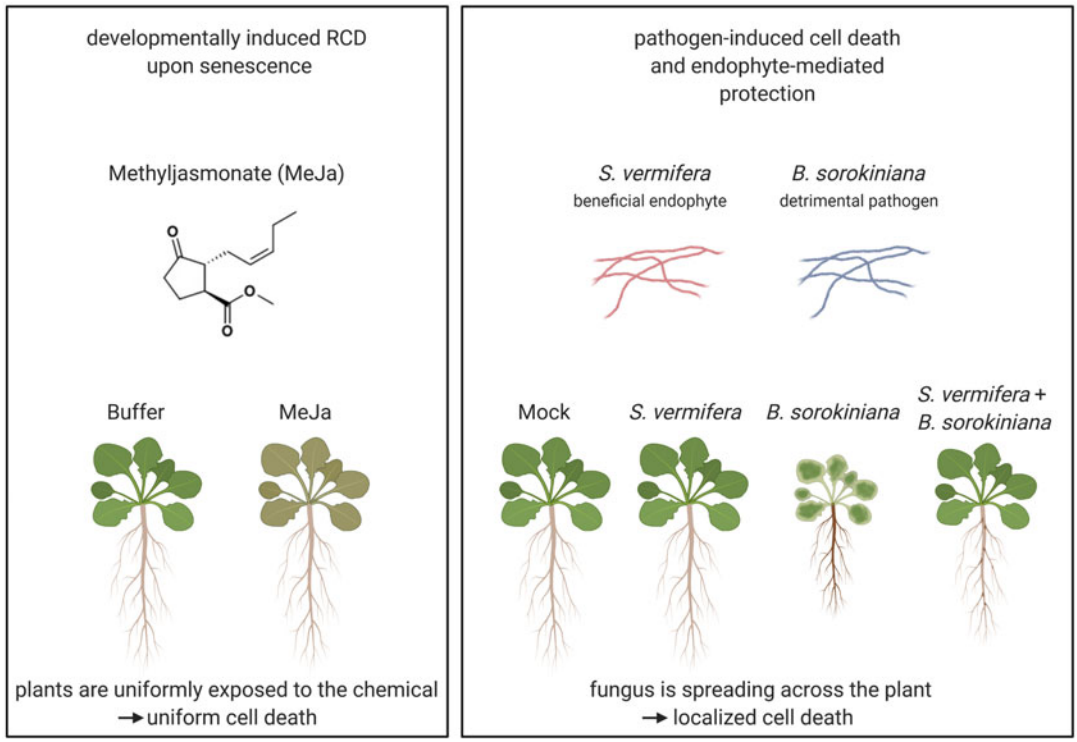

Fig. 1 Experimental setup depicting a chemically induced developmental cell death via methyl jasmonate (MeJA) (left) and a pathogen-induced cell death via *B. sorokiniana* colonization in a tripartite system with the beneficial endophyte *S. vermifera* (right)

PAM analysis revealed consistent results. Interestingly, *B. sorokiniana* colonized plants do not display a uniformly decreased F_V/F_M ratio (Fig. 2b). Instead, there is a local reduction of photosynthetic activity and cell death that slowly spreads across the whole shoot. This in turn leads to a constant reduction in the photosynthetic active leaf area over time reflecting the proceeding cell death.

2 Materials

2.1 Equipment

1. 24-Well culture plates.
2. Square plates.
3. Forceps.
4. Conductivity meter.
5. Imaging PAM fluorometer (e.g., Imaging-PAM M-Series Chlorophyll Fluorescence System (Walz)) able to measure culture plates (e.g., 24-well plates).

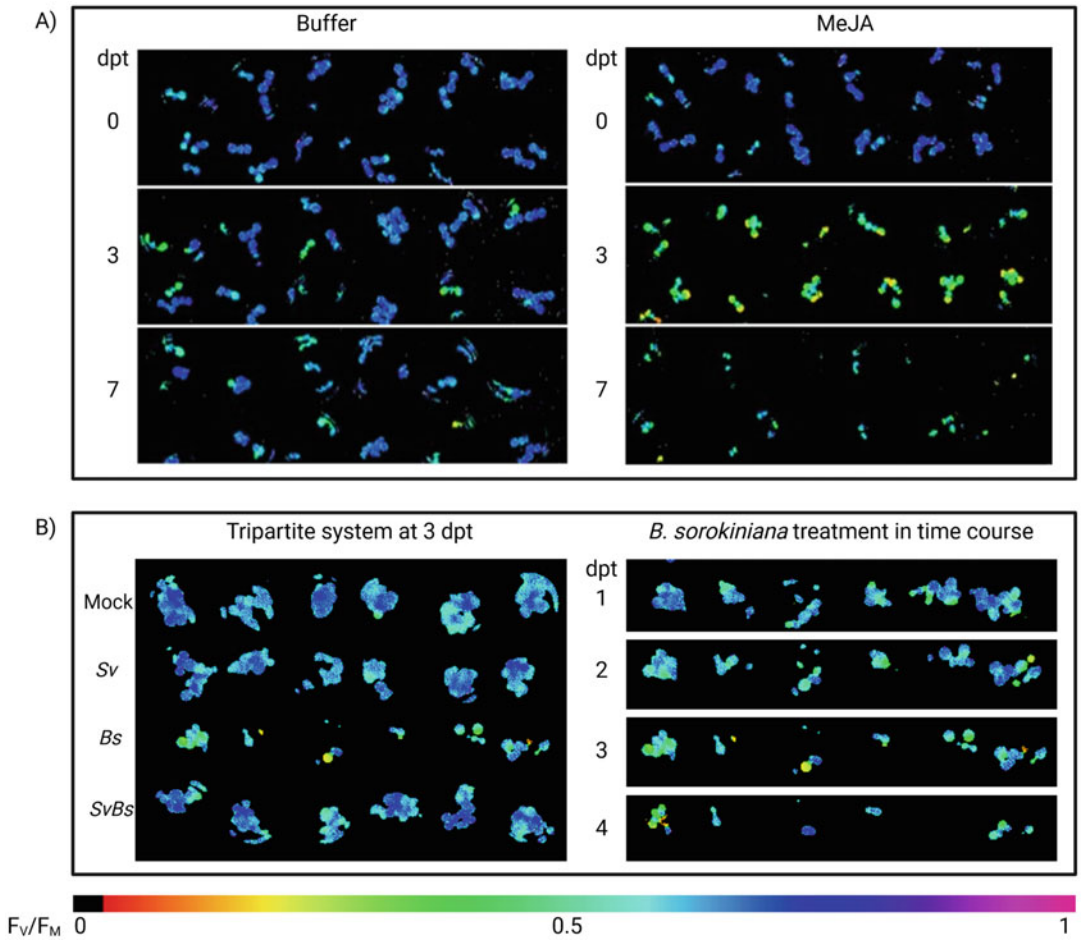

Fig. 2 PAM fluorometry pictures of (**a**) 7-day-old *Arabidopsis thaliana* seedlings treated with 50 mM MES buffer (pH 5.6) or 500 mM MeJA, respectively, at 0, 3, and 7 days post transfer (dpt). (**b**) 14-day-old *Arabidopsis thaliana* seedlings inoculated with sterile water (Mock), the beneficial fungal endophyte *S. vermifera* (Sv), the fungal pathogen *B. sorokiniana* (Bs) or a combination of both fungi (SvBs). At 7 days post inoculation (dpi) the plant roots were washed and the seedlings were transferred to deionized water. Pictures were taken every 24 h for 4 days. Blue color represents high photosynthetic activity while lighter colors represent a lower photosynthetic activity

6. Personal computer with a software suitable for PAM evaluation (e.g., ImagingWin, Walz, Germany) installed.
7. Growth cabinet or growth chamber for plant growth under controlled conditions.

2.2 Reagents and Consumables

1. *Arabidopsis thaliana* seeds.
2. Murashige-Skoog (MS) medium including vitamins.
3. Sucrose.
4. 70% Ethanol.

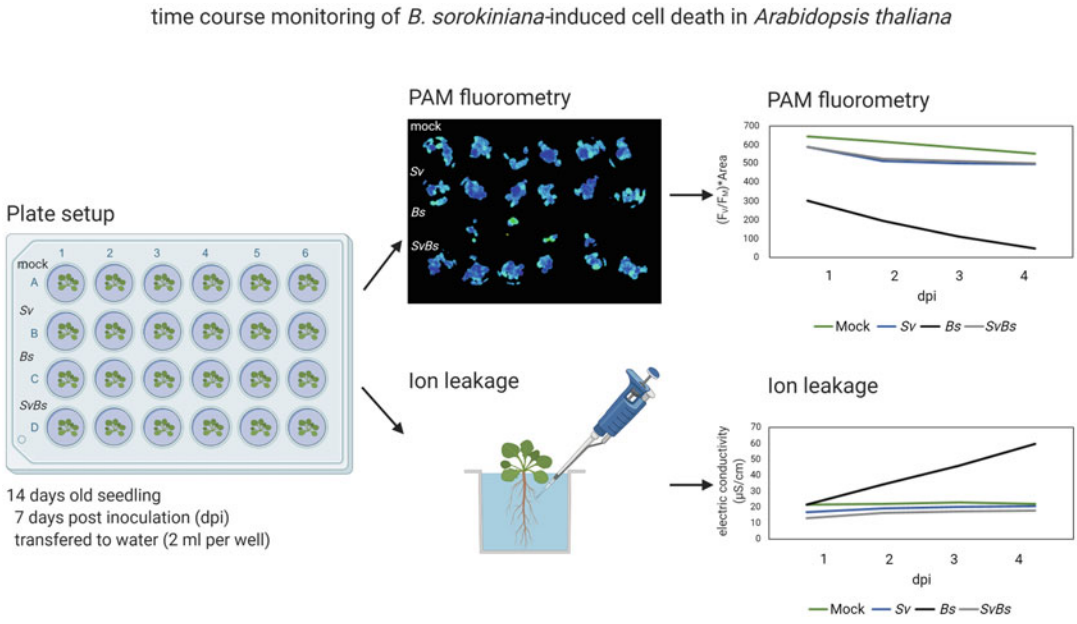

Fig. 3 Evaluation of *B. sorokiniana*-induced cell death via PAM fluorometry and ion leakage analysis. Both readouts are noninvasive and thus suitable to measure cell death progression over time on the same samples. While ion leakage represents a direct proxy, its measurement is more time-consuming. PAM fluorometry visualizes plant cell death indirectly via F_V/F_M-ratios but can represent a more efficient screening strategy and can be enhanced by additional analysis of the photosynthetic active area

5. 100% Ethanol.
6. Plant Agar.
7. Deionized water or sterile 2.5 mM 2-(N-morpholino) ethanesulfonic acid (MES) adjusted to pH 5.6 (*see* **Note 1**).
8. Chemical or organism to be tested, here: 10 mM MeJA solved in 2.5 mM MES (pH 5.6) or fungal inoculum in water.

3 Method

3.1 Seedling Sterilization, Germination, and Treatment

1. Sterilize *Arabidopsis thaliana* seeds by washing them two times with 70% EtOH for 5 min followed by one washing step with 100% EtOH for 5 min.
2. Let the seeds dry under sterile conditions.
3. Sow sterilized seeds on ½ MS, 4% plant agar, and 1% sucrose square plates.
4. Stratify the seeds for 2 days at 4 °C in darkness.
5. Then transfer plates to a growth chamber at a day/night cycle of 8/16 h at 22/18 °C, 60% humidity and a light intensity of 125 µmol m^{-2} s^{-1}.

6. Let seedlings germinate for 7 days in these conditions prior to the treatment.
7. *For chemical treatment*: Fill each of the 24 wells of a sterile culture plate with 1.9 mL of 2.5 mM MES buffer (pH 5.6).
8. Transfer 3–5 7-day-old seedlings to each well and let them regenerate in the growth chamber at the abovementioned conditions (*see* **Note 2**).
9. After regeneration, take first measurements of ion leakage and PAM fluorometry as a time point 0.
10. Treat the 8-day-old seedlings with 100 μL of 20× chemical solution (e.g., 10 mM MeJA; end concentration: 500 μM) per well.
11. *For tripartite treatment*: transfer 20 seedlings per plate to ½ MS plates, 4% plant agar without added sucrose.
12. Inoculate the plants with the respective fungi or fungal combination, here with 1 mL of a 1 g/L *S. vermifera* mycelium solution per plate, 1 mL of a 5000 spores/L *B. sorokiniana* spore solution per plate, or a combination of both fungi.
13. Distribute the fungal solution on the roots and the plate area below the roots. For this, distribute the solution on the roots and let it run down the plate. Recollect it with a pipette and repeat until a homogenous inoculation is achieved.
14. Incubate the plates in the growth chamber (conditions see above) for 7 days.
15. At 7 days post inoculation, when the seedlings are 14 days old, fill each well with 2 mL deionized water, wash the colonized roots to remove the extraradical hyphae, and transfer 3–5 seedlings to each well.
16. For washing, hold the seedlings carefully with forceps, dip the roots into autoclaved water, and slip the wet root over tissue paper. Extraradical hyphae will stick to the tissue paper.

3.2 Time Course PAM and Ion Leakage Measurement

1. Measure ion leakage for each well according to the manufacturer instructions. Be careful not to touch the seedlings while measuring (*see* **Note 3**).
2. Before measuring PAM, transfer the plates to darkness for 15 min to enable measurement of the maximum fluorescence (F_M) as well as the ground fluorescence (F_0) in the dark-adapted leaves (*see* **Note 4**). For dark adaption, the plates can be kept in a closed drawer or covered with aluminum foil. Afterward, measure the fluorescence by taking pictures with the fluorometer plate by plate in darkness according to the manufacturer's instructions.
3. Continue the measurements every 24 h for 4–8 days.

3.3 Evaluation of PAM with and Without Area

1. The evaluation differs depending on the Imaging PAM fluorometer and the respective evaluation-software. Here, we explain the evaluation with ImagingWin (Walz) in more detail.
2. Open the first PAM file (.pim or .xpim) in ImagingWin. Photosynthetically active leaf tissues are visualized in blurry colors (Fig. 2).
3. Click on Options and uncheck "Mean over Area of interest (AOI)."
4. Reset the AOI and calculate the F_V/F_M value of each well by adding AOI circles around each well.
5. Extract the F_V/F_M values (denoted as Y(II) by the program) using the "Report" tab. The resulting F_V/F_M values represent the mean PSII activity of living leaves of the 3–5 seedlings in each well.
6. Export a JPEG file of the F_V/F_M channel and measure the photosynthetic active leaf area via a suitable software (e.g., ImageJ [12]) to include the dimension of the living leaf area into the evaluation (*see* **Notes 5** and **6**).
7. Multiply the F_V/F_M value for each well with the photosynthetic active leaf area.

4 Notes

1. For chemical treatments, the buffer is of high importance for the experiment. Many chemicals have a strong effect on the pH of the solution. The buffer ensures that the plant health is not affected by an extremely acidic or basic pH.
2. When transferring and washing the seedlings, be careful not to injure the plants, as this induces stress and tampers the results. An additional recovery day between picking and treatment is thus recommended (*see* Subheading 3.1).
3. 24 h of regeneration before starting measuring are recommended to avoid artifacts in ion leakage measurements due to wounding during the seedling transfer.
4. Be sure not to expose the dark adapted plants to light before measuring. Also, do not measure the plates twice without repeating the adaption process.
5. During evaluation, be aware that dead cells are no longer photosynthetic active and are thus excluded from the program calculation. Including the area in your calculation will allow you to take the dead leaf area into account. For a cell death response such as observed upon *B. sorokiniana* treatment (Figs. 1, 2b and 3) the area is essential for quantification. For a cell death that spreads and proceeds uniformly on the plant

such as in case of MeJA (Figs. 1 and 2a), the area calculation is not necessarily needed. However, it still increases the measurement sensitivity.

6. As it is hard to perform absolute size measurements of the photosynthetic active leaf area due to the lack of a size standard, relative sizes can be compared. For this, the plates have to be continuously measured in the same setup (same height, zoom, etc.)

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Chapter 15

Measuring and Perturbing Ferroptosis in Plants

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Abstract

Ferroptosis is an oxidative iron-dependent cell death that was recently described in vertebrates, invertebrates, fungi, plants, and bacteria. In plants, ferroptosis has been reported in response to heat shock in roots of 6-day-old *Arabidopsis thaliana* seedlings. Generally, all biochemical and morphological ferroptosis hallmarks are conserved between animals and plants. Here, we describe a protocol to induce and quantify ferroptosis in plants based on the analysis of dead cells with a Sytox Green stain. Furthermore, heat shock induced cell death is prevented by using specific ferroptosis inhibitors.

Key words Ferroptosis, Cell death, Plants, Root hairs, Heat stress, Sytox Green

1 Introduction

Ferroptosis is an iron-dependent mechanism of cell death characterized by the accumulation of lipid reactive oxygen species and particular morphological, biochemical, and genetic features. Ferroptosis was first described in human tumor cells [1] and later in several models of human diseases [2]. Also, there are reports on ferroptosis in diverse mammals and non-mammalian vertebrates, invertebrates (reviewed in [3]), fungi [4], plants [5], and bacteria [6].

By far, human disease models are the biological system where ferroptosis has been studied the most. Much of what we know to date comes from pharmacological studies using the canonical ferroptosis inhibitors liproxstatin-1 (Lip-1), ferrostatin-1 (Fer-1), deuterated polyunsaturated fatty acids (D-PUFAs)—which suppress lipid peroxidation—the iron chelators Ciclopiroxoleamine (CPX) and Deferoxamine (DFO) [3, 7]. The cystine/glutamate antiporter (system Xc-) involved in glutathione (GSH) synthesis [1], the glutathione peroxidase 4 (GPX4) involved in the detoxification of lipid peroxides [8], phosphatidylethanolamines (PEs)

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containing poly-unsaturated fatty acids (PUFAs), lipoxygenases (LOXs) [9], and an iron labile pool, seem to be essential players in human ferroptosis [10].

In 2017, our laboratory reported the first evidence of ferroptosis in plants [11]. We studied the effect of ferroptosis inhibitors on specific cell death events occurring during development (particularly during reproductive development or xylem differentiation) and on different abiotic stresses known to trigger cell death. While the canonical ferroptosis inhibitors (Fer-1, CPX, or D-PUFA) did not prevent developmental cell death, they were able to suppress cell death in 6-day-old Arabidopsis seedlings exposed for 10 min at 55 °C (heat shock-) [11]. Heat stress induces ferroptosis through iron-dependent reactive oxygen species (ROS) accumulation, lipid peroxidation, and glutathione depletion. At the morphological level, retracted cytoplasm, lytic vacuoles, and mitochondria shrinkage were also observed in dying root cells, while nuclei were conserved without changes [11]. Additional reports showed that Arabidopsis cell suspensions treated with the GPX inhibitor (RSL3) also show cell death, which can be prevented by Lip-1 and Fer-1 [12] as occurs in animal cells [13]. Ferroptosis in plants can also be triggered by biotic stresses as it was reported for two different plant-pathogen interactions [14, 15].

We designed a protocol to robustly induce ferroptosis triggered by heat stress in 6-day-old Arabidopsis seedlings. This method offers several advantages: (1) it allows to work in vivo with intact plant tissues; (2) plant roots constitute a great system to perform microscopic analyses of treated cells, because of low autofluorescence, allowing the use of different probes and to visualize the location of proteins and subcellular structures; (3) it uses roots from 6-day-old seedlings, so material can be obtained in a short period of time. Here, we describe this method in detail, explaining cell death induction and how to perform assays to test the effect of ferroptosis inhibitors in Arabidopsis seedlings submitted to heat stress.

2 Materials

2.1 Plant Material and Growth Conditions

1. *Arabidopsis thaliana* seeds (ecotype Columbia).
2. 1.5 mL Microfuge tubes.
3. Sterile square plates 120 × 120 × 17 mm.
4. 10, 200, and 1000 μL pipettes and pipette tips.
5. Sterile deionized water.
6. 50% (v/v) Ethanol.
7. Bleach solution: 50% (v/v) commercial bleach, 1% (w/v) SDS.

8. Murashige and Skoog (MS) growth medium: MS half-strength basal salt mixture (2.2 g/L), 1% (w/v) sucrose, pH 5.7 (KOH), 0.8% (w/v) agar. Autoclave and pour approximately 40 mL of growth medium per plate.
9. Hypoallergenic 3 M, Micropore tape.

2.2 Ferroptosis Assays

1. 1.5 mL Microfuge tubes.
2. Forceps.
3. 1, 10, 200, and 1000 μL pipettes and pipette tips.
4. Dry bath at 55 °C.
5. Sterile deionized water.
6. 0.1% DMSO solution to use as vehicle control.
7. 10 mM Stock solution of Ferrostatin-1 (Fer-1), prepared in DMSO, stored at −20 °C. It is used as 1 μM final concentration. We suggest to prepare fresh as needed.
8. 10 mM Stock solution of Ciclopiroxolamine (CPX), prepared in DMSO, stored at −20 °C. It is used as 10 μM final concentration. We suggest to prepare fresh as needed.
9. 3.5 M Stock solution D4 Linoleate (D4-Lin), prepared in water stored at room temperature. It is used as 8 μM final concentration. We suggest to prepare fresh as needed.

2.3 Quantification of Cell Death

1. 24-Well plates.
2. Sterile deionized water.
3. 5 mM Stock solution Sytox Green, prepared in DMSO, stored at −20C. As Sytox Green is used at a final concentration of 5 μM, we suggest to dilute the stock solution with water to 0.5 mM which can be stored at −20C (working solution).
4. Microscope: Zeiss Axio Imager A2 microscope equipped with an Axiocam HRC CCD camera (Zeiss) using the Axiovision program (version 4.2), or similar.
5. Microscope slides and cover slips.

3 Methods

3.1 Plant Growth

1. In a laminar flow hood, place *A. thaliana* (ecotype Columbia) seeds in a 1.5 mL tube. Do not exceed 100 μL of the tube in order to have efficient sterilization.
2. Add 1 mL of 50% ethanol solution for 1 min. Discard the ethanol solution. Add 1 mL of bleach-SDS solution (50% bleach, 1% SDS) and incubate for 10 min with constant rotation or shaking. Keep the tube still (seeds will drop to the bottom) and carefully remove the solution. Perform 4 washes

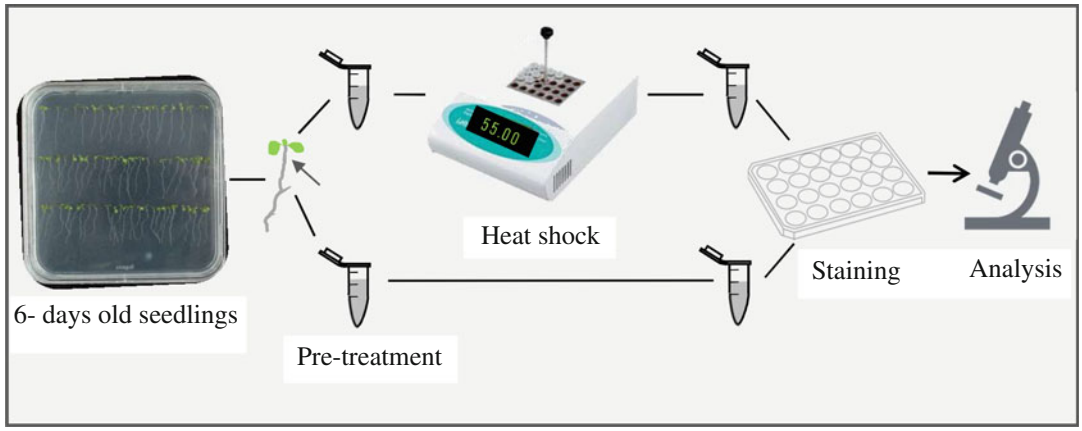

Fig. 1 Scheme showing the protocol used to induce and measure ferroptosis in 6-day-old Arabidopsis seedlings. A square plate containing 3 rows of seedlings is shown. The gray arrow indicates the region of root junction from where seedlings should be handled using fine-tipped tweezers, without pressing. The heat treatment is performed using 1.5 mL tubes and a multiwell plate is recommended for staining. Fluorescent microscopy is used for the analyses

with sterile distilled water, shaking in between of each wash to remove bleaching solution. To stratify seeds, keep the tube with fresh sterile water at 4 °C in the dark for 2 days.

3. In a laminar flow hood, sow the stratified seeds one by one on top of the MS growing medium following a single line using a pipette and a 200 μL tip. In one square plate of 12 × 12 cm, three lines of seedling can be sown (Fig. 1). Finally, close and seal the plate with micropore tape (*see* **Note 1**).
4. Place the plate in a vertical orientation to allow roots to grow down for 6 days at 23 °C, 16 h light-8 h dark. Only 6-day-old *A. thaliana* seedlings should be used, no younger or older ones (*see* **Note 2**).

3.2 Ferroptosis Assay

1. From this step the work can be done at the bench. Pipette 1 mL of each freshly prepared 0.1% DMSO, 1 μM Fer-1, 10 μM CPX, or 8 μM D4-Lin in 1.5 mL tubes. Use two tubes with 1 mL for each of the solutions. Use forceps to remove carefully seedlings one by one from the growing plate and place them in the 1.5 mL tubes (*see* **Note 3**). Place at least 5 6-day-old seedlings in each tube. Finally, incubate the tubes with ferroptosis inhibitors or control solution in a growing chamber (23 °C, 16 h light-8 h dark) (*see* **Note 4**) for 17 h (*see* **Note 5**).
2. Keep half of the tubes in the growing chamber. Use the other half to induce ferroptosis by incubation at 55 °C for 10 min in a dry bath (*see* **Note 6**). After heat stress, return the tubes containing the treated seedlings to the growing chamber room for 6 h (*see* **Note 7**).

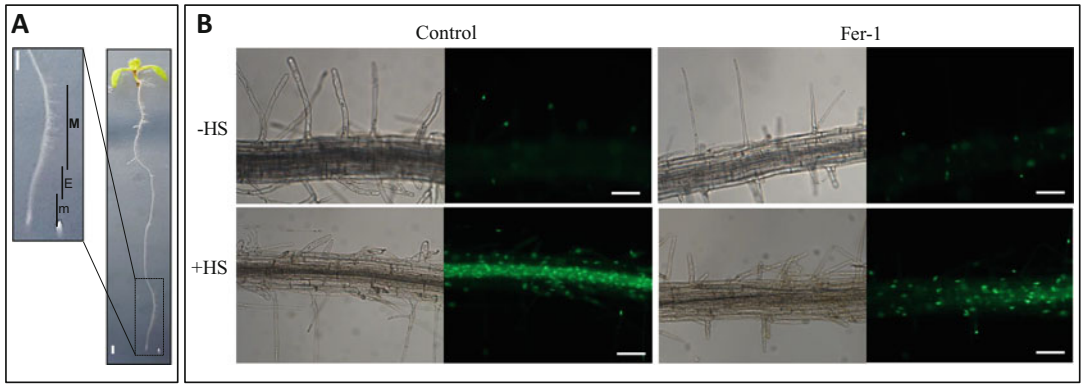

Fig. 2 Cell death in Arabidopsis roots. (**a**) *A. thaliana* seedling (right) showing the principal regions of development (left). From bottom to top: meristematic zone (m), elongation zone (E), and maturation zone (M). Maturation zone is selected for the analysis of cell death (bold). Bars: 1 mm (**b**) Six-day-old seedlings are pre-incubated with DMSO (control) or 1 μM Fer-1 (ferroptosis inhibitor). Cell death is induced by heat shock at 55 °C for 10 min. Seedlings are then stained with Sytox Green 6 h after heat stress. Representative pictures are shown. Green dots correspond to the nuclei of dead cells. Bars: 50 μm

3.3 Analysis of Cell Death

1. Pipette 400 μL of distilled water and 4 μL of 0.5 mM Sytox Green working solution (*see* **Note 8**) in each well of a 12-multiwell plate. Transfer each seedling present in the 1.5 mL tubes into one well of the 12-multiwell plate (*see* **Note 3**). Incubate for 10 min at room temperature in the dark. Then, wash the seedlings twice, by transferring them to another well containing distilled water.
2. Take the seedlings from the wells, place them on a drop of water on top of a microscope slide and cover the sample with a thin glass coverslip without pressing. Analyze the maturation zone of treated roots (Fig. 2a) on a fluorescence microscope (*see* **Note 9**). First, using bright field, count the number of root hairs visible in the region to be analyzed. Adjust the fine focus of the microscope so most root hairs appear in the field of view. Under UV light and using a proper filter, count the number of Sytox Green positive nuclei (as indicator of dead cells, Fig. 2). Register the ratio of dead cells/total cells. Repeat this procedure in three different fields from each root sample. Analyze at least three roots for each treatment. Finally, plot the results as percentage of dead cells (*see* **Note 9**).

4 Notes

1. It is preferable to use square plates to analyze a high number of seedlings. It is important not to leave water around the seeds when they are placed on the MS-agar plate to avoid contamination. Plates should remain open inside the flow hood until

the water evaporates. Do not replace micropore tape with parafilm. The tape is effective in blocking particles of bacteria size and above, and avoids the excess of humidity [16].

2. Six-day-old Arabidopsis roots are well developed, and the number and length of root hairs are optimal to analyze cell death. At this stage of development, manipulation of the seedlings is easy, and thus less stressful for the seedlings**.** In order to work with healthy roots, it is also necessary to handle the seedlings with extreme care as roots can be easily damaged. Growing plates must be completely free of any type of contamination (bacteria or fungi). Also, take into consideration that changes in growth media or environmental conditions would modify root hairs density [17, 18]. As it was explained by Kacprzyk and coworkers [19], it is important to obtain moderate density and short root hairs to simplify the analysis.
3. To handle seedlings we used fine-tipped tweezers, hooking the seedlings by placing the tweezers around the root junction, lifting the seedling upward by levering, supporting the cotyledons on the tweezers without pressing.
4. Incorporation of negative controls is highly recommended. Stressors such as higher temperatures (77 °C for 10 min) or salt stress (150 mM NaCl) induced a type of cell death that cannot be prevented by ferroptosis inhibitors [11]. Besides, inhibitors of enzymes involved in events related to ferroptotic cell death can also be considered. One example is DPI (diphenyleneiodonium—NADPH inhibitor).
5. We do the pre-treatments with ferroptosis inhibitors in the middle of the light period. Heat shock is done around 2 h after light turns on.
6. In our hands, ferroptosis inhibitors prevent cell death triggered by a treatment of 10 min at 55 °C. Due to the fact that small variations in temperature could produce different outcomes [20, 21], it is important to always use the same device to perform the heat treatment and to reproduce the same conditions for the heat stress.
7. While Sytox Green is impermeant to live cells, it easily penetrates cells with compromised plasma membrane so it can be used as an indicator of cell death.
8. Almost 60% of root hairs die 6 h after the heat stress. However, intermediate times could be considered to perform extra analysis and measurements such as ROS production, MDA content, GSH levels, among others.
9. As we mentioned before, almost 60% of root hairs die 6 h after the heat stress. However, in the presence of ferroptosis inhibitors root hairs death ratio remains close to control levels.

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Chapter 16

Detection and Quantification of the Hypersensitive Response Cell Death in *Arabidopsis thaliana*

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Abstract

In plants, the hypersensitive response (HR) is a programmed cell death modality that occurs upon recognition of harmful non-self. It occurs at the site of pathogen infection, thus preventing pathogens to live off plant tissue and proliferate. Shedding light on the molecular constituents underlying this process requires robust and quantitative methods that can determine whether plants lacking functional genes are defective in HR execution compared to wild-type controls. In this chapter, we provide two quantitative protocols in which we measure cell death from *Arabidopsis thaliana* leaves infected with avirulent HR-causing bacterial strains. Firstly, we use trypan blue staining to quantify the stained area of leaves upon bacterial infection using a personalized macro in the Image J (Fiji) software. Alternately, we incorporate an electrolyte leakage protocol in order to measure HR caused by different avirulent bacterial strains at different bacterial titers. We encourage users to perform a combination of both methods when assessing HR in different plant genotypes.

Key words *Arabidopsis thaliana*, Hypersensitive response, *Pseudomonas syringae* pv *tomato* DC3000, Trypan Blue Staining, Cell death quantification by Image J, Electrolyte leakage

1 Introduction

As a means of restricting pathogen growth, plants deploy a tightly regulated form of immune cell death at the attempted pathogen ingress site, traditionally known as the hypersensitive response (HR) [1, 2]. Upon recognition of harmful non-self, host intracellular immune receptors of the nucleotide-binding leucine rich repeat (NLR) type recognize pathogen effector molecules triggering an amplified immune response named effector-triggered immunity (ETI), which usually culminates in HR cell death [3]. When plant cells undergo HR as a consequence of pathogenic infection, the following hallmarks are generally displayed: cytoplasmic

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shrinkage, mitochondrial swelling, chromatin condensation, chloroplast and plasma membrane disruption, and vacuolization [4, 5].

A thorough understanding of the molecular players and mechanisms regulating HR-cell death is still lacking. With the advent of the genomic era, numerous HR regulators have been reported [6]. Consequently, robust methods for quantitative analysis of HR cell death are of utter importance to effectively evaluate whether mutations in certain genes render a plant unable to execute HR.

Trypan blue staining of infected plant tissue has been extensively used as a qualitative method for visualization of dead cells [7–9]. Since live cells possess intact membranes, the Trypan Blue dye is excluded from the cells, whereas in dead cells the dye transverse the plasma membrane as a consequence of the loss of its integrity [10]. Hence, dead cells are stained and appear in a distinctive blue color when imaged under a microscope. Subsequently, stained cells can be quantified in order to precisely determine whether differences exist between distinct plant genotypes in terms of HR cell death.

Loss of plasma membrane integrity in dying cells also results in the release of electrolytes to the extracellular milieu. The degree of electrolyte leakage from dying cells can also be used as a readout of the extent to which cell death is taking place in the infected tissue [11]. Currently available conductivity meters allow measurements of electrolyte leakage in relatively small volumes (2 mL), which facilitate accurate and rapid quantification of a larger number of samples.

On the one hand, we provide a detailed method for the quick and automated quantification of cell death using trypan blue staining. For this, we use *Arabidopsis thaliana* plants (Arabidopsis) belonging to the Columbia-0 ecotype (Col-0) inoculated with the HR-causing bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 carrying the effector *avrRpm1* (*Pto* DC3000 *avrRpm1*) using the syringe-infiltration method. In Col-0 HR is triggered upon recognition of avrRpm1 by the NLR receptor RPM1 [12]. Upon trypan blue staining of leaves at different time points after infection, we quantify stained cells in the infiltrated leaves using the image processing package Fiji (built upon the ImageJ2 free software) [13], using a newly developed macro that allows automated quantification of the stained area.

On the other hand, we describe a robust method for quantification of electrolyte leakage of dying cells from Arabidopsis Col-0 leaves infiltrated with both *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) using different bacterial titers adapted from a previously described protocol [11]. avrRpt2 also causes HR in Col-0, as this effector is recognized by the NLR RPS2 [14]. As a negative control for our experiments, we use the Arabidopsis Col-0 *rpm1–3* and *rps2* mutants, which do not display HR triggered by

Pto DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*), respectively, since they are defective in the cognate NLRs RPM1 and RPS2 [12, 14].

2 Materials

2.1 Plant Material and Growth Conditions

1. *Arabidopsis* thaliana Col-0 seeds from the following phenotypes: wild-type, *rpm1–3* (N68739) and *rps2* (N6196) from the Nottingham Arabidopsis Stock Centre (NASC) based in the University of Nottingham, UK (*see* **Note 1**).
2. Soil mix: 5 parts peat soil + 2 parts vermiculite + 1 part perlite.
3. A growth chamber with controlled temperature (22 °C), photoperiod (9 h light, 15 h dark), humidity (70% relative humidity) and white LED light intensity of 150 μmol/m^2/s.
4. Small size plastic pots.
5. Flat polypropene trays.

2.2 Bacterial Strains, Preparation of Inoculum and Infection

1. *Pseudomonas syringae pv tomato (Pto)* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) avirulent strains (*see* **Note 2**).
2. Solid King's Broth medium (KB medium): For 500 mL: 10 g peptone from meat, 0.75 g K_2HPO_4, 0.75 g $MgSO_4 \bullet 7H_2O$, 5 ml glycerol, 7.5 g bacteriological agar, and Milli Q sterilized water.
3. Antibiotics for selection of avirulent *Pto* DC3000 strains (*see* **Note 3**).
4. Microwave.
5. Water bath with adjustable temperature.
6. Resuspension buffer: Autoclaved 10 mM magnesium chloride ($MgCl_2$).
7. 50 and 15-mL centrifuge tubes.
8. Petri dishes.
9. Polystyrene disposable cuvettes.
10. 1 mL Needleless syringes.
11. Spectrophotometer.
12. Laminar flow hood.
13. Plastic wrapping paper/plastic dome.
14. Marker pen (black).

2.3 Trypan Blue Staining and Microscopy Slide Preparation

1. Stock of trypan blue staining solution: 100 mg phenol (solid), 100 mL lactic acid, 100 mL glycerol, 100 mL Milli Q sterilized water.
2. Trypan blue staining working solution: 1 part trypan blue staining solution +3 parts 96% ethanol.

3. Destaining solution: 1 kg chloral hydrate dissolved in 400 mL Milli Q sterilized water.
4. Magnetic stirrer with adjustable temperature.
5. Grid cloth mesh.
6. Tilt shaker.
7. Fume hood.
8. Slide preparation: 50% glycerol, fine painting brush, microscopy glass slides, and coverslips.
9. Optivisor lenses 3.5×.
10. Clear glue.
11. Microdissection microscope.

2.4 Electrolyte Leakage

1. Scissors.
2. Cork borer.
3. Forceps.
4. Milli Q sterilized water.
5. Sterile 12-well plate.
6. LAQUAtwin EC-11 Conductivity meter (HORIBA Advanced Techno Co., Ltd).

2.5 Quantification of Cell Death by Trypan Blue Staining and Electrolyte Leakage

1. Image J (Fiji) software for trypan blue staining quantification [13].
2. R software for graph plotting of conductivity measurements and statistical analysis.

3 Methods

3.1 Sowing of Arabidopsis Seeds and Plant Growth

1. Fill small plastic pots with soil peat, vermiculite, and perlite mix ($5/1/1_{/2}$). Compress the mix without exerting too much pressure into the pot, place the pots on a middle size flat polypropene tray, and wet the mixture to field capacity with tap water.
2. Sow 4–5 seeds in each pot. Fill 6–8 pots per genotype for electrolyte leakage experiments and 8 pots per genotype for a time course of infected leaves (2 plants per time point) stained with trypan blue staining (*see* **Note 4**).
3. Randomize the previously labeled pots on the tray.
4. Cover the tray with plastic wrapping paper or a plastic dome in order to maintain humidity required for germination.
5. Stratify the seeds by placing the tray on a cold room/refrigerator at 4 °C for 2 days.

6. Transfer the tray to a growth chamber with a photoperiod of short-day conditions: 9 h light/15 h dark (*see* **Note 5**), 22 °C, 70% relative humidity, and light intensity of 150 μmol/m^2/s.
7. Remove the plastic wrapping paper or dome after 3 days and let the seedlings grow for 5–6 more days.
8. With the help of thin forceps, remove unwanted seedlings from each pot and leave only one seedling growing.
9. Water plants two to three times per week without overwatering to avoid stress on the plants.
10. On the second to third week of growth, use a marker pen to mark leaf eighth of the Arabidopsis plant, which will be the one infected (*see* **Note 6**).
11. Four- to five-week-old plants grown in these conditions are ideal for bacterial infection by syringe infiltration.

3.2 Preparation of Bacterial Inoculum and Syringe Infiltration

1. Sterilize a laminar flow cabin by cleaning surfaces with 70% ethanol and switch on the UV light for 5 min.
2. Prepare the KB medium and add appropriate antibiotics for selection of avirulent bacterial strains.

3.2.1 Growth of Bacteria in KB Medium Plates

3. Pour 25 mL of KB + antibiotics into each plate.
4. Three days before infecting Arabidopsis, streak avirulent bacteria from a −80 °C glycerol stock with a sterile tip. Place the plate on a still 28 °C incubator. Bacteria will grow after 2 days of incubation.
5. One day before infecting Arabidopsis, collect all bacteria grown on the initial plate and re-streak them on the surface of a new KB plate using a sterile inoculating loop.

3.2.2 Preparation of Bacterial Inoculum

1. On the day of the infection, add 10 mL of autoclaved 10 mM Mg_2Cl inside the plate and wait 10 min in order for the bacteria to detach from the surface of the plate.
2. Re-suspend bacteria with the help of a 10 mL Pasteur pipette by gently pipetting up and down in order to detach as much bacteria as possible from the plate.
3. Take 1 mL of bacteria from the plate and mix it with 9 mL of 10 mM $MgCl_2$ in a 15 mL tube.
4. Make a 1:10 dilution in 10 mM $MgCl_2$ and measure bacterial optical density at 600 nm (OD_{600}) using a spectrophotometer. Calculate the volume needed from undiluted bacteria in the previous step and dilute it in 10 mM $MgCl_2$ in order to reach the OD_{600} desired for infection (*see* **Note 7**).

3.3 Trypan Blue Staining

1. Label the time point after infection at which each plant leaf will be collected on each pot.

2. Pressure infiltrate the eighth leaf of Arabidopsis plant with avirulent bacteria using a needleless syringe (*see* **Note 8**).
3. After infiltration, gently dry the excess of liquid on the surface of the leaf and collect the leaves corresponding to each time point by cutting through the petiole with the aid of small scissors.
4. Place the leaves in a 50 mL tube containing 15 mL of trypan blue staining working solution. Always work in a fume hood when handling trypan blue staining solution and destaining solution.
5. Pour boiling water into a plastic box and submerge the sealed tubes inside the water for 5 min or until the leaves turn blue.
6. Pour the trypan working solution along with the leaves onto a sieve and transfer the leaves carefully with tweezers to a new 50 mL tube containing 20 mL of destaining solution (*see* **Note 9**). From this step onward, the leaves will stay in the same tube in order to avoid damage caused by transferring leaves from one tube to another.
7. Let the tubes rotate on a tilt shaker at 80 rpm for 1 h.
8. Use a mesh grid in order to sieve and discard the destaining solution and replace it with fresh 20 mL destaining solution (*see* **Note 10**). Let the 50 mL tubes rotate overnight.
9. The following day, sieve the destaining solution using a new mesh grid and add 20 mL of 50% glycerol. Leaves can be stored for prolonged periods in this solution.

3.4 Mounting Microscopy Slides

1. Pour the 20 mL of 50% glycerol containing eight leaves into a petri dish.
2. With the aid of a fine painting brush, gently transfer a single leave onto a microscopy glass slide.
3. Place 500 μL of 50% glycerol on top of the glass slide.
4. Gently expand the leaf on the surface of the glass slide with fine touches using a paint brush (*see* **Note 11**).
5. Once the leaf is correctly expanded on the glass slide, place a coverslip on top of the leaf by gently dropping the coverslip from the top of the leaf to the bottom. Try to avoid bubbles forming in between the leaf and the coverslip (*see* **Note 12**).
6. Gently brush clear glue at the edges of the coverslip so that it adheres to the glass slide and coverslips do not detach.

3.5 Microscopy Imaging

1. Image individual leaves with a microdissection microscope at 5× magnification. Always use the same settings for all samples.

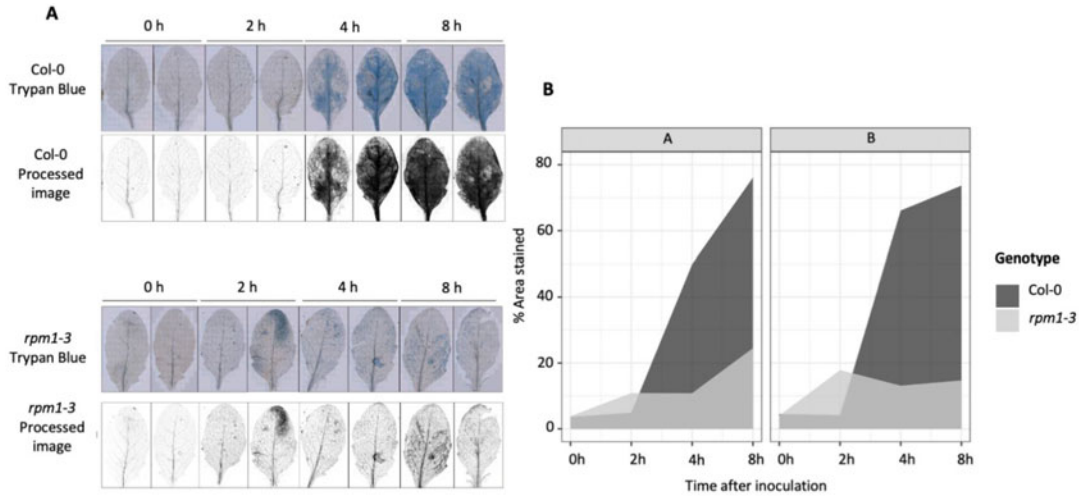

Fig. 1 Quantification of trypan blue stained area of Arabidopsis leaves infected with an HR-causing avirulent bacterial strain. (**a**) Four- to five-week-old Arabidopsis leaves of either Col-0 or *rpm1–3* were syringe-infiltrated with *Pto* DC3000 (*avrRpm1*) at 2.5×10^7 CFUs/O.D_{600} = 0.05. Two independent leaves were stained in trypan blue at different time points after infiltration (0, 2, 4 and 8 h) and subsequently imaged under the microscope. (**b**) Image J software was used for quantification of stained area which is represented as a percentage (*see* **Note 12**)

3.6 Quantification of Cell Death Using Image J

1. Open the image files obtained in the microscope using the Fiji software (Image J distribution).
2. Install the cell death quantification macro (*see* **Note 13**).
3. Select process image for cell death macro and follow the instructions for quantification.
4. Plot the percentage of stained leaf as a function of time (Fig. 1).

3.7 Electrolyte Leakage Assay

1. Pressure infiltrate the seventh and eighth leaf of an Arabidopsis plant with avirulent bacteria. Four plants per genotype are required for the experiment.
2. After infiltration, gently dry the excess of liquid on the surface of the leaf.
3. Collect the leaves by cutting through the petiole with the aid of small scissors.
4. Place the infiltrated leaves on top of a flat surface and punch out discs (one disc per leaf) using a cork-borer (size 4, diameter = 7.5 mm) (*see* **Note 14**).
5. Immediately after punching out leaf discs, place two leaf discs from a single plant into one well of a 12-well plate containing 2 mL Milli Q sterilized water.

6. Use as many 12-well plates as required depending on the number of genotypes included in the experiment.
7. Cover the plate with the lid and place it on a tilt shaker at 90 rpm for 1 h (*see* **Note 15**).
8. In the meantime, perform a one-point calibration of the LAQUAtwin EC-11 Conductivity meter (HORIBA Advanced Techno Co., Ltd) using the conductivity standard solution to 1.41 mS/cm.
9. Replace the 2 mL water from the wells with new 2 mL Milli Q sterilized water. Once the water is replaced, a time series of measurements of water conductivity start (*see* **Note 16**).
10. Record water conductivity by pipetting 100 μL of water per well into the conductivity meter. Ions released from dying cells during the course of HR correlate with the conductivity of the solution. The unit used to measure conductivity is microSiemens per centimeter (μS/cm) where cm denotes the distance between the two electrodes sensors of the conductivity meter.
11. Return the water from the device to the well in order to maintain the same volume of water in the wells throughout the experiment (*see* **Note 17**).
12. Record conductivity at each time point. Meanwhile leave the 12-well plate rotating on the tilt shaker.

3.8 Data Representation and Statistical Analysis

1. Plot conductivity in μS/cm as a function of time (Fig. 2).
2. For statistical analysis, compare the conductivity (in μS/cm) of two genotypes at a given time point by a two tailed Student's t-test. For comparison of more than one genotype, use a one-way analysis of variance (ANOVA).

4 Notes

1. While NASC distributes seeds to Europe, the Biological Resource Center (ABRC) based at Ohio State University (USA) delivers seeds to North and South America. Laboratories located in other parts of the world may order stocks from either of both stock centers. Arabidopsis Col-0 accessions carry the resistance (R) genes *RPM1* and *RPS2,* which encode for the NLRs RPM1 and RPS2, respectively. In contrast, *rpm1–3* and *rps2* mutants are not equipped with functional RPM1 and RPS2, respectively [12, 14].
2. *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) avirulent strains overexpress the effector molecules avrRpm1 and avrRpt2, respectively. Plant NLRs RPM1 and RPS2 recognize

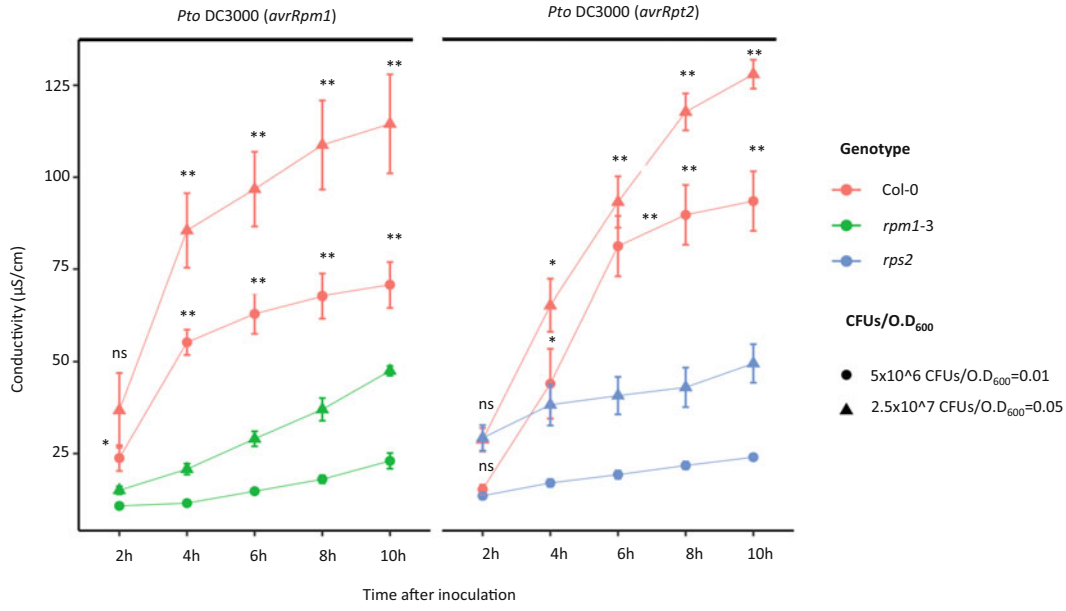

Fig. 2 Electrolyte leakage from Col-0, *rpm1–3* and *rps2* leaf discs after bacterial inoculation. Four- to five-week-old Arabidopsis leaves were syringe-infiltrated with either *Pto* DC3000 (*avrRpm1*) or *Pto* DC3000 (*avrRpt2*) with two independent bacterial titers: 2.5×10^7 CFUs/$O.D_{600} = 0.05$ (triangles) or 5×10^6/$O.D_{600} = 0.01$ (circles). Conductivity measurements of electrolyte leakage from dying cells were recorded from 2 to 10 h after inoculation. Standard error bars represent four biological replicates. Asterisks denote significant differences (**, *P* value <0.01 or *, *P* value<0.05, NS, *P* value >0.05) from independent Student's t-tests for comparisons between two genotypes at each time point and $O.D_{600}$. *NS* non-significant

perturbations in the host cell caused by the aforementioned effectors eliciting an ETI response that is accompanied by HR.

3. For selection of *Pto* DC3000 (*avrRpm1*) and *Pto* DC3000 (*avrRpt2*) in KB media. Kanamycin is added for selection of the construct that carries the *avrRpm1* and *avrRpt2*, whereas resistance to rifampicin comes inherently in *Pto* DC3000. Working concentrations for kanamycin and rifampicin are 50 µg/mL.
4. We recommend including at least eight pots per time point and genotype in the trypan blue experiment to have robust and consistent results when comparing genotypes that show mild differences.
5. We recommend avoiding walk-in chambers for pathogenesis-related experiments in order to avoid stresses from other pathogens (i.e., insect infestations) that can be present in a chamber where other plants are growing or where users come in and out on a regular basis. We suggest a photoperiod of 9 h light/15 h dark that resembles short-day conditions (8 h light/16 h dark) but adds an extra hour of light, allowing plants to be at their optimal stage for infiltration earlier than the classical short-day

photoperiod (in between the 4th and 5th week of growth). This extended short-day cycle is used by many laboratories working on molecular plant pathology.

6. In order to have comparable results between different plants, we always infiltrate the seventh and eighth leaf of the plant [15]. In this way leaves of comparable developmental stages that may respond similarly to the pathogen are chosen for infiltration.
7. The CFUs/O.D.$_{600}$ (OD_{600} = 1.0 correlate to $3.55*10^8$ CFU mL^{-1} determined by serial dilutions and plating) of the bacterial inoculum may be adapted depending on the genotype being infected [16], bacterial strain used in the experiment, or time points at which samples are collected after infection.
8. Gently exert pressure on the abaxial side of the leaf with a needleless syringe and infiltrate the leaf thoroughly. If users are not experienced, we recommend practicing beforehand with water on plants that will not be used in the experiment. Besides including mutants impaired in pathogen effector recognition as negative controls (i.e., *rpm1–3* and *rps2*) when available, we encourage users to include leaves infiltrated with 10 mM $MgCl_2$ as an additional negative control.
9. Pouring trypan blue working solution along with the already stained leaves onto a sieve will allow you to grab the leaves from the petiole and transfer them easily to a new 50 mL tube containing destaining solution.
10. Once leaves are incubated in destaining solution, they need to be handled very carefully to avoid damage. Furthermore, since leaves will lose the green color due to the loss of chlorophyll, it will become harder to identify where the petiole is. As a result, we recommend working always in the same tube, once the destaining solution has been added to the leaves.
11. The abaxial side of the leaf faces the coverslip. Use Optivisor lenses in order to aid vision when handling the leaves.
12. Gently drop the coverslip on top of the leaf very slowly from top to bottom of the leaf by sliding a 1000 μL pipette tip below the coverslip really slowly. Avoiding as many bubbles as possible at this step is critical so that they do not appear in the images and do not affect quantification.
13. Follow the instruction guide for running the cell death processing macro located in the GitHub platform: https://github.com/Celldeathquantification/Cell-death-quantification.
14. When punching out leaf discs, we recommend users to excise the leaf disc from the center part of the leaf. Exerting strong pressure toward a flat surface covered with a fine layer of tissue paper allows neat excision of discs.

15. The first hour of incubation of leaf discs under constant rotation is intended to remove electrolytes leaked from damaged cells on the edges of the leaf discs as a consequence of the excision caused by the cork-borer.
16. Time points selected for conductivity measurements can vary depending on the bacterial inoculum used. We recommend a time series of measurements from 0 h to 10 h once the water from **step 9** has been replaced, with measurements being taken every 2 h.
17. Always clean the sensor of the conductivity meter with Milli Q sterilized water in between samples.

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Chapter 17

Monitoring of ATG4 Protease Activity During Autophagy in the Model Microalga *Chlamydomonas reinhardtii*

José L. Crespo and M. Esther Pérez-Pérez

Abstract

Deciphering the molecular mechanisms underlying the regulation of the ATG4 protease is essential to understand the regulation of ATG8 lipidation, a key step in the biogenesis of the autophagosome and hence in autophagy progression. Here, we describe two complementary approaches to monitor ATG4 proteolytic activity in the model green alga *Chlamydomonas reinhardtii*: an in vitro assay using recombinant ATG4 and recombinant ATG8 as substrate, and a cell-free assay using soluble total protein extract from Chlamydomonas and recombinant Chlamydomonas ATG8 as substrate. Both assays are followed by non-reducing SDS-PAGE and immuno-blot analysis. Given the high evolutionary conservation of the ATG8 maturation process, these assays have also been validated to monitor ATG4 activity in yeast using Chlamydomonas ATG8 as substrate.

Key words ATG4, Protease, Autophagy, Microalgae, Chlamydomonas, Non-reducing SDS-PAGE, Western-blot

1 Introduction

Autophagy is a degradative process by which eukaryotic cells degrade and recycle intracellular material such as proteins, lipids, membranes, or even organelles [1]. A hallmark of autophagy is the formation of autophagosomes, that is, double-membrane vesicles where the material to be degraded in the vacuole/lysosome is engulfed [2–4]. This catabolic process and the proteins involved (autophagy-related or ATG proteins) are highly conserved in all eukaryotes including the green lineage [5, 6]. Autophagy occurs through a sequential and regulated series of events that can be summarized in five main stages: (1) induction and nucleation of the phagophore membrane; (2) expansion of the phagophore; (3) closure and maturation of the autophagosome; (4) fusion of the autophagosome to the vacuole/lysosome; and (5) degradation and efflux of breakdown products [4, 7]. Up to now, 42 ATG

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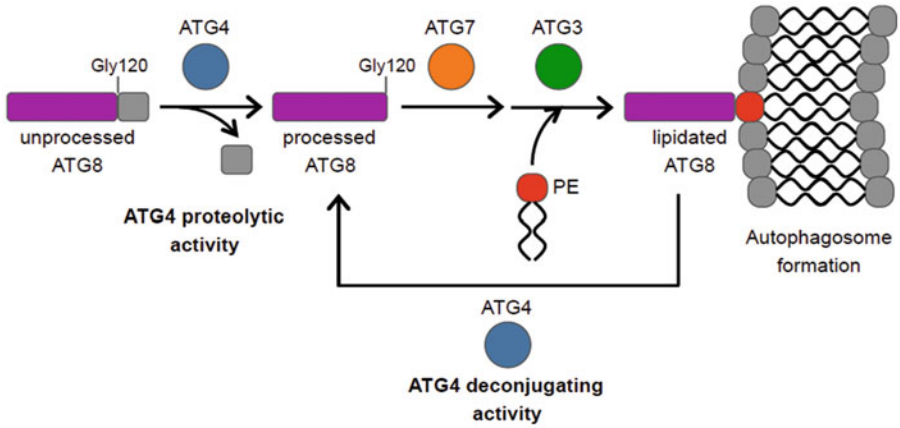

Fig. 1 The dual function of ATG4. The lipidation of ATG8 is a sequential reaction involving three consecutive steps: (1) Nascent ATG8 is processed at the highly conserved glycine (Gly120 in Chlamydomonas ATG8) by ATG4 (ATG4 proteolytic activity). (2) Processed ATG8 is first transferred to ATG7 (an E1-activating enzyme) and then to ATG3 (an E1-activating enzyme). (3) Finally, ATG8 is conjugated to phosphatidylethanolamine (PE). ATG4 can also cleave lipidated ATG8, releasing free ATG8 for recycling (ATG4 deconjugating activity)

proteins have been described, mainly in the model yeast *Saccharomyces cerevisiae* [7]. Among these ATG proteins, the ATG8 and ATG12 systems, two conserved ubiquitin-like systems, act coordinately to accomplish vesicle expansion, autophagosome formation, cargo recognition and autophagosome targeting to the vacuole/lysosome, and therefore their function is essential for autophagy [4]. The ATG8 system is composed by ATG3, ATG4, ATG7, and ATG8. First, nascent ATG8 is processed at the C-terminus by the cysteine protease ATG4, exposing a highly conserved Gly. Second, the E1-activating enzyme ATG7 transfers ATG8 to the E2-activating enzyme ATG3. Then, ATG3 catalyzes the binding of the membrane lipid phosphatidylethanolamine (PE) to the conserved Gly of ATG8 (Fig. 1). The ATG12 system includes ATG5, ATG7, ATG10, ATG12, and ATG16, and leads to the formation of the heterotrimeric complex ATG12-ATG5-ATG16, which may function as an E3-like enzyme for the ATG8 conjugation system. Finally, the ATG4 protease can also release ATG8 from the autophagosome membranes for recycling (Fig. 1) [7]. Therefore, ATG4 plays a major role in autophagosome formation and thus its activity must be highly regulated.

The detection and quantification of ATG4 activity is essential for understanding the balance between free/unmodified ATG8 and lipidated ATG8. A proteolytic assay to monitor ATG4 activity both in vitro (Fig. 2) and in *cell-free total protein extracts* (Fig. 3) allowed the study of ATG4 regulation in two model unicellular eukaryotes, the green alga *Chlamydomonas reinhardtii* [8, 9] and the budding yeast *Saccharomyces cerevisiae* [10]. By using these assays, it has been shown that the cysteine protease ATG4

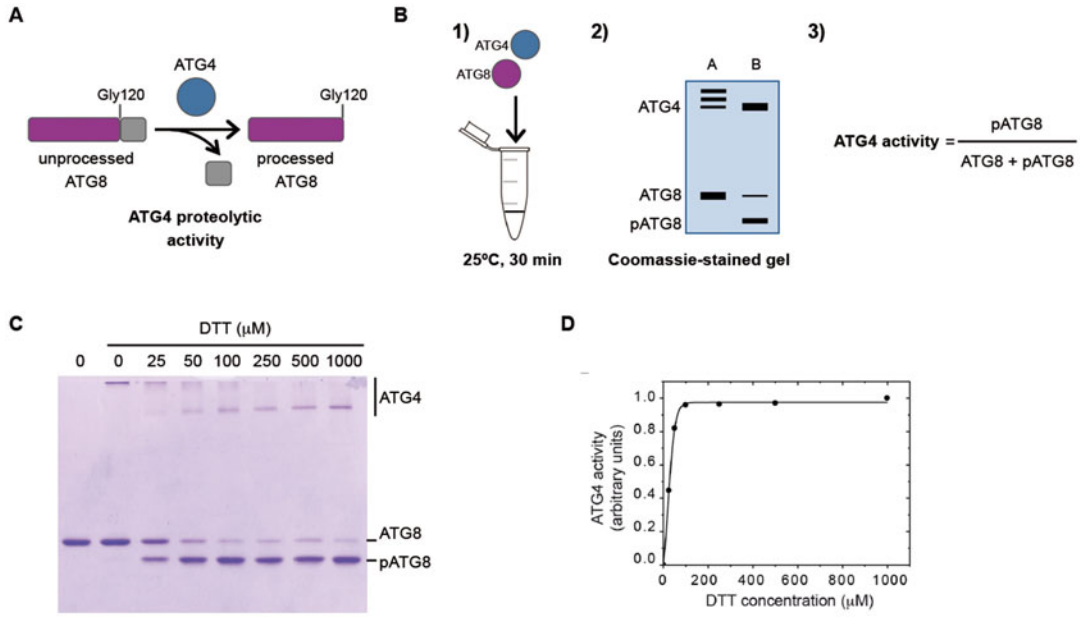

Fig. 2 In vitro assay to monitor ATG4 proteolytic activity. (**a**) ATG4 catalyzes the processing of ATG8 at the highly conserved Gly120, releasing a 14-amino acid sequence (gray square) from the C-terminus. (**b**) Steps of the in vitro assay: (1) Reaction mixture including ATG8 and ATG4 proteins. (2) 15% non-reducing SDS-PAGE to resolve unprocessed (ATG8) and processed (pATG8) forms of ATG8. Lane *A* represents inactive ATG4 unable to process ATG8 while lane *B* represents active ATG4 that cleaves ATG8 to produce pATG8. (3) ATG4 proteolytic activity can be determined as the ratio pATG8/(ATG8 + pATG8). (**c**) Effect of DTT on ATG4 proteolytic activity. Chlamydomonas $ATG4^{WT}$ was incubated with Chlamydomonas $ATG8^{WT}$ for 1 h in the presence of different DTT concentrations ranging from 25 μM to 1 mM. Lane 1 does not contain ATG4. Image adapted from [9]. (**d**) Quantification of ATG4 activity from (**c**). The sample with the highest DTT concentration (1 mM) was used as reference for quantification (activity = 1, in arbitrary units). Imaged adapted from [9]

undergoes redox regulation through the formation of a single disulfide bond under oxidizing conditions generated during stress [9, 10]. This regulatory disulfide bond can be efficiently reversed by the thioredoxin system according to the intracellular redox potential [9, 10]. Moreover, Chlamydomonas ATG4 (CrATG4) and Saccharomyces ATG4 (ScATG4) have an additional grade of redox regulation since higher ROS levels result not only in the oxidation but also in the oligomerization of both ATG4 proteins [9]. Similar to monomeric and oxidized ATG4, the oligomeric form is inactive and cannot process ATG8 [9].

Here, we describe a method to analyze ATG4 proteolytic activity in vitro using recombinant ATG4 protease from different organisms and recombinant Chlamydomonas ATG8 (CrATG8) as substrate (Fig. 2). The C-terminus of the CrATG8 protein contains 14 amino acids after the conserved Gly, which allows easy detection of processed and unprocessed forms (Fig. 2a) following ATG4 cleavage by a Coomassie-stained SDS-PAGE (Fig. 2b and c). The

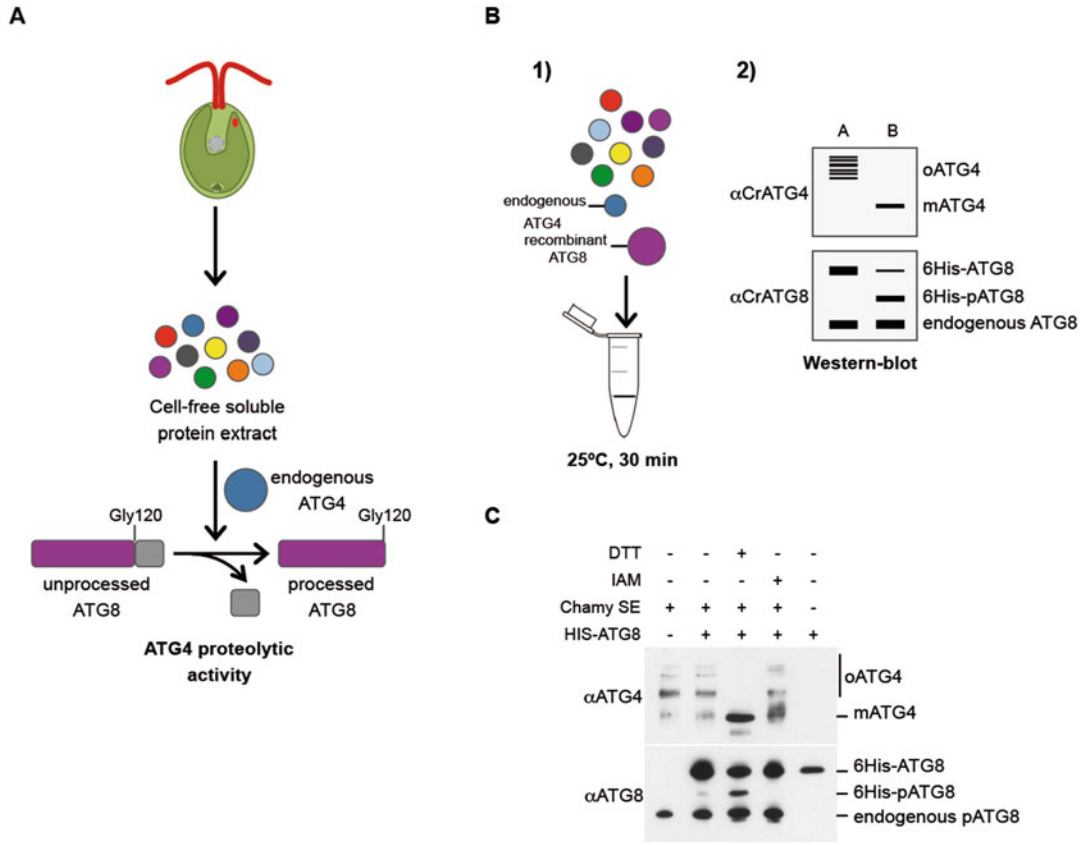

Fig. 3 ATG4 protease activity in cell-free extracts from Chlamydomonas. (**a**) Cell-free total extract from Chlamydomonas is incubated with unprocessed 6His-tagged recombinant ATG8 to analyze ATG8 cleavage by endogenous ATG4 activity. (**b**) Steps of the cell-free ATG4 assay: (1) Reaction mixture including recombinant 6His-tagged ATG8 and total protein extract from Chlamydomonas. (2) Immunoblot with anti-CrATG8 (bottom panel) and anti-CrATG4 (top panel) antibodies after 15% non-reducing SDS-PAGE. Lane *A* represents inactive ATG4 unable to process 6His-ATG8 while lane *B* represents active ATG4 that cleaves 6His-ATG8 to produce 6His-pATG8. (**c**) Illustration of cell-free ATG4 assay. Ten nanograms of recombinant 6His-tagged ATG8 protein (6His-ATG8) were incubated with 50 μg of cell-free soluble extracts (SE) of Chlamydomonas for 30 min at 25 °C. When indicated (+), cell-free soluble extracts were previously incubated with a reducing (25 mM DTT) or alkylating (20 mM IAM) agent for 30 min on ice. The reaction was stopped by the addition of β-mercaptoethanol-free loading buffer and 5 min boiling at 100 °C. Proteins were resolved by 15% SDS-PAGE and analyzed by western blotting with anti-CrATG4 (top panel) and anti-CrATG8 (bottom panel) antibodies. Full-length (6His-ATG8) and processed (6His-pATG8) recombinant forms of 6His-tagged ATG8 can be distinguished from endogenous ATG8 by their different electrophoretic mobility as previously described [8]. The oligomeric (oATG4) and monomeric (mATG4) ATG4 forms are also indicated. Imaged adapted from [9]

oligomeric/monomeric proportion of ATG4 can be also analyzed in these assays using non-reducing gels (Fig. 2b and c). ATG4 activity can be determined as the ratio between processed and total (unprocessed and processed) ATG8 forms (Fig. 2b and d). The effect of oxidizers and reducers on ATG4 activity can also be analyzed by incubating the protein with these compounds prior to

the proteolytic assay (Fig. 2c). This assay is versatile since a range of concentrations, time-course or different compounds can be easily tested [9, 10]. We also describe a modification of this assay using recombinant CrATG8 and total protein extracts from Chlamydomonas cells subjected to different stress conditions to examine endogenous ATG4 activity (Fig. 3). Purified CrATG8 contains a 6His-tag at its N-terminus and can be clearly resolved from endogenous CrATG8 by SDS-PAGE. The ratio between processed 6His-CrATG8 and total (unprocessed and processed) 6His-CrATG8 corresponds to proteolytic activity of endogenous CrATG4. The different CrATG8 forms can be detected by western-blot analysis with an anti-CrATG8 antibody [8] (Fig. 3b and c, bottom panel). In parallel, western-blot analysis with an anti-CrATG4 antibody [9] can be used to detect the different isoforms of endogenous CrATG4 (Fig. 3b and c, top panel). The cell-free assay described here is designed to test Chlamydomonas ATG4 activity but total protein extracts from other organisms such as yeasts and plants might be also analyzed with minor modifications of the protocol. The combination of both assays will provide insights about the regulation of a key protein essential for autophagosome biogenesis and autophagy progression.

2 Materials

Prepare all solutions and buffers using distilled water or ultrapure water quality (obtained by purifying deionized water to reach a sensitivity of 18.2 MΩ cm at 25 °C) as indicated. Prepare and store all reagents at the indicated temperature (unless indicated otherwise).

2.1 Growth Media and Components

Prepare and sterilize the stock solutions and media before using.

1. LB (Luria-Bertani) liquid growth media for *Escherichia coli*: dissolve 10 g triptone, 5 g yeast extract, and 10 g NaCl in 1 L distilled water in 1 L graduated cylinder. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle and store at room temperature.
2. LB (Luria-Bertani) solid growth media for *Escherichia coli*: dissolve 10 g triptone, 5 g yeast extract, and 10 g NaCl in 900 mL distilled water in 1 L graduated cylinder. Add 15 g agar to the previous mix. Adjust to 1 L with distilled water. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle. Allow the medium to cool until around 50 °C. Add sterilized antibiotic (*see* **Note 1**). Mix by gently swirling to homogenously distribute the melted agar and the antibiotic throughout the solution. Pour the solution on sterilized Petri

plates. Let the medium to cool and solidify. Store at 4 °C no longer than 2 weeks (*see* **Note 2**).

As indicated above, when required, kanamycin is added to the medium from a sterilized 1000× stock. To prepare this kanamycin stock (50 mg/mL, 1000×): weigh 500 mg of kanamycin sulfate powder and dissolve in 10 mL of ultrapure water. Mix until the solution is totally clear and filter with a 10 mL-syringe and a 0.2 μm filter under sterile conditions. Aliquot in 1.5 mL sterilized Eppendorf tubes and store at −20 °C.

3. Tris-acetate phosphate (TAP) medium for *Chlamydomonas reinhardtii*: prepare stock solutions of 100 × Tris-Acetate, 40 × Beij solution, 1 M potassium phosphate buffer, and Mineral traces solution. For 100 × Tris-Acetate dissolve 242 g Tris in 900 mL distilled water, then add 100 mL glacial acetic acid. Store at room temperature. For 40 × Beij solution: add about 400 mL of distilled water to a 500-mL graduated glass beaker. Weigh and mix in 2 g $CaCl_2 \times 2H_2O$. Add about 400 mL of distilled water to another 500-mL graduated glass beaker. Weigh and mix in 16 g NH_4Cl and 4 g $MgSO_4 \times 7H_2O$. Transfer everything to a 1 L graduated cylinder, mix and make up to 1 L with water. Sterilize and store at room temperature (*see* **Note 3**). For 1 M potassium phosphate buffer (pH 7.0) mix 250 mL 1 M K_2HPO_4 with 170 mL 1 M KH_2PO_4. Store at 4 °C. To prepare Mineral traces solution first prepare Solution 1 by dissolving the following minerals in the indicated order in 550 mL ultrapure water (11.4 g H_3BO_3, 22 g $ZnSO_4$ $7H_2O$, 5.06 g $MnCl_2 \times 4H_2O$, 4.99 g $FeSO_4 \times 7H_2O$, 1.61 g $CoCl_2 \times 6H_2O$, 1.57 g $CuSO_4 \times 5H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24} \times 4H_2O$), then heat at 100 °C to dissolve well. Prepare also solution 2: dissolve 50 g Na_2EDTA in 250 mL ultrapure water by heating, and add to the solution 1 at 100 °C. Heat the combined solutions to 100 °C, cool to 80–90 °C, and adjust to pH 6.5–6.8 with 20% KOH. The pH meter should first be calibrated at 75 °C; the temperature should remain above 70 °C. Adjust to 1 L, and allow a rust-colored precipitate to form, during 2 weeks at room temperature, in a 2 L Erlenmeyer flask loosely stoppered with cotton. The solution will change from green to purple. Then, the solution is filtered several times through three layers of Whatman paper, and a clear purple solution is obtained. Finally, the mineral traces are aliquoted in 50 mL tubes and stored at −80 °C. It is stable at 4 °C for 4–6 weeks. This protocol is based in the one described by [11].

To prepare the TAP medium add about 900 mL of distilled water to a 1 L graduated cylinder. Add 10 mL 100 × Tris-Acetate, 25 mL 40 × Beij solution, 1 mL 1 M potassium phosphate buffer,

and 1 mL Mineral Traces. Mix and check pH is 7.0. Make up to 1 L with distilled water. Sterilize and store at room temperature (*see* **Note 4**).

2.2 ATG4-Activity Assay Components

1. TBS buffer: 50 mM Tris base, 138 mM NaCl, and 27 mM KCl (pH 8.0): add about 800 mL of distilled water to a 1-L glass beaker. Weigh 6.1 g Tris base, molecular biology grade, and dissolve it in the water. Add 8.1 g NaCl and 2 g KCl, molecular biology grade, and dissolve them in the water. Mix and adjust pH with 37% HCl (v/v). Transfer to a 1-L graduated cylinder and adjust to 1 L with water. Sterilize and store at room temperature.
2. Lysis buffer: 50 mM Tris–HCl (pH 7.5): add about 800 mL of distilled water to a 1-L glass beaker. Weigh 6.1 g Tris base, molecular biology grade, and dissolve it in the water. Mix and adjust pH with 37% HCl (v/v). Transfer to a 1-L graduated cylinder and adjust to 1 L with water. Sterilize and store at room temperature.

2.3 SDS-PAGE Components

1. Resolving gel buffer: 1.5 M Tris–HCl (pH 8.8): add about 400 mL of distilled water to a 500 mL glass beaker. Weigh 90.9 g Tris base, molecular biology grade, and dissolve it in the water. Mix and adjust pH with 37% HCl (v/v). Transfer to a 500-mL graduated cylinder and adjust to 500 mL with water. Sterilize and store at 4 °C.
2. Stacking gel buffer: 0.5 M Tris–HCl (pH 6.8): add about 400 mL of distilled water to a 500-mL glass beaker. Weigh 30.3 g Tris base, molecular biology grade, and prepare a 500 mL solution as described above for the resolving buffer. Sterilize and store at 4 °C.
3. Acrylamide 40% (w/v) solution (Acrylamide:Bis-acrylamide, 29:1), electrophoresis grade (Fisher Scientific). Store at 4 °C.
4. N,N,N,N′-Tetramethyl-ethylenediamine (TEMED) (Sigma-Aldrich). Store at room temperature.
5. 10% Ammonium persulfate (APS) (w/v) solution in ultrapure water. Prepare 10 mL, aliquot in 1.5 mL tubes, and store at −20 °C.
6. SDS-PAGE running buffer: 0.025 M Tris–HCl, 0.192 M glycine and 0.1% (w/v) SDS. Prepare a 10× stock solution of running buffer (0.25 M Tris, 1.92 M glycine, 1% SDS). Add about 900 mL of distilled water to a 1-L graduated cylinder. Weigh and add 30.3 g Tris and 144 g glycine, mix and make up to 950 mL with water. Add 50 mL of 20% SDS (Sigma-Aldrich). SDS should be added last since it is a detergent and makes bubbles. Store at room temperature. Use this stock solution to prepare a 1× running buffer solution in distilled water when required.

7. Bio-Rad protein assay dye reagent for protein quantification as described by the manufacturer.
8. Loading buffer β-mercaptoethanol free: 0.125 M Tris–HCl (pH 6.8), 4% (w/v) SDS, 0.025% (w/v) bromophenol blue, 20% (v/v) glycerol (*see* **Note 5**). This is a 4× stock solution; prepare 5 mL, aliquot in 1.5 mL tubes, and store at −20 °C. Use it as a 1× solution with the protein sample.
9. Bromophenol blue solution: prepare a 0.1% (w/v) solution in ultrapure water.
10. Electrophoresis unit: SE260 Mighty Small II (GE Healthcare) (*see* **Note 6**) or Mini-Protean Tetra Cell (BioRad).
11. Container: we use square, plastic Petri dishes (120 mm × 120 mm) to stain, destain, and wash SDS-PAGE gels.
12. Coomassie staining solution: dissolve 2.5 g of brilliant blue powder in 100 mL acetic acid. Add 400 mL ethanol and mix gently. Adjust to 1 L with distilled water and mix gently until the solution is homogenous. Store in dark at room temperature (*see* **Note 7**).
13. Destaining solution: mix 40% ethanol, 10% acetic acid, and 50% distilled water. Store at room temperature.
14. ChemiDoc Imaging System from Bio-Rad or similar system to capture gel images. Image Lab software from Bio-Rad or similar software to quantify protein bands.

2.4 Immunoblot Components

1. Blotter system: TE 77 PWR Semi-Dry transfer unit (GE Healthcare). Follow the manufacturer's instructions.
2. Nitrocellulose membranes: hybridization nitrocellulose filter (0.45 μm HATF) by GE Healthcare.
3. Blotting paper: grade 3MM Chr cellulose chromatography papers by GE Healthcare.
4. Western blot transfer buffer: 0.025 M Tris–HCl, 0.192 M glycine, 20% (v/v) ethanol, and 3.75% (w/v) SDS. Prepare and store at 4 °C.

   Prepare a 10× stock solution of blotting buffer (0.025 M Tris, 0.192 M glycine, and 3.75% (w/v) SDS) without 20% (v/v) ethanol. Prepare and store at 4 °C. Add about 900 mL of distilled water to a 1-L graduated cylinder. Weigh and add 58.1 g Tris and 29.3 g glycine, mix until the powder is totally dissolved. Add 18.8 mL of 20% SDS (Sigma-Aldrich) and make up to 1 L with distilled water. SDS should be added last since it is a detergent and makes bubbles. Use this stock solution to prepare a 1× blotting buffer solution in distilled water and add 20% (v/v) ethanol just before using.

5. Phosphate buffered saline (PBS): 0.136 M NaCl, 0.027 M KCl, 0.010 M Na_2HPO_4, 0.009 M KH_2PO_4 at pH 7.4.

   Prepare a 10× PBS stock solution buffer (1.36 M NaCl, 0.27 M KCl, 0.10 M Na_2HPO_4, 0.09 M KH_2PO_4). Add about 900 mL of distilled water to a 1-L graduated cylinder. Weigh and add 80 g NaCl, 2 g KCl, 36.3 g Na_2HPO_4, 2.4 g KH_2PO_4. Mix and adjust pH with 10 M KOH. Sterilize and store at room temperature. Use this stock solution to prepare a 1× PBS solution in distilled water when required.
6. PBS containing 0.1% (v/v) Tween-20 (PBS-T): prepare 1 × PBS from 10 × PBS and add 0.1% (v/v) Tween-20 (Sigma-Aldrich) (*see* **Note 8**).
7. Red Ponçeau solution: dissolve 1 g red Ponçeau in 50 mL acetic acid and dilute to 1 L with distilled water. Mix gently and store in dark at room temperature.
8. Blocking solution: PBS-T with 5% (w/v) milk powder (*see* **Note 9**).
9. Container: we use square, plastic Petri dishes (120 mm × 120 mm) to incubate and wash nitrocellulose membranes.
10. Anti-CrATG8 polyclonal antibody: the antibody was produced as described in Pérez-Pérez et al. [8]. Dilute to a final concentration of 1:3000 in blocking solution (*see* **Note 10**).
11. Anti-CrATG4 polyclonal antibody: the antibody was produced as described in Pérez-Pérez et al. [9]. Dilute to a final concentration of 1:5000 in blocking solution. Both antibodies, anti-CrATG8 and anti-CrATG4, were produced in rabbit.
12. Secondary antibody: horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (Sigma-Aldrich) was used to a final dilution of 1:10,000 in blocking solution.
13. ChemiDoc Imaging System from Bio-Rad or similar system to capture western-blot chemiluminiscence. Image Lab software from Bio-Rad or similar software to quantify protein bands.

3 Methods

3.1 Expression and Purification of ATG8 and ATG4 from Chlamydomonas in E. coli

1. Clone the cDNA coding sequence of the *ATG8* and *ATG4* genes from Chlamydomonas (or other organism) into pET28a (+) or a similar plasmid to express the corresponding protein in *E. coli* (*see* **Note 11**).
2. The different ATG8 or ATG4 site-directed mutants were synthetized by GeneCust Europe and cloned into pET28a (+) for expression in *E. coli* (*see* **Note 12**).

3. The nucleotide sequences of all clones were verified and used to transform *E. coli* BL21 (DE3) cells.
4. Recombinant proteins were expressed by inducing *E. coli* cells at exponential phase (OD_{600nm} 0.4–0.6) with 0.5 mM isopropyl-b-D-thiogalactopyranoside (Sigma-Aldrich) for 2.5 h at 37 °C.
5. Purification of ATG8 wild-type and mutant versions: recombinant $ATG8^{WT}$, $ATG8^{G120A}$, and $ATG8^{G120*}$ proteins were purified by affinity chromatography on a His-Select IMAC Nickel-charged resin (Bio-Rad) following the manufacturer's instructions. Briefly, proteins were bound on the column; then, the unbound proteins were removed by washing the column consecutively with 5, 10, and 50 mM imidazol-containing buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl); finally, ATG8 was eluted with 150 mM imidazol-containing Tris–HCl buffer. Imidazole was removed by using Amicon Ultra-4, membrane PLGC Ultracel-PL from Millipore according to the manufacturer (*see* **Note 13**).
6. Purification of ATG4 wild-type and mutant versions: recombinant $ATG4^{WT}$ and the Cys-to-Ser mutant ATG4 versions were purified by affinity chromatography on a His-Select IMAC Nickel-charged resin (Bio-Rad) following the manufacturer's instructions. ATG4 proteins were purified by affinity chromatography as described above for ATG8.

3.2 In Vitro Assay to Monitor ATG4 Proteolytic Activity Using Recombinant Proteins

To have an overview of the workflow see Fig. 2.

1. Determine the purity of ATG8 and ATG4 proteins by SDS-PAGE gels.
2. Quantify the protein amounts using the Bio-Rad protein assay dye reagent as described by the manufacturer.
3. In vitro assay: the typical reaction mixture contains 1 μM ATG4, 5 μM ATG8, 1 mM DTT, and 1 mM EDTA in TBS buffer (*see* **Note 14**). When required, ATG4 can be pre-incubated with different compounds to test their effect on its activity (*see* **Note 15**). The typical incubation time is around 30 min, but it could be shorter or longer based on specific studies (*see* **Note 16**). Some examples of the application of this assay can be found in [8–10].
4. The reaction mixture is incubated at 25 °C for the indicated time.
5. The reaction is stopped by addition of β-mercaptoethanol-free Laemmli sample buffer (see SDS-PAGE components in the Materials Section) followed by 5 min of boiling.
6. Resolve the proteins on 15% SDS-PAGE gels (as described in Subheading 3.5).

7. Incubate the SDS-PAGE gels with the Coomassie staining solution (prepared as described in SDS-PAGE components in the Materials Section) with gently shacking for 30 min at room temperature.
8. After removing the Coomassie staining solution and washing once with distilled water, incubate the SDS-PAGE gels with the destaining solution for 30 min. Repeat this step until the protein bands are visualized. The SDS-PAGE gels can be kept in distilled water (*see* **Note 17**).
9. Scan the gels in a ChemiDoc Imaging System (Bio-Rad).
10. For ATG4 quantification, quantify the signals corresponding to unprocessed (ATG8) and processed (pATG8) with the software Image Lab (Bio-Rad). The ATG4 activity is considered as the ratio between processed ATG8/total ATG8 (pATG8/(ATG8 + pATG8)). Several examples of quantification can be found in [9, 10].

3.3 Preparation of Soluble Cell Extracts from Chlamydomonas

1. Grow Chlamydomonas cells under continuous illumination (20–30 μE/m^2s) at 100 rpm and 25 °C in TAP liquid medium. Typically, a 250-mL flask containing 50 mL of TAP medium is inoculated with Chlamydomonas cells to a final density of 2 10^5 cells/mL. Allow cells to grow to a cell density of 1–2 10^6 cells/mL; usually this takes about 24 h (*see* **Note 18**). The cell density is measured using a Counted II FL Automated Cell from Invitrogen.
2. Collect Chlamydomonas cells by centrifugation (4000 × *g*, 5 min), wash once in lysis buffer, and resuspend in a minimal volume (around 200 μL) of the same buffer (*see* **Note 19**).
3. Lyse cells by two cycles of freezing at −80 °C followed by thawing at room temperature. Separate the soluble cell extract from the insoluble fraction by centrifugation (15,000 × *g*, 20 min) in a microcentrifuge at 4 °C (*see* **Note 20**).

3.4 Cell-Free Assay to Monitor Endogenous ATG4 Activity from Soluble Cell Extracts

To have an overview of the workflow see Fig. 3.

1. Prepare soluble cell extracts from Chlamydomonas as described above (Subheading 3.3).
2. Quantify the proteins from total extracts using the Bio-Rad protein assay dye reagent as described by the manufacturer.
3. For incubation of Chlamydomonas soluble cell extracts with recombinant His-tagged CrATG8 mix 50 μg of soluble extract from Chlamydomonas and 10 ng of recombinant His-tagged CrATG8 in TBS buffer. Incubate for 30 min at 25 °C (*see* **Note 16**). When required, the soluble extract can be pre-incubated with different compounds, including reducing, oxidants or alkylating agents among others.

4. Stop the reaction by the addition of β-mercaptoethanol-free Laemmli sample buffer followed by 5 min of boiling.
5. Resolve the proteins on a 15% SDS-PAGE gel as described in Subheading 3.5.
6. Perform Western-blot analysis with anti-ATG8 or anti-ATG4 antibodies as described below in Subheading 3.6.

3.5 Separation and Analysis of Proteins by Electrophoresis Under Non-reducing Conditions

1. Sample preparation: typically, 15 μL from the in vitro or the cell-free assays are used. These samples contain the β-mercaptoethanol-free loading buffer and are boiled to stop the reaction in the assay.
2. Assemble the glass plates of the SE260 Mighty Small system following the manufacturer's instructions (GE Healthcare).
3. Prepare 15% acrylamide resolving gel. Leave sufficient space for the stacking gel and carefully overlay the acrylamide solution with isopropanol.
4. After polymerization is complete, pour the 5% acrylamide stacking gel onto the surface of the polymerized resolving gel. Immediately insert a Teflon comb, being careful to avoid trapping air bubbles.
5. Load protein samples and pre-stained protein standards on the gel.
6. Apply a voltage of 150 V to the gel and let the dye front run out of the gel. Stop electrophoresis when the 6 kDa marker reaches the bottom of the gel (*see* **Note 21**).

3.6 Western-Blot and CrATG8 Detection

1. After non-reducing SDS-PAGE, prepare the transfer unit with three pieces of 10 × 10 cm blotting paper and a nitrocellulose membrane of the same size, previously humidified with transfer buffer.
2. Gently lay the gel on the nitrocellulose membrane and place three pieces of humidified blotting paper on the gel.
3. Electrotransfer proteins from the gel to the membrane by applying a maximum current of 1 mA/cm^2 of the gel surface during 1 h 15 min.
4. After the transfer, submerge the membrane in blocking solution for at least 1 h.
5. Cut the membrane horizontally at the 36 kDa marker. Use the bottom part of the membrane to incubate it with the anti-CrATG8 antibody.
6. Add anti-CrATG8 antibody to the blocking solution to a final dilution of 1:3000 and incubate overnight at 4 °C.
7. Wash the membrane four times with PBS-T, 5 min each.

8. Incubate the membrane with the secondary antibody (1: 10,000 final dilution) in blocking solution at room temperature for at least 45 min.
9. Wash the membrane four times with PBS-T, 5 min each.
10. Develop the signal using the Luminata Crescendo Western HRP Substrate (Millipore) according to manufacturer's instructions.
11. Remove excess reagent from the membrane by keeping the membrane in a vertical position for a few seconds.
12. Scan the membranes using ChemiDoc system from Bio-Rad according to manufacturer instructions.

3.7 Western-Blot and CrATG4 Detection

1. Follow the same protocol described above for anti-CrATG8 western-blot (see Subheading 3.6).
2. Use the top part of the nitrocellulose membrane (see Subheading 3.6, **step 5**) to incubate with the anti-CrATG4 antibody.
3. Add the anti-CrATG4 antibody to the blocking solution to a final dilution of 1:5000 and incubate overnight at 4 °C.
4. Follow the same protocol after primary antibody than the one described above for anti-CrATG8 western-blot (see Subheading 3.6).

4 Notes

1. Chlamydomonas ATG4 and ATG8 proteins can be overexpressed and purified in *E. coli* after transformation of BL21 (DE3) cells with p28ATG4 [9] and p28ATG8 [8] plasmids, respectively. Both plasmids are based on pET28a (+), which confers kanamycin resistance. LB medium for expression contains 50 μg/mL kanamycin for clone selection, that is obtained by adding 1 mL of kanamycin stock solution (50 mg/mL) to 1 L of LB solid medium before plating.
2. To keep the antibiotic selection, fresh antibiotic containing LB plates should be used, or at least not older than 2 weeks.
3. To prepare Beij solution, dilute $CaCl_2$ and $MgSO_4$-NH_4Cl separately.
4. Add 1.2% of agar to prepare TAP plates. When required, antibiotics, vitamins, amino acids, or drugs can be added before plating.
5. It is important to notice that this is a non-reducing loading buffer since it does not contain the reducing agent β-mercaptoethanol.

6. Both electrophoresis systems can be used but it is important to highlight that the SE260 system from GE Healthcare allows running of 10 × 10.5 cm gels, which compared to standard 10 × 8 cm mini-gels from BioRad gives more than 25% higher resolution, especially for low molecular weight proteins, including CrATG8 recombinant or endogenous proteins.
7. The Coomassie staining solution can be reused several times.
8. Due to the high density of Tween 20 it is better to prepare a 50% (v/v) stock solution in distilled water and use it to prepare PBS-T. The 50% stock solution is stored in dark at room temperature.
9. Mix thoroughly before using. Store at 4 °C, no longer than one day.
10. After using it, the blocking solution containing the CrATG8 antibody can be stored at −20 °C and reused several times.
11. In the case of Chlamydomonas ATG8, the corresponding gene was cloned into pET28a (+) plasmid (from Novagen) at NdeI and XhoI sites for expression of the corresponding 6His-tagged ATG8 protein. A similar procedure was performed for Chlamydomonas ATG4.
12. The different ATG8 ($ATG8^{G120A}$ and $ATG8^{G120*}$) and ATG4 ($ATG4^{C84S}$, $ATG4^{C400S}$, and $ATG4^{C473S}$) mutants were cloned into pET28a (+) plasmid (from Novagen) at NdeI and XhoI sites for expression of the corresponding 6His-tagged protein.
13. The cutoff of Amicon Ultra-4 should be carefully chosen based on the molecular weight of the protein. For instance, a cutoff of 3 kDa was used for all ATG8 variants and 30 kDa for all ATG4 variants.
14. To analyze the role of an amino acid or a domain on ATG4 activity, the mutant version of ATG4 lacking the specific amino acid or domain can be used in the reaction mixture instead of $ATG4^{WT}$.
15. To investigate the effect of a compound on ATG4 activity, the ATG4 protein can be pre-incubated with different concentrations of this compound before addition to the reaction mixture.
16. The incubation time of the reaction mixture can be modified according to the objectives. For instance, a time-course assay is useful to analyze activation or inhibition kinetic parameters.
17. When it is required to keep the SDS-PAGE gels longer, they can be stored in a 5% acetic acid solution.

18. When an autophagy-activating treatment is required, the treatment or stress have to be applied in cells growing exponentially since the stationary growth phase (4–6 10^6 cells/mL) activates autophagy [8].
19. Typically, 50 mL cells of a density of 2–4 10^6 cells/mL are resuspended in 300–400 μL of lysis buffer. Optionally, a cocktail of protease inhibitors can be added to the lysis buffer.
20. Total soluble extracts obtained by this method usually are colorless or display a pale yellow color.
21. We use SeeBlue Pre-Stained standard (Invitrogen) that contains low molecular weight markers of 14, 6, and 3 kDa. To get a better resolution of the different Chlamydomonas ATG8 forms, we let the pre-stained 6 and 3 kDa markers to run out of the gel.

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Chapter 18

Oxidative Stress in Roots: Detection of Lipid Peroxidation and Total Antioxidative Capacity

Katarina Šoln and Jasna Dolenc Koce

Abstract

Various abiotic and biotic agents disturb the fine balance between cellular oxidants and antioxidants. The resulting oxidative stress occurs either due to the increasing levels of reactive oxygen species (ROS) or weak antioxidative system that cannot scavenge ROS burst. In addition to their harmful role, ROS can also act as signaling molecules, and oxidative stress is often the initial step in the programmed cell death. Here we describe two parameters of oxidative stress that can be measured spectrophotometrically: lipid peroxidation via the content of the by-product malondialdehyde, and the amount of all non-enzymatic antioxidants named as total antioxidative capacity. Both methods are presented using young radish (*Raphanus sativus*) seedlings after treatment with extract from the invasive plant species Japanese knotweed (*Fallopia japonica*).

Key words Lipid peroxidation, Malondialdehyde, Non-enzymatic antioxidants, Oxidative stress, Reactive oxygen species, Root, Spectrophotometer, Total antioxidative capacity

1 Introduction

Abiotic [1–3] and biotic [4, 5] environmental stress can lead to a strong burst of reactive oxygen species (ROS) in plant cells, which is known as oxidative stress. ROS are highly reactive molecules that oxidize other macromolecules in cells and thus contribute to their degradation [6, 7]. The cellular defense mechanism are antioxidants, which quickly scavenge ROS. However, an imbalance between ROS and antioxidants leads to an excessive production and accumulation of ROS [2], which then stimulate the activity of several groups of plant proteases, such as cysteine proteases, vacuolar processing enzymes, and metacaspase, promote a hypersensitive response, and ultimately lead to programmed cell death [8]. Here we demonstrate a simple and quick method for spectrophotometric determination of two oxidative stress parameters in plants: lipid peroxidation and total antioxidative capacity.

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Methods in Molecular Biology, vol. 2447, https://doi.org/10.1007/978-1-0716-2079-3_18,

Lipid peroxidation is one of the methods frequently used to estimate oxidative stress, as ROS cause the degradation of membrane lipids by peroxidation. Polyunsaturated fatty acids are particularly susceptible to reactions with ROS because of the possible abstraction of an electron from the double bond [9]. With this method it is possible to determine the level of lipid peroxidation indirectly by measuring the content of malondialdehyde (MDA). MDA is a by-product of lipid peroxidation which reacts with thiobarbituric acid in a colorimetric reaction [10]. It has been suggested that MDA content can be used as a biomarker for oxidative damage caused by environmental stress, abiotic stress such as metals [1] and drought [3], and biotic stress [5]. The method is relatively simple and fast and for these reasons it is widely used, which also leads to some artifacts in the methodology and interpretation of the results [11]. The original method, as described by Heath and Packer [12], was improved by Hodges et al. [13] to avoid overestimating the MDA in plant samples with high carbohydrate and anthocyanin content. Here we present the method that is suitable for determining the MDA content in young roots of seedlings a few days after germination as well as for plants grown for more than 3 weeks [4]. The preparation of the samples also allows other biochemical analyses such as enzyme activity.

The second method for evaluating oxidative stress in plants is total antioxidative capacity (TAC). The TAC allows us to estimate the concentration of non-enzymatic antioxidants in plants [4, 14], including ascorbic acid (vitamin C), α-tocopherol (vitamin E), reduced glutathione, carotenoids, flavonoids, and proline [6, 7]. Several methods for determining TAC have been reported, i.e., the Ferric reducing/Antioxidative power (FRAP) assay [15], the horseradish peroxidase-based TAC assay [16], and the reaction with 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical [4, 14, 17]. Here we present a simple method for measuring the free radical scavenging of the antioxidants with the DPPH radical. Due to its free electron form, DPPH becomes a stable radical and absorbs light at a wavelength of 515 nm when in the active state [14, 17]. Antioxidants can be reduced by releasing an H^+ atom to the DPPH molecule. The newly formed DPPH radical has different absorption properties, and the reaction can be measured spectrophotometrically as the color changes from purple to yellow. With the reduction of further DPPH radicals, the absorbance decreases until a stationary state is reached [14]. The lower the absorption of DPPH and the faster this process occurs, the higher the concentration of non-enzymatic antioxidants [17]. The TAC concentration is calculated according to the standard curve with Trolox, the synthetic analog of vitamin E [4, 17].

Both methods, lipid peroxidation and TAC, can easily be adapted for small amounts of plant material (i.e., seedling root) and can be used to detect oxidative stress in young or mature

plants. We describe the method using young radish (*Raphanus sativus*) seedlings after treatment with extract from the invasive plant species Japanese knotweed (*Fallopia japonica*). Some studies reported simultaneously increased TAC and decreased lipid peroxidation [4, 14], because higher level of non-enzymatic antioxidants represents a removal mechanism for ROS [2] and therefore less cell damage, i.e., lipid peroxidation, occurs.

2 Materials

2.1 Treatment of Radish Seeds with Knotweed Extract

1. Seeds of radish (*Raphanus sativus* L.) stored at 4 °C (*see* **Note 1**).
2. Fresh rhizomes of Japanese knotweed (*Fallopia japonica* (Hout.) Ronse Decr.).
3. Knife.
4. Lyophilizer.
5. Cutting mill (cut to 1 mm pieces or smaller).
6. Vacuum filtration unit.
7. Orbital shaker.
8. Filter paper.
9. Round plastic Petri dish with lid (14 cm diameter, one for each treatment replicate).
10. Scalpel.
11. Analytical scale.
12. Liquid nitrogen in a Dewar.
13. Aluminum foil.

2.2 Root Tissue Homogenization

Prepare all solutions using distilled water. Prepare and store all reagents at room temperature (RT) unless otherwise specified.

1. Mortar and pestle.
2. 100 mM Potassium phosphate buffer: prepare separately 100 mL of 100 mM dipotassium hydrogen phosphate (K_2HPO_4) and 100 mL of 100 mM potassium hydrogen phosphate (KH_2PO_4). Mix 61.5 mL 100 mM K_2HPO_4 and 38.5 mL 100 mM KH_2PO_4 to prepare 100 mL of 100 mM potassium phosphate buffer (*see* **Note 2**). Check the pH value to 7.0 with a pH meter. Store at 4 °C.
3. 100% Methanol.
4. Eppendorf tubes (2 mL).
5. Tabletop centrifuge.

2.3 MDA Reaction

1. Glass test tubes (6 mL).
2. TBA/TCA acetic reagent: 0.5% (w/v) thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) (*see* **Note 3**). Add 10 g TCA to 50 mL distilled water. Dissolve by stirring on a magnetic stirrer, approximately 20 min. Add 0.25 g TBA. Dissolve by stirring on magnetic stirrer, approximately 20 min. Acetic reagent can be prepared in advance and stored at 4 °C before the analysis.
3. Aluminum foil.
4. Drying oven.
5. Ice.

2.4 Spectrophotometric Measurement of MDA

1. Polystyrene (PS) cuvette for visible light (Vis).
2. Distilled water.
3. Spectrophotometer.

2.5 TAC Reaction

1. 12 mM DPPH solution: dilute 47.3 mg DPPH (2,2-diphenyl-1-picryl-hydrazyl) in 10 mL methanol (*see* **Note 4**). The color of the DPPH solution is purple (*see* **Note 5**). Store in 15 mL plastic Falcon tube wrapped in aluminum foil at RT and use within a week (*see* **Note 6**). For the TAC analysis, dilute the DPPH stock solution with methanol at a ratio of 1:100 (v/v) to prepare 120 μM DPPH solution. Store at RT in a bottle wrapped in aluminum foil. Prepare fresh (*see* **Note 7**).
2. Potassium phosphate buffer (*see* Subheading 2.2).
3. Eppendorf tubes (2 mL).

2.6 Spectrophotometric Measurement of TAC

1. Glass cuvette.
2. 100% Methanol.
3. Spectrophotometer.
4. 10 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in methanol (*see* **Note 4**): in 2 mL Eppendorf tubes prepare 2 mM, 1.5 mM, 0.8 mM, 0.6 mM, 0.4 mM, 0.2 mM Trolox by diluting 10 mM Trolox with methanol. Mix on vortex. Use methanol for 0.0 mM Trolox. Prepare at least three replicates for each concentration. Perform TAC reaction (*see* Subheading 2.5) by adding 30 μL Trolox of each standard in Eppendorf tube and add 2 mL 120 μM DPPH. Incubate in the dark for 15 min at RT. Measure the absorbance of each standard at 515 nm against 100% methanol. Use a glass cuvette.
5. Create a standard curve with spectrophotometer software by plotting absorbance at 515 nm on the y-axis and the Trolox concentration on the x-axis and define the equation of the linear function (*see* **Note 8**).

3 Methods

Treatment of radish seeds with knotweed extracts, root homogenization, measurement of MDA content and TAC are described in the following sections and presented as graphical protocols in Figs. 1 and 2, respectively.

3.1 Treatment of Radish Seeds with Knotweed Extract

1. Collect rhizomes of Japanese knotweed (*Fallopia japonica*), wash them to remove soil, cut with a knife to app. 1 cm wide pieces, lyophilize (0.003 mbar, −98 °C, 5 days), and grind to 1 mm particles with cutting mill.
2. Add 10 g of ground rhizome material to 100 mL distilled water, incubate for 24 h at room temperature (RT) on an orbital shaker (175 rpm). Vacuum filter the suspension through filter paper. Store the filtered extract at 4 °C and use within 3 days.
3. Place radish (*Raphanus sativus*) seeds on the filter paper in plastic Petri dish. Water with 10 mL of extract and allow the seeds to grow for 3 days at RT. For control treatment use distilled water only. Petri dish must be covered with a lid to prevent evaporation.
4. Separate the root from the shoot with a scalpel. Weigh 100 mg roots per sample, wrap in aluminum foil, and freeze the package in liquid nitrogen.
5. Store at −20 °C.

3.2 Root Tissue Homogenization for Determination of the MDA Content

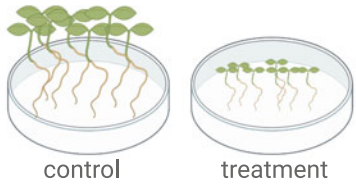
1. Homogenize 100 mg of root tissue in 1.5 mL 100 mM potassium phosphate buffer in a mortar with pestle (*see* **Note 9**).
2. Transfer the homogenate into Eppendorf tube.
3. Centrifuge (22,000 × *g*) for 20 min at 4 °C.
4. Transfer the supernatant to a new Eppendorf tube and discard the pellet (*see* **Note 10**).

3.3 Root Tissue Homogenization for Determination of TAC

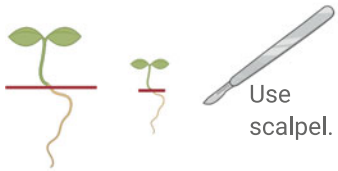
1. Homogenize 100 mg of root tissue (*see* **Note 11**) in 1 mL methanol in a mortar with pestle (*see* **Note 4**).
2. Add another 1 mL methanol and continue with homogenization.
3. Transfer the homogenate into Eppendorf tube.
4. Centrifuge (22,000 × *g*) for 5 min at 4 °C.
5. Transfer the supernatant to a new Eppendorf tube and discard the pellet (*see* **Note 10**).

Determination of lipid peroxidation

1. Germination test with treatment.



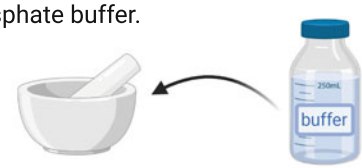
2. Separate root from shoot.



3. Weight 100 mg of fresh root tissue.

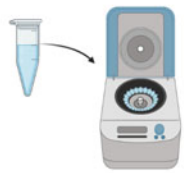
4. Freeze in liquid nitrogen and store at -20 °C before use.

5. Homogenize in 1.5 ml 100 mM potassium phosphate buffer.

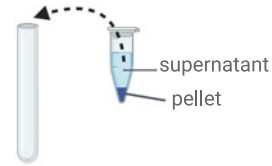


6. Transfer homogenate in the Eppendorf tube.

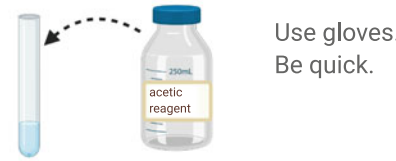
7. Centrifuge for 20 min (14000 rpm) at 4 °C.



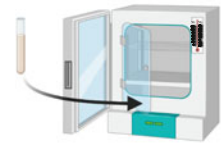
8. Add 200 µl supernatant into glass test tube.



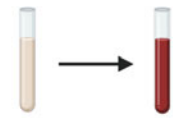
9. Add 800 µl acetic reagent.



10. Incubate for 30 min at 95 °C.

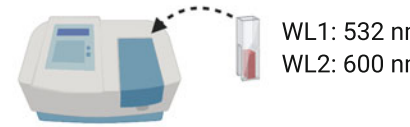


Note the colour change:



11. Cool in ice-cold water.

12. Measure the absorbance with spectrophotometer.



13. Calculate the MDA content.

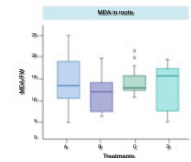


Fig. 1 Schematic presentation of the protocol for the content of malondialdehyde (MDA) in roots

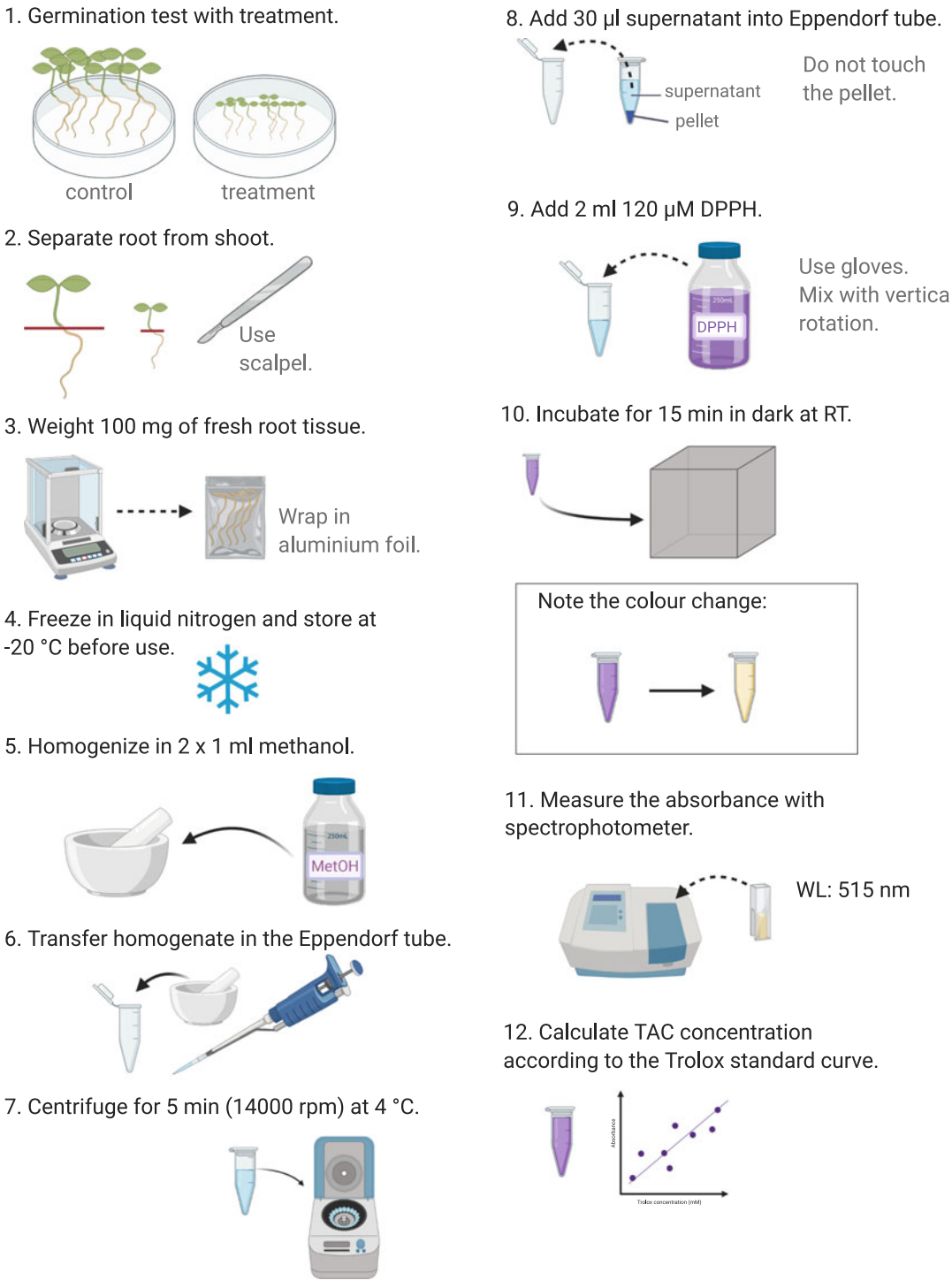


Fig. 2 Schematic presentation of the protocol for total antioxidative capacity (TAC) in roots

3.4 MDA Reaction

1. Add 200 μL supernatant into glass test tube. For a blank sample, use 100 mM potassium phosphate buffer instead of the supernatant.
2. Add 800 μL acetic reagent (*see* **Note 12**).
3. Cover the test tube with aluminum foil to avoid evaporation during incubation (*see* **Note 13**).
4. Incubate for 30 min at 95 °C (*see* **Note 14**). The color should change from light yellow to dark brownish red (*see* **Note 15**).
5. Cool in ice-cold water to stop the reaction (*see* **Note 16**).

3.5 Spectrophotometric Measurement of MDA

1. Transfer the MDA reaction mixture from the test tube into a PS cuvette. Use distilled water for the reference cuvette (*see* **Note 17**).
2. Spectrophotometrically measure the absorbance at the wavelengths 532 and 600 nm.

3.6 Calculation of MDA Content

Calculate the content according to the equation:

$$\mathrm{MDA/FW} = (\mathrm{A}_{532} - \mathrm{A}_{600})/(\mathcal{E} \times 1000 \times m)$$

Legend:

MDA/FW: MDA content per sample's fresh biomass [nMg^{-1}].

$\mathrm{A}_{532} - \mathrm{A}_{600}$: the difference between the sample's absorbance at 532 nm and absorbance at 600 nm.

$\mathcal{E}$: MDA extinction coefficient at the wavelength 532 nm, $\mathcal{E} = 155\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$.

m: fresh biomass of the sample [g].

3.7 TAC Reaction

1. Add 30 μL supernatant from the root tissue homogenization (*see* Subheading 3.3) to a 2 mL Eppendorf tube. For a blank sample, use 100 mM potassium phosphate buffer (*see* Subheading 2.2) instead of the supernatant.
2. Add 2 mL of 120 μM DPPH (*see* **Note 18**).
3. Mix gently with vertical rotation.
4. Incubate in the dark for 15 min at RT (*see* **Note 19**). The color should change from purple to light yellow (*see* **Note 20**).

3.8 Spectrophotometric Measurement of TAC

1. Transfer the TAC reaction mixture into a glass cuvette. Use methanol for the reference cuvette. Use methanol also to wash the cuvette between measurements of consecutive samples.
2. Spectrophotometrically measure the absorbances of samples at the wavelength 515 nm.
3. Use the Trolox standard curve to calculate the TAC concentration in mM (*see* Subheading 2.6).

4 Notes

1. The protocol can also be used for other plants. We used corn (*Zea mays*) seeds.
2. For the preparation of other volumes of 100 mM potassium phosphate buffer, the volumes of 100 mM K_2HPO_4 and 100 mM KH_2PO_4 must be adjusted.
3. Use gloves as TBA and TCA are toxic, corrosive, and irritating to the skin. Waste solutions containing TBA and TCA must be collected separately and sent to an appropriate disposal facility.
4. Methanol is toxic (use gloves) and volatile (work in fume hood if possible). Waste solutions containing methanol must be collected separately and sent to an appropriate disposal facility.
5. Care should be taken when preparing DPPH, as it is purple colored. Use gloves.
6. DPPH is sensitive to light and must be stored in the dark.
7. Always prepare fresh solution to avoid DPPH degradation.
8. The Trolox standard curve can be created once and then saved in the spectrophotometer software for subsequent analyses. In our setup, the standard curve equation was $y = -0.3\ x + 1.0$.
9. Tissue homogenization in potassium phosphate buffer allows the same supernatant to be used for several biochemical analyses, which is important when small biomass of plant material is available (i.e., 100 mg). We stored the excess supernatant at −20 °C and within a few days used it for other spectrophotometric analyses, such as protein concentration and enzyme activity of catalase and guaiacol peroxidase.
10. Should you accidentally touch the pellet, repeat the centrifugation to ensure a clear supernatant.
11. This method can be adapted according to the biomass of the sample. For example: we also used 50 mg root and homogenized twice in 500 μL methanol. However, if possible, 100 mg biomass is optimal for the TAC determination.
12. First transfer the supernatants to each test tube and add acetic reagent afterward. This allows better control of the start of the reaction.
13. Covering the test tubes with aluminum foil prevents evaporation during incubation without increasing the pressure in the test tube. When the test tubes are tightly closed, the pressure increases and opens the cap.
14. For the incubation in the oven dryer always use the iron stand, as the plastic stand melts at 95 °C.

15. The blank sample with potassium phosphate buffer should remain light yellow after the incubation.
16. Ensure that no water or ice gets into the test tubes.
17. Gently tap the cuvette to remove air bubbles.
18. The reaction starts when supernatant and DPPH are mixed; therefore it is important to first transfer the supernatants into each Eppendorf tube and add DPPH afterward. This allows better control of the start of the reaction.
19. Incubation in the dark can be performed in a closed box. Caution: incubation should last at least 15 min, but no longer than 30 min.
20. The blank sample with potassium phosphate buffer should remain purple after incubation. However, if the color of other samples turns yellow before incubation, the analysis should be repeated from scratch as this indicates that all DPPH has been used and therefore quantification is no longer possible.

Acknowledgements

The authors are thankful to Dr. Željka Vidaković Cifrek and Dr. Mirta Tkalec from the University of Zagreb, and Dr. Edith Stabentheiner and Dr. Maria Müller from the University of Graz for their supervision and support of the MDA and TAC methods, respectively. This work was financially supported by the Slovenian Research Agency (grant no. P1-0212). The figures were created with BioRender.com.

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Chapter 19

Imaging of Potassium and Calcium Distribution in Plant Tissues and Cells to Monitor Stress Response and Programmed Cell Death

Katarina Vogel-Mikuš and Paula Pongrac

Abstract

In plants, the response to stress, such as salinity, pathogen attack, drought, high concentration of metals, hyperthermia, and hypothermia, is usually accompanied by potassium ion (K^+) leakage from the cytosol to the cell wall, mediated by plasma membrane cation conductivity. Stress-induced electrolyte leakage co-occurs with accumulation of reactive oxygen species (ROS) and calcium ions (Ca^{2+}) and often results in programmed cell death (PCD). The development of X-ray and mass spectrometry (MS) based imaging techniques has enabled insight into the spatial tissue and cell-specific redistribution of major and trace elements during the stress response. In this chapter a workflow for sample preparation, imaging, and image analysis by X-ray and MS based techniques is presented.

Key words Environmental stress, Imaging, X-ray fluorescence, PIXE, Synchrotron, Particle accelerator, Ions

1 Introduction

Environmental stresses have an adverse effect on crop productivity and present a major constraint to global food security. As a consequence of global climate change, some stress factors such as heat, drought, salinity, tropospheric ozone, and excess UV radiation are foreseen to become even more prevalent in the coming decades. Therefore, better understanding of the plant cell response to various stress factors is crucial to work toward combating the decline of the crop yields.

As sessile organisms, plants lack the ability of animals, to efficiently evade various environmental stressors by moving to more favorable places. Instead, plants have developed complex strategies to overcome and withstand environmental stressors. At moderate stress levels acclimatization responses are usually triggered, while at high or prolonged low doses the stress can induce localized cell

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Methods in Molecular Biology, vol. 2447, https://doi.org/10.1007/978-1-0716-2079-3_19,

death. Stress-induced death occurs rapidly (in the range of seconds or minutes) via physical or chemical damage, or more slowly (in the range of hours or days) via a process known as programmed cell death (PCD) [1]. PCD is an active, genetically controlled process, initiated to isolate and remove damaged tissues, thereby ensuring the survival of the organism [2]. PCD is especially beneficial for survival of the plant in the case of the pathogen attack, but occurs also during the differentiation of certain tissues through growth and development (e.g., xylogenesis) [1]. Positive effects of PCD in the case of plant responses to abiotic stress may include aerenchyma formation [3] or death of the main root and formation of a new root system, more tolerant to stresses such as high concentration of salt or metals [2].

Based on cytological events and activation of biochemical markers accompanying cell death, van Doorn et al. (2011) [4] proposed division of PCD into "vacuolar" (also referred as "autophagic" or "autolytic") and "necrotic" ("non-autolytic") types. Vacuolar type of PCD is accompanied by increased activities of autophagic proteins, formation of autophagosomes, and their fusion with vacuoles, and terminates by a rupture of vacuole through stimulation of the vacuolar processing enzymes (VPE), specific cysteine proteases residing in the vacuole [1, 5]. When vacuolar PCD is induced during development (e.g., development of xylem vessels), cell compartments almost completely disappear before the plasma membrane collapses. In contrast, vacuolar PCD induced by biotic stresses is accompanied by less severe degradation of organelles and can lead to the plasma membrane permeabilization. The necrotic PCD may or may not involve rupture of the vacuole. It relies on induction of cell death proteases and endonucleases via receptor-activated and ionic mechanisms as well as on release of cytochrome c from mitochondria. In a long term, it leads to collapse of membrane potential, shrinkage of protoplast, and irreversible and nonspecific permeabilization of the plasma membrane [2].

Potassium ion leakage is an indicator of stress response in intact plant cells [6]. The redistribution of potassium can be used as a test for the stress-induced injury of plant tissues and a criterion to evaluate the level of stress tolerance. Potassium leakage as a response to stress occurs in all plant cell types and tissues and can be triggered by pathogen attack [7], high concentration of salt or metals [8, 9] (Fig. 1), high soil acidity, wounding, drought, or heat [2].

With development of X-ray fluorescence (XRF) and mass spectrometry (MS) based imaging techniques [10] it became possible to get insight into spatial element distribution at tissue, cell, and subcellular levels. First studies were performed in metal hyperaccumulating and metal tolerant plants [11–14], identifying sites of metal localization and deciphering the metal tolerance mechanisms

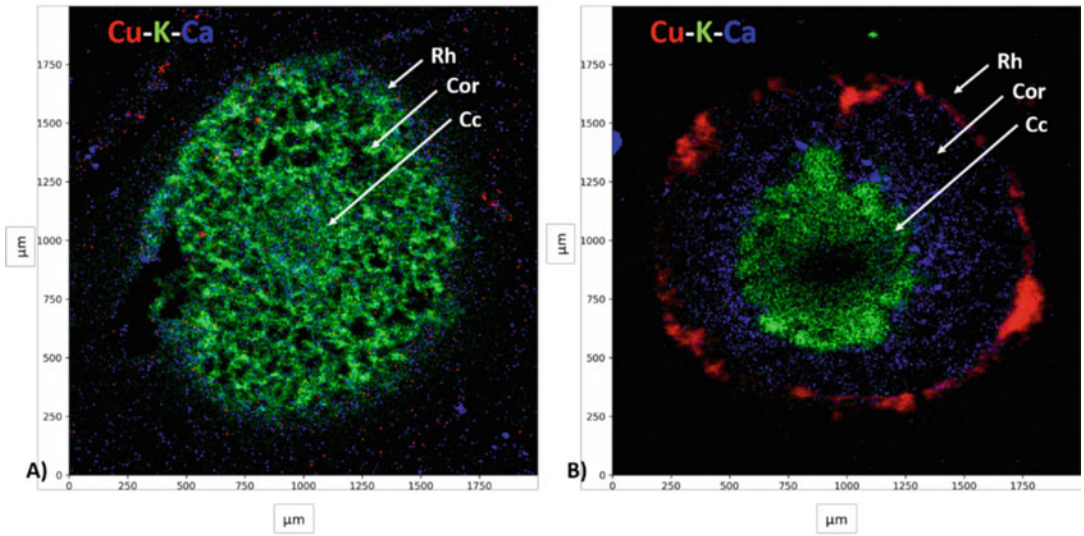

Fig. 1 Copper (Cu, red), potassium (K, green), and calcium (Ca, blue) localization in the control (**a**) and Cu-exposed sunflower root cross section (**b**). Samples were prepared by cryofixation and freeze-drying and analyzed by micro particle induced X-ray emission. Depletion of K is seen in the root cortex of Cu-exposed plant [9]. Rh-rhizodermis, C-cortex, CC-central cylinder

[15], and further in (pseudo)cereal grain to better resolve element storage pools in different tissues related to grain processing and different milling fractions [16–19].

2 Materials

2.1 Sample Preparation

1. Aluminum thermo-block, for rapid freezing of specimens (or Vitrobot).
2. Cryo-microtome.
3. Freeze-drier.
4. Scalpels and forceps.
5. Filter paper.
6. Stainless steel tubes with polished tips.
7. Aluminum foil for making tissue-freezing medium containers.
8. Aluminum pots with stainless steel fitting cover.
9. Tissue-freezing medium (tissue-tek from different providers, e.g., Leica).
10. Liquid nitrogen.
11. Gaseous propane.

2.2 Sample Mounting

1. Pioloform solution (75 g of pioloform dissolved in 100 mL of chloroform) (*see* **Note 1**).

2. Magnetic stirrer.
3. A 100 mL volumetric flask made from dark glass.
4. Microscope object-glass.
5. Aluminum sample holders.
6. Glue (two-component Araldite).

2.3 Measurements

1. Particle accelerator beamline for micro proton induced X-ray emission (micro-PIXE).
2. Synchrotron-based beamline for X-ray fluorescence (SR-micro XRF).
3. Laser ablation inductively plasma coupled mass spectrometer (LA-ICP-MS).

2.4 Data Analysis

1. For PIXE data: GEOPIXE software [20].
2. For SR-micro XRF data: PyMCA software [21].
3. For LA-ICPMS data: Image J (https://imagej.nih.gov/ij/download.html), PyMCA (RGB correlator).

3 Methods

3.1 Sample Preparation

Sample preparation is one of the most important steps in element localization studies. The ultimate goal is to maintain the element distribution and morphology of the tissue, cellular, and subcellular structures as close as possible to the in vivo stage [20–22]. Sample preparation by cryofixation involves plunge freezing of fresh plant material, followed by measurements in cryo-conditions or freeze-drying. Air drying or chemical fixation followed by resin embedding (including freeze-substitution) can cause elemental redistribution and should therefore be avoided in physiological investigations related to concentration and distribution of elements [21]. Sample preparation of root, stem, and leaf tissues by cryofixation can be performed following either of three protocols:

Protocol #1 (Fig. 2)

1. Excise pieces/strips of plant organ with a sharp scalpel or a razor blade (*see* **Note 2**).
2. Leaf strips are folded slightly by hand along the midrib, taking care to avoid snapping the sample, and placed into 2 mm stainless steel tube with a polished tip, with the strip partially protruding. Pieces of fine roots, with diameter much smaller than the stainless steel tube hole, are inserted into the needle in a cladding of filter paper, the root protruding up to 5 mm (*see* **Note 3**).

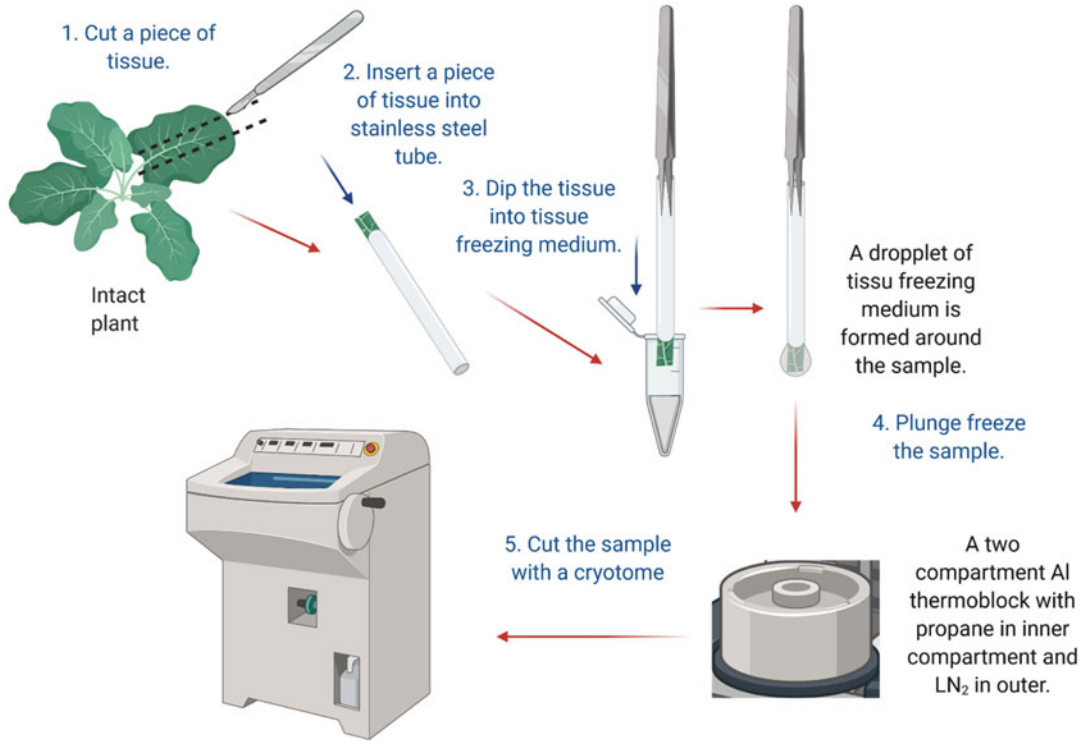

Fig. 2 Schematic presentation of sample preparation via cryofixation. Image is not to scale

Fig. 3 A two compartment Al thermo-block filled with liquid nitrogen in outer and propane in inner compartment

3. Dipping the stainless steel tube and the protruding plant material in tissue-freezing medium for 2–3 s, so that a layer of the tissue-freezing medium is formed around the sample.
4. Cryofixation: The two-compartment Al thermo-block is used (Fig. 3). Liquid nitrogen is poured into the outer compartment, while propane gas is led into the inner compartment.

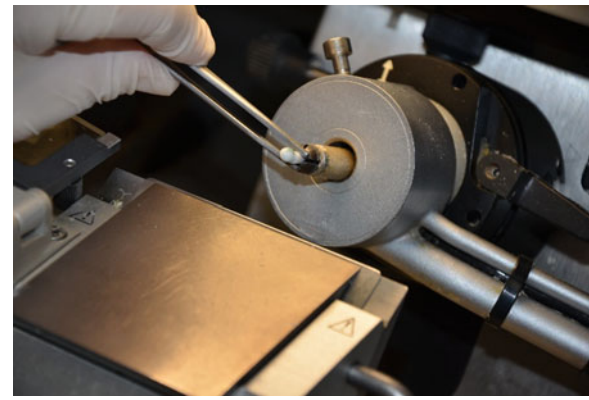

Fig. 4 Mounting of the stainless steel tube into the adapter on the cryotome head

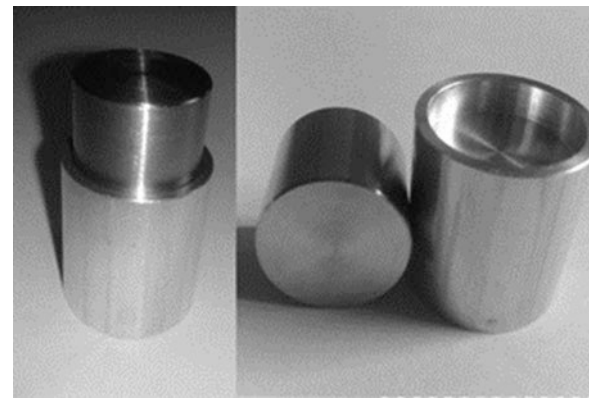

Fig. 5 Custom made pots for sample freeze-drying (bottom part: aluminum, cover: stainless steel)

Liquid nitrogen in the outer compartment liquifies the propane (*see* **Note 4**). The sample in the stainless steel tube is plunged into the liquid propane.

5. Mounting the stainless steel tube to the cryo-microtome head (Fig. 4) and sectioning of the protruding plant material to the desired thickness according to the thickness range of the cryo-microtome and the cell size of the sample (*see* **Note 5**).
6. Placing the cryo-section to the custom holder for cryo-analysis and transfer into the instrument (*see* **Note 6**).
7. Alternatively, freeze-drying of cryo-sections: Fresh cryo-sections are placed in cooled custom-made Al pots lined with filter paper and weighed down with stainless steel lids (Fig. 5) (see **Note 7**). The sections in the pots are transferred into the freeze-dryer; it is essential to ensure the sections do not defrost. Optimally, pots are submerged in liquid nitrogen and transferred immediately.

8. Freeze-dried sections are placed onto the sample support on a suitable holder for the measurement (depending on the technique) (*see* **Note 6**).

Protocol #2

1. Excise the plant organ (*see* **Note 2**).
2. Hand cutting by a razorblade (use of polystyrene for support if necessary).
3. Placing the sections onto Al foil, making a pouch and freezing the piece in liquid propane, when dealing with smaller sections or in liquid nitrogen when dealing with larger samples (*see* **Note 8**). (The size is determined by the size of the inner Al thermo-block compartment).
4. Mounting onto a sample support in a suitable holder for cryo-measurements; or freeze-drying (*see* **Note 6**).

Freeze-dried sections are placed onto a sample support in a suitable holder (*see* **Note 6**).

Protocol #3

1. Cryofixation of an entire plant organ (if not too big or too thick; good vitrification is obtained with plant leaves of up to 300 μm thick), for example, whole leaf placed flatly onto the Al foil, pouched in the foil and frozen in liquid nitrogen (*see* **Note 8**).
2. Freeze-drying the pouch with the sample kept as flat as possible to preserve the morphology of the specimen.
3. Dried material is cut using a sharp razor blade into >300 μm thick cross sections.
4. Mounting the sections onto the support (double sided carbon tape, Scotch tape, of XRF-transparent foils such as pioloform, Mylar, Ultralene) on a suitable sample holder (*see* **Note 6**).

3.2 Sample Mounting

Depending on the imaging technique (e.g., PIXE, SR-micro-XRF), the sample can be mounted between two pioloform foils stretched over an Al frames of appropriate shapes and sizes and glued with a two component Araldite.

Preparation of pioloform foils: Object glass is briefly dipped into pioloform solution. After drying in air for few seconds a foil is formed on either side of the object glass. Foil should be cut along the edges, floated off onto the water surface, and collected with a sample holder (Fig. 6).

For Laser ablation inductively coupled mass spectrometry (LA-ICPMS) the freeze-dried samples can be deposited directly on a microscope object glass.

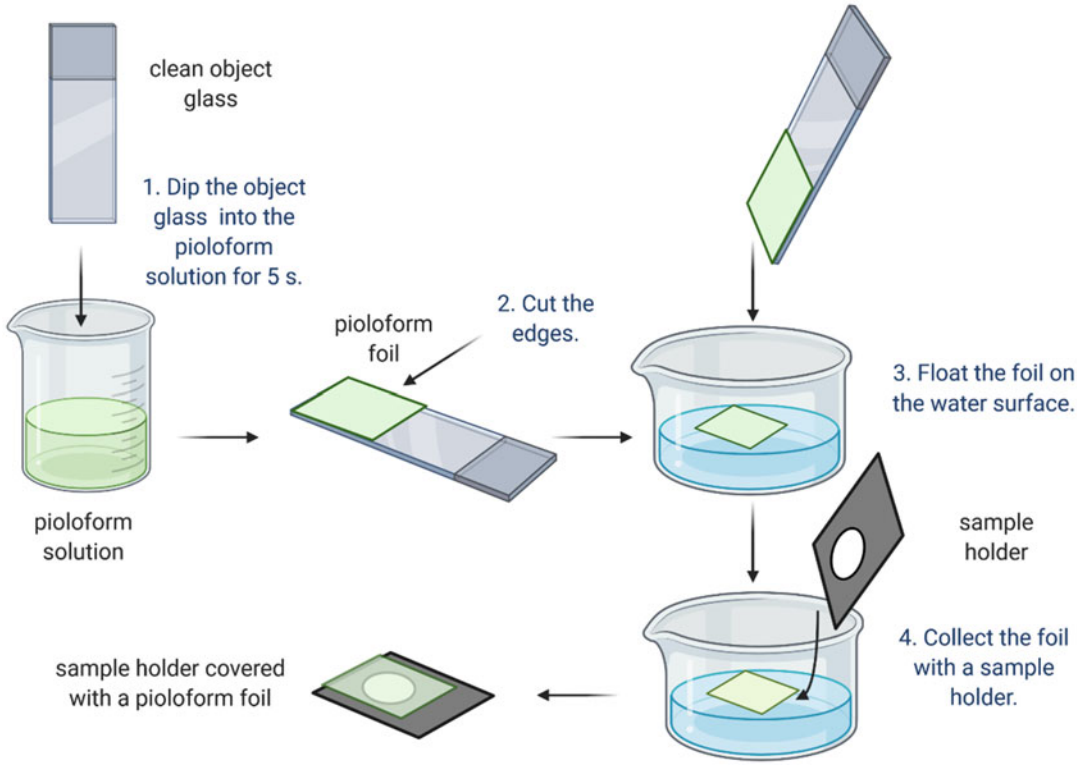

Fig. 6 Schematic presentation of preparation of pioloform foil stretched over the sample holder. Image is not to scale

3.3 Imaging

Various **X-ray fluorescence-based techniques** can be employed in imaging the element distribution of plant tissues:

Particle induced X-ray emission (PIXE) with a focused beam is performed on a particle accelerator. The sample is raster scanned with a proton beam with energy of 2–3 MeV, X-rays emitted from the sample after Coulomb reaction are collected with an energy dispersive X-ray detector (e.g., SiLi or Ge). The beam can be focused with magnetic lenses down to 1 μm and the measurements are performed in vacuum [10, 22, 23]. This technique is suitable for tissue/cellular localization studies [11, 24], its advantage is a wide elemental range. In plants it is possible to detect and image the distribution of elements from Na to U with sensitivity from 1 μg g^{-1} for middle Z elements to few 100 μg g^{-1} for light (Na, Al) and heavy (Cd, Ag) elements [25]. In some instruments the measurements under cryo-conditions are also possible [26], but in frozen hydrated samples the sensitivity for light elements such as Al, P, S, Cl, K, and Ca is lower due to absorption of fluorescent X-rays in water. PIXE spectra can be processed by GeoPIXE software [27] with possibility of fully quantitative analysis and results. Access to the technique is possible through https://www.ionbeamcenters.eu/radiate/.

Micro-X-ray fluorescence (micro-XRF) imaging is a synchrotron radiation-based technique. The sample is raster scanned with a monochromatic photon beam and X-rays emitted from the sample during photoelectric effect are collected with an energy dispersive X-ray detector (e.g., SDD). The monochromatic X-ray beam can be focused down to 1 μm [28, 29] and in nano-beamlines even to a few 10 nm. The technique is suitable for tissue, cellular, and subcellular imaging of element distribution. The advantage is better sensitivity as in PIXE due to possibility of element excitation just above the absorption edge, but this limits the range of elements to be imaged. For imaging of K and Ca a beamline should enable to excite and detect energies around 4.0 keV. XRF spectra can be processed by PyMCA software [30], providing qualitative and, in certain beamlines, also quantitative [29] information. Access to the technique is possible through semi-annual calls for projects at synchrotron facilities worldwide. The list of beamlines can be found through http://www.wayforlight.eu/en/catalogue?FacilityType=700.

Tabletop X-ray fluorescence scanners are also available (producers Bruker, Horiba), with lateral resolution of 10–100 μm, still suitable for tissue specific imaging [31].

3.3.1 Mass Spectrometry-Based Imaging Techniques

Laser ablation inductively coupled mass spectrometry (LA-ICPMS) is based on laser ablation of the sample, aerosol formation, burning aerosol in plasma, and detection of ionized elements by mass spectrometer. LA-ICPMS is characterized by its superior chemical sensitivity below μg kg^{-1} and is therefore suitable for imaging of trace element distributions. Furthermore, it is a benchtop laboratory technique with significantly lower operating costs than accelerator- or synchrotron-based techniques. The lateral resolution reaches down to 1 μm thanks to small ablation cells [32, 33]. In contrast to micro-PIXE and micro-XRF techniques in LA-ICPMS the samples are usually completely ablated during the measurement [34] and after LA-ICPMS no other analytical technique can be applied on the sample.

3.4 Image Analysis

The main goal of the image analysis is to correlate morphological sample characteristics with element distribution images. In this way tissues and cells with altered ionome could be identified and correlated with the on-going physiological processes. In the case study presented in Fig. 7 the effects of accumulation of iron oxide (Fe_3O_4) nanoparticles (NP) [35] in sunflower roots exposed for 1 week to 50 μg g^{-1} of NPs in hydroponic solution were studied. Iron nanoparticles accumulated in the root rhizodermis, which resulted in depletion of K and accumulation of Ca. Metal and metal oxide nanoparticles have a higher tendency to generate excessive amounts of reactive oxygen species (ROS). Due to the strong oxidation potential, the excess ROS induced by

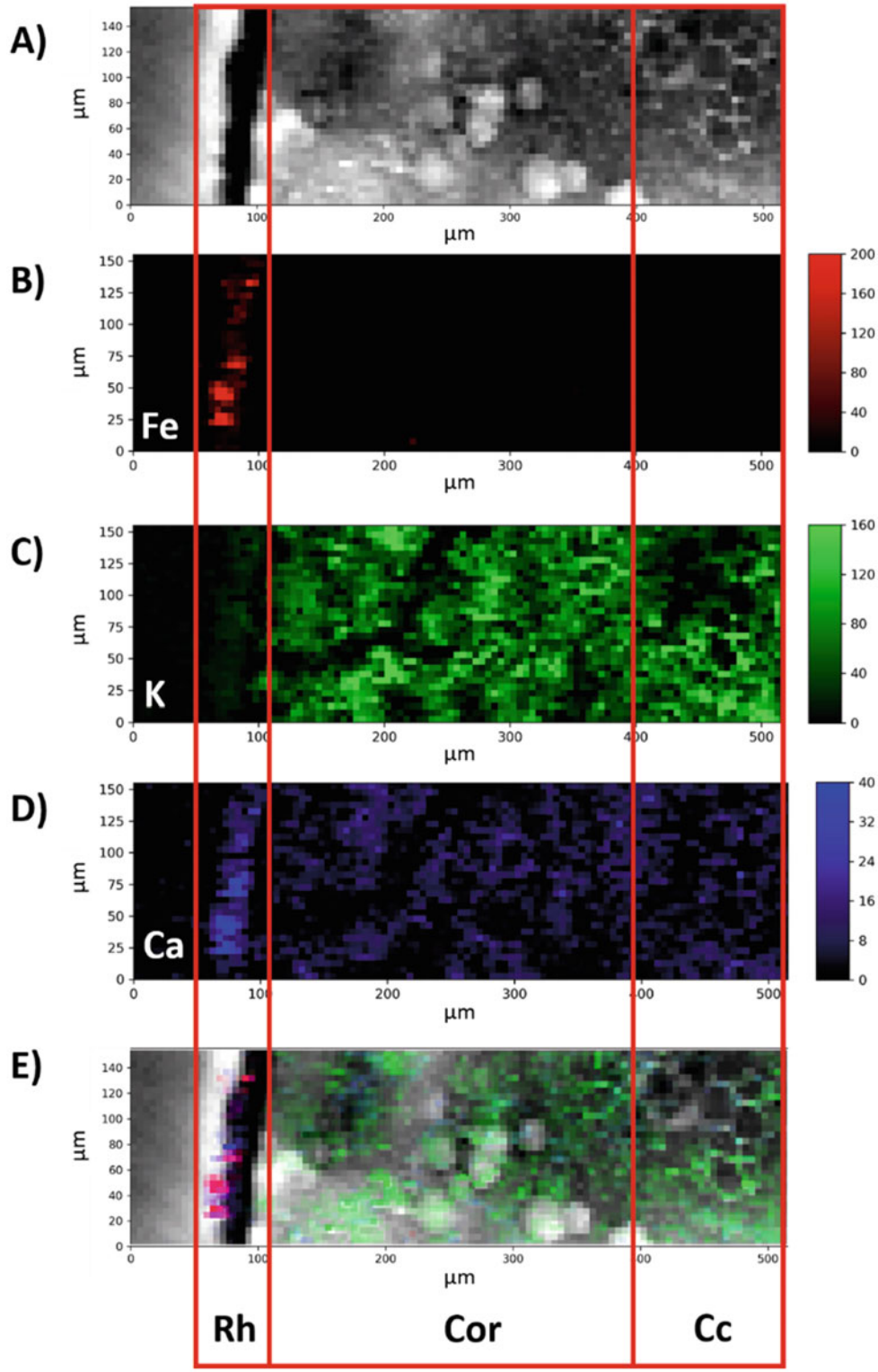

Fig. 7 Iron (Fe) nanoparticle accumulation in rhizodermis of sunflower roots imaged by synchrotron light (Energy = 7.0 keV) at the ID21 beamline of ESRF. (**a**) an image obtained with detection of transmitted X-ray photons with the energy of 7.0 keV behind the freeze-dried sunflower root sample. Darker areas represent less

nanoparticles can result in the damage of biomolecules and organelle structures and lead to protein oxidative carbonylation, lipid peroxidation, DNA/RNA breakage, and membrane structure destruction, which further cause apoptosis and necrosis and even mutagenesis [36].

4 Notes

1. Work in a fume hood. Pour 50 mL of chloroform into a flask made of brown glass (pioloform solution is photosensitive) and add pioloform (available as powder). Stir and slowly add chloroform up to 100 mL. To dissolve pioloform completely, stir with a magnetic stirrer (it takes about 2 h for the pioloform solution to be ready for use). Store the pioloform solution in the dark. For best results, use freshly prepared pioloform solution.
2. Small pieces of plant tissue (up to 2 × 2 mm) are frozen more efficiently. The freezing speed of larger samples is much lower and prevents vitrification of the sample. When freezing is slow, crystal ice forms in the cytosol, which can damage the plasma membrane and the cell walls, leading to a redistribution of elements.
3. If the protruding material is longer, the sample may break and fall off during cryo-sectioning.
4. This step should be performed with great care. Personal safety equipment (safety goggles and gloves suitable for handling liquid nitrogen) must be worn during the entire procedure. In addition, the mixture of air and propane is explosive; take utmost care when introducing gaseous propane into the inner compartment of the thermo-block. The use of automatic vitrobot is strongly recommended.
5. A special clasping mechanism (adapter) is required to hold the stainless steel tube with the cryo-fixed sample in place during cryo-sectioning. The use of anti-roll plate over the cryotome knife to prevent curling of the cryo-section is advised.

Fig. 7 (continued) transmitted X-ray photons and higher density of the sample; (**b–d**) X-ray fluorescence imaging: (**b**) the relative intensity of Fe-Ka line (counts s^{-1}), (**c**) the relative intensity of potassium (K)-Ka line (counts s^{-1}), and (**d**) the relative intensity of calcium (Ca)-Ka line (counts s^{-1}); (**e**) Colocalization image of X-ray fluorescence (distribution of Fe in red, of Ca in blue and of K in green) superimposed on X-ray transmission image (gray). Iron enrichment in rhizodermis colocalizes with Ca enrichment and K depletion. Rh-rhizodermis, Cor-Cortex, Cc-central cylinder

6. Each imaging instrument has its own specific sample holder (s) and system of transferring the sample; therefore, it is crucial to consult the beamline scientists prior to the measurements.
7. The use of custom-made Al pots with a fitting cover keeps the sections flat and prevents curling of the sections during freeze-drying.
8. Ensure that the sample is in good contact with Al foil. This creates a "metal mirror" freezing effect, whereby heat is rapidly transferred from the sample through a metal object (in our case Al foil).

Acknowledgments

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Chapter 20

Monitoring *Pseudomonas syringae* Growth in Agroinfiltrated Leaves: The "Agromonas" Assay

Pierre Buscaill and Renier A. L. van der Hoorn

Abstract

We recently developed a simple method called "agromonas" which facilitates the detection of *Pseudomonas syringae* growth in agroinfiltrated leaves expressing genes of interest. This protocol consists of agroinfiltration followed by inoculation of *Pseudomonas syringae* by either infiltration or spray inoculation. Next, bacterial growth is measured a few days later by plating leaf extracts out on *P. syringae*-selective medium. In this protocol, we describe all the steps required for agroinfiltration, inoculation of *P. syringae* by both injection and spray infection and explain how to quantify *in planta* live bacteria.

Key words Agroinfiltration, Disease assay, *Pseudomonas syringae*, *Agrobacterium tumefaciens*, Infiltration, Spray-inoculation

1 Introduction

The plant-pathogenic bacterium *Pseudomonas syringae* is one of the best-studied plant pathogens and serves as a model for understanding bacterial pathogenicity, molecular mechanisms of plant-microbe interactions, as well as microbial ecology and epidemiology [1]. To evaluate the level of resistance of a plant or the virulence of bacterial pathogens, the quantification of bacterial growth (i.e., colony count assay) is routinely performed on stable transformant plants. However, generation of stable transgenic lines is lengthy process limited to plant species that are amenable to genetic transformation. Therefore, there is a need for rapid disease assays to monitor the growth of *P. syringae* in tissue overexpressing or depleted for immune components.

Agrobacterium tumefaciens-mediated transient expression (agroinfiltration) facilitates rapid overexpression and transcript depletion of various exogenous and endogenous genes. However, agroinfiltrated *N. benthamiana* leaves are not routinely used for disease assays with *P. syringae* because selective isolation of

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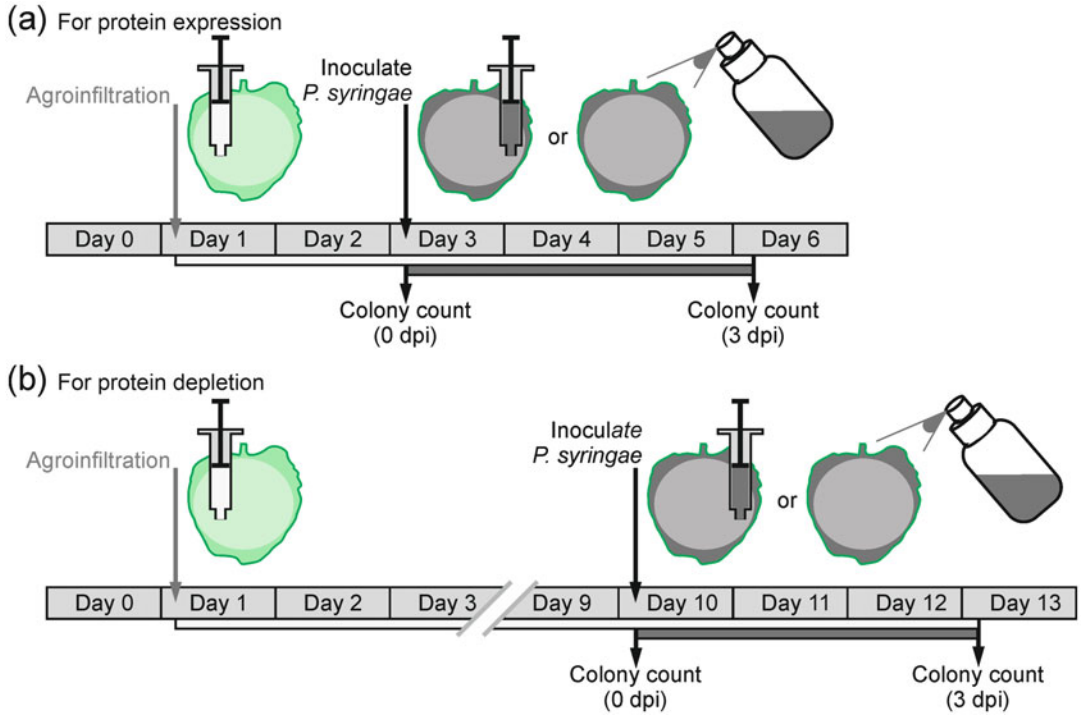

Fig. 1 Experimental procedure of agromonas assay. (**a**) Experimental procedure for agromonas assay upon protein expression. At 2 days after agroinfiltration, agroinfiltrated leaves are inoculated with *P. syringae* bacteria by injection or spray infection. Bacterial growth is measured after inoculation and 3 days later (3 days post-infiltration, 3 dpi) by a classic colony count on LB agar plates containing CFC. (**b**) Experimental procedure for agromonas assay on leaves depleted for endogenous proteins by RNAi. Ten days after agroinfiltration with a silencing construct, agroinfiltrated leaves are inoculated with *P. syringae* bacteria by injection or spray infection. Bacterial growth is measured 3 days later by a classic colony count on LB agar plates containing CFC

P. syringae from agroinfiltrated tissue is rather challenging by the overlap of endogenous or introduced antibiotics resistance.

Recently, we developed a method called "agromonas" to study the growth of *Pseudomonas* species in agroinfiltrated tissues using bacterial endogenous resistance to antibiotics (Fig. 1) [2]. We perform separate quantification of these two bacterial populations by exploiting the combination of cetrimide, fucidin, and cephaloridine (CFC) or gentamycin for the selective isolation of *Pseudomonas* species [3] or Agrobacterium, respectively, from agroinfiltrated tissues.

There are two strategies in agromonas assays to study genes that confer immunity. The first one is to overexpress a protein (Fig. 1a), the other is to deplete a host component (Fig. 1b) by RNA interference (RNAi) using hairpin (hp) constructs [4]. Agromonas assay is also adaptable to study pre-invasive and post-invasive immunity by using spray inoculation and infiltration, respectively.

Previously, we have demonstrated that agromonas assay can be used to study components that enhance immunity (e.g., FLS3, EFR, and BGAL1) and reduce immunity (e.g., *hpFLS2* and AvrPto) [2]. The following protocol contains all the information to perform agroinfiltration followed by inoculation of *P. syringae* by either infiltration or spray inoculation and the determination of *in planta* bacterial growth.

2 Materials

2.1 Agroinfiltration of N. benthamiana Plants

1. Liquid LB medium: For 1 L, dissolve 10 g NaCl, 10 g tryptone, and 5 g of yeast extract in 950 mL of MilliQ water. Adjust the pH to 7.0 with 5 M NaOH. Adjust the final volume of the solution to 1 L with MilliQ water. Sterilize by autoclaving the medium for 15–20 min at 121 °C and 15 psi (1.05 kg/cm^2).
2. LB agar medium: same procedure as liquid LB medium with 12 g agar. Adjust the pH to 7.0 with 5 M NaOH. Adjust the final volume of the solution to 1 L with MilliQ water. Sterilize by autoclaving the medium for 15–20 min at 121 °C and 15 psi (1.05 kg/cm^2) on liquid cycle.
3. Rifampicin (50 mg/mL): dissolve 250 mg of rifampicin in 5 mL dimethyl sulfoxide (DMSO), vortex well, and store at −20 °C in 500 μL aliquots.
4. Gentamicin (50 mg/mL): dissolve 250 mg of gentamicin in 5 mL sterile water. Filter sterilize the solution into a sterile falcon tube, using a syringe with an 0.22 μm filter sterilization unit. Store at −20 °C in 500 μL aliquots.
5. Kanamycin (50 mg/mL): dissolve 250 mg of kanamycin in 5 mL sterile water, filter sterilize the solution with 0.22 μm filter unit, and store at −20 °C in 500 μL aliquots.
6. 50 mL sterile falcon tubes (Corning, New York, USA).
7. Spectrophotometer (Jenway 7200, Cole-Parmer, Illinois, USA) and clean UV cuvettes (Brand, Wertheim, Germany) for measuring optical density (OD) at 600 nm.
8. *Nicotiana benthamiana* plants grown in a growth chamber at 21 °C and ~60% relative humidity with a 16 h photoperiod and a light intensity of 2000 cd.sr.m^{-2}.
9. 1 mL syringe without needle (Terumo, Tokyo, Japan).
10. 500 mM MES buffer pH 5.0: dissolve 4.881 g of 2-(N-morpholino)ethanesulfonic (MES) in a 100 mL Duran bottle containing 40 mL of MilliQ water. Adjust the pH to 5.0 by adding a few drops of 5 M NaOH. Add MilliQ water to a final volume of 50 mL.
11. 1 M $MgCl_2$: dissolve 10.165 g in 50 mL of MilliQ water.

12. 1 M acetosyringone: dissolve 196.19 mg in 1 mL of DMSO.
13. *Agrobacterium tumefaciens* strain GV3101 is used in this protocol. Other *A. tumefaciens* strains are also suitable.

2.2 Infection by Syringe-Infiltration

1. *Pseudomonas syringae* stock frozen in 25% Glycerol (*see* **Note 1**).
2. 1 M $MgCl_2$: dissolve 10.165 g in 50 mL of MilliQ water.
3. 50 mL falcon tubes.
4. 2 mL tubes (Eppendorf, Hamburg, Germany).
5. Benchtop centrifuge (Heraeus Biofuge Pico Microlitre centrifuge, Hanau, Germany).
6. 1 mL syringe without needle (Terumo, Tokyo, Japan).
7. Plant growth chamber or cabinet and microbiological incubator (New Brunswick Innova 4230, Edison, NJ, USA) with Class II pathogen licence.

2.3 Infection by Spray Inoculation

1. *Pseudomonas syringae* stock frozen in 25% Glycerol (*see* **Note 1**).
2. 1 M $MgCl_2$: dissolve 10.165 g in 50 mL of milliQ water.
3. 50 mL falcon tubes.
4. Large centrifuge (Allegra X-15R, BeckmanCoulter, CA, USA).
5. Spectrophotometer (Jenway 7200, Cole-Parmer, Illinois, USA) and clean UV cuvettes (Brand, Wertheim, Germany) for measuring optical density (OD) at 600 nm.
6. Silwet-77 (PlantMedia, USA).
7. 50 mL spray bottle (Carl Roth, Germany).
8. Laminar flow cabinet (Gelaire, Sydney, Australia) with Class II pathogen licence.
9. Plant growth chamber or cabinet with Class II pathogens licence.

2.4 Determination of In Planta Bacterial Growth

1. Square petri dishes (120 × 120 × 7 mm) (Greiner Bio-One, Kremsmünster, Austria).
2. LB agar medium: dissolve 10 g NaCl, 10 g tryptone and 5 g of yeast extract, 10 g agar in 950 mL of MilliQ water. Adjust the pH to 7.0 with 5 N NaOH. Adjust the final volume of the solution to 1 L with MilliQ water. Sterilize by autoclaving the medium for 15–20 min at 121 °C and 15 psi (1.05 kg/cm^2) on liquid cycle.
3. Cetrimide, Fucidin, Cephalosporin (CFC; Oxoid, Basingstoke, UK) at 1× concentration: prepared CFC antibiotics by resuspending the powder of one vial with 1 mL of sterile water and 1 mL of ethanol.

4. Gentamicin (50 mg/mL): dissolve 250 mg of gentamicin in 5 mL sterile water, filter sterilize with 0.22 μm filter unit, and store at −20 °C in 500 μL aliquots.
5. Cork-borer (1 cm diameter).
6. 24-well plate (Greiner Bio-One, Kremsmünster, Austria).
7. 15% H_2O_2: mix 30% (w/v) H_2O_2 (Sigma-Aldrich, St. Louis, MI, USA) with equal amount of sterile water.
8. Tissue-lyser (Qiagen 85210, Hilden, Germany).
9. 5.0 mm metal beads (Biospec Products, Bartelsville, OK, USA).
10. 1.5 mL Eppendorf safe-lock tubes.
11. Laminar flow cabinet (Gelaire, Sydney, Australia) with Class II pathogen licence.
12. 96-well plate (Greiner Bio-One, Kremsmünster, Austria).
13. 12-channel 20–200 μL electronic multichannel pipette (Eppendorf, Hamburg, Germany).
14. Microbiological incubator (New Brunswick Innova 4230, Edison, NJ, USA) with Class II pathogen licence.

3 Method

3.1 Transient Expression or Depletion of Proteins by Agroinfiltrating N. benthamiana *Leaves*

Day 0

1. Inoculate 10 mL of liquid LB medium containing 50 μg/mL rifampicin, 10 μg/mL gentamicin, and 50 μg/mL kanamycin in a 50 mL falcon tube with the *Agrobacterium tumefaciens* strain GV3101 (*Atum*GV3101) carrying the desired plasmid directly from the glycerol stock. For overexpression, you need the RNA silencing suppressor p19, an empty vector (EV), and your gene of interest. For depletion, you need a hairpin construct targeting your gene of interest and a negative control hairpin construct targeting GFP (*hp:GFP*).
2. Incubate the cultures in a microbial incubator at 28 °C under agitation (220 rpm) and allow to grow for 24 h.

Day 1

1. Harvest the *Atum*GV3101 cells by centrifugating the overnight cultures at 3500 × *g* for 10 min at 21 °C and discard the supernatant (*see* **Note 2**).
2. Prepare the induction buffer (10 mM $MgCl_2$, 10 mM MES pH 5.0, and 150 μM acetosyringone): i.e., for 10 mL of infiltration buffer: in 700 μL of water add 100 μL of 1 M $MgCl_2$, 200 μL of 500 mM MES pH 5.0, and 1.5 μL of 1 M acetosyringone.

3. Resuspend each bacterial pellet in 1 mL of induction buffer.
4. Measure the OD_{600} of the *Agrobacterium* suspension with a spectrophotometer by diluting 20 μL of the culture in 980 μL water.
5. To overexpress a protein, mix each Agrobacterium suspension carrying the desired plasmid with Agrobacterium suspension carrying silencing inhibitor p19 to a final $OD_{600} = 0.5$ (e.g., p19 + EV = 0.5). To deplete a protein, dilute the Agrobacterium suspension carrying the desired hairpin construct to $OD_{600} = 0.2$ (e.g., *hp:GFP*) (*see* **Note 3**).
6. Incubate for 1 h at 21 °C.
7. Infiltrate the cells with a needless syringe into the abaxial side of three leaves of 3–4-week-old *N. benthamiana* plants (*see* **Notes 4–10**).
8. Use a permanent maker to mark the infiltrated area if the leave was not fully infiltrated.

3.2 N. benthamiana *Plants Infection by Syringe-Infiltration*

Day 1

1. Revive the desired *P. syringae* strains from the glycerol stock on LB agar medium plate (e.g., for *Pto*DC3000 strains, on LB medium containing 50 μg/mL rifampicin).
2. Incubate the plate at 28 °C for 24 h.

Day 2

1. Pick a single bacterial colony from the plate and inoculate 10 mL of liquid LB containing 10 mM $MgCl_2$ in a 50 mL falcon tube.
2. Incubate the culture at 28 °C under agitation (220 rpm) for 24 h to allow growth.

Day 3

1. Centrifugate 2 mL of the overnight grown bacterial culture in a 2 mL Eppendorf tube at 4000 × *g* for 2 min at 21 °C (*see* **Note 2**).
2. Remove the supernatant and resuspend the pellet in 2 mL of sterile water.
3. Wash the overnight culture with sterile water by repeating **steps 1** and **2**.
4. Measure the OD_{600} of the *P. syringae* suspension with a spectrophotometer by diluting 100 μL of the culture in 900 μL of water.
5. Dilute the bacteria in sterile water to a final $OD_{600} = 0.001$ (equivalent of 1×10^6 CFU/mL) and infiltrate the solution into agroinfiltrated leaves using a needleless syringe via the abaxial side of the leaves (*see* **Notes 11**, **12** and **14**).

3.3 N. benthamiana Plants Infection by Spray Inoculation

Day 1

1. Revive the desired *P. syringae* strains from the glycerol stock on LB agar medium plate (e.g., for *Pto*DC3000 strains, on LB medium containing 50 μg/mL rifampicin).
2. Incubate the plate at 28 °C for 24 h.

Day 2

1. Cover the plants with propagator lid and place them in the plant growth chamber for one day.
2. Pick a single bacterial colony from the plate and inoculate 40 mL of liquid LB containing 10 mM $MgCl_2$ in a 200 mL flask or in four sterile 50 mL falcon tubes.
3. Incubate the bacterial culture at 28 °C under agitation (220 rpm) for 24 h to allow growth.

Day 3

1. Centrifugate the overnight culture bacteria culture at 4000 × *g* for 5 min at 21 °C (*see* **Note 2**).
2. Resuspend the bacterial pellet in 16 mL of sterile water.
3. Measure the OD_{600} of the *P. syringae* suspension by diluting 100 μL of the solution with 900 μL water.
4. Dilute the bacteria with sterile water to a final $OD_{600} = 0.5$ (equivalent of 5×10^8 CFU/mL).
5. Add 0.04% (v/v) of Silwet-77 to the *P. syringae* solution just before spraying the plants.
6. Spray the *P. syringae* suspension onto the adaxial surfaces of agroinfiltrated leaves. Spray equal amount for each plant (*see* **Notes 11** and **12**).
7. After spray infection, re-cover the plants with the propagator lid and keep the plants for 3 days in the plant growth cabinet at 21 °C (*see* **Note 14**).

3.4 Determination of In Planta Bacterial Growth

Day 6

1. Prepare the LB agar medium with 1× CFC by adding 800 μL of rehydrated CFC supplement in 200 mL of LB agar based medium cooled to 50 °C. Mix well, pour 40–45 mL into sterile square Petri dishes, and solidify for 20–30 min at room temperature without lids.
2. Prepare LB agar medium with 10 μg/mL gentamycin by adding 40 μL of 50 mg/mL gentamycin in 200 mL of LB agar based medium cooled to 50 °C. Mix well, pour 40–45 mL into sterile square Petri dishes, and solidify for 20–30 min (*see* **Note 14**).
3. From three infected leaves per plant, excise 1 leaf disc per leaf using a cork-borer (1 cm diameter) (*see* **Note 15**) (Fig. 2).

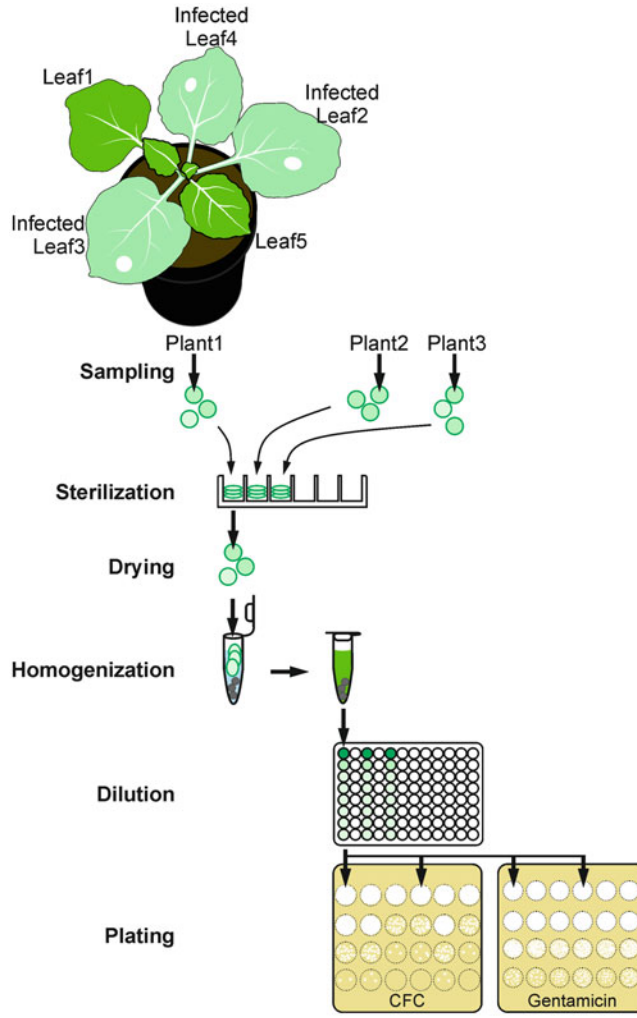

Fig. 2 Experimental procedure of colony count. Experimental procedure of colony count for one treatment in agromonas assay (e.g., EV or *hp:GFP*) with $n = 3$ biological replicate with two technical replicates. Leaf discs are surface sterilized, homogenized in water. Dilutions of three biological replicates ($n = 3$) are plated on selective media (e.g., CFC or gentamicin) with two technical replicates

4. Combine the three leaf discs from the same plant (same treatment) to a 24-well plate (1 well = 1 plant).
5. Add 1.5 mL of 15% H_2O_2 into each well of the 24-well plate containing leaf discs. Soak the leaf discs in the solution for 2 min and shake the plate gently to sterilize the leaf surface.
6. Remove the sterilization solution and wash the leaf discs twice with 1.5 mL of sterile water.

7. Dry the leaf discs on a clean surface under the laminar flow cabinet for 20–30 min.
8. Add 3 metal beads in each 1.5 mL Eppendorf safe-lock tube. Prepare same number of tubes as the number of infected plants used in this experiment.
9. Place the surface-dried leaf discs into the 1.5 mL tube with metal beads using a clean tweezers without crushing the leaf discs. Place the leaf discs of the same plant in one tube. (Fig. 2).
10. Add 1 mL of sterile water into each 1.5 mL Eppendorf safe-lock tube and seal the tubes.
11. Homogenize the leaf discs in sterile water using the tissuelyser for 5 min at 30/s (*see* **Note 16**).
12. Prepare the 96-well plate to dilute the lysates in series. Add 180 µL of sterile water to every well from rows B to H (*see* **Note 17**).
13. Add 200 µL of the homogenate into wells in row A. Plants expressing the same construct should be in alternative wells to ensure that the droplets are spaced apart when plating the serial dilution on the LB agar medium plate (Fig. 2).
14. Dilute in series the homogenate in the 96-well plate. Take 20 µL of the solution in row A, mix carefully by pipetting up and down (i.e., 20 times), add 20 µL to well B. Discard the tips.
15. Take 20 µL of the solution in row B, mix 20 times, add 20 µL of the solution to well C. Discard the tips.
16. Repeat until serial dilutions are complete (until row H).
17. Add three tips onto the odd channels of the multi-channel pipette, to take only samples from the same treatment in the plate (Fig. 2) (e.g., EV control).
18. Put 20 µL of each dilution, starting from the highest dilution to the lowest (from row H to row A) onto the LB agar plate supplemented with either 1× CFC for selection of *P. syringae* strains or 10 µg/mL gentamicin for selection of *Atum*GV3101.
19. Repeat alongside on the same agar plate to obtain two technical replicates.
20. Repeat for row G, F, E, D, C, B, and A from the 96-well plate (*see* **Note 18**).
21. Allow the serial dilution to dry on the plate under the laminar flow chamber before handling.
22. Incubate the plates at 28 °C (*see* **Note 19**).

Day 8

1. After 36 h incubation at 28 °C, count colonies from the dilution of which there are between 3–30 colonies per droplet (*see* **Notes 20–22**). Count the bacteria before the colonies grow together.
2. Enter the data into excel file to calculate CFU/cm^2 as indicated below. Calculate the significance *p*-value using the two-tailed Student's *t*-test to compare bacterial growth between two different treatments.

CFU	Dilution factor	CFU/extract	CFU/cm^2	Log_{10}CFU/cm^2
X	Y	(X*Y*1000)/Z	(X*Y*1000)/Z/A	Log10(X*Y*1000/Z/A)

X = number of colonies; Y = dilution factor; Z = volume plated onto the LB agar plate in µl; A = total area of leaf discs in cm^2

4 Notes

1. CFC permits the selection of *Pseudomonas* species [3]; therefore any *Pseudomonas* species pathogenic on *N. benthamiana* can be used in Agromonas assays. We mostly used *P. syringae* pv. *tomato* DC3000, the causative agent of the bacterial speck disease of tomato lacking the type III effector gene *hopQ1-1* [*Pto*DC3000(Δ*hQ*)]; *P. syringae* pv. *tabaci* 6605 (*Pta*6605), the causative agent for wildfire disease in tobacco; and *P. syringae* pv. *syringae* B728a (*Psy*B728a), the causative agent of bacterial brown spot of bean. Among them, only *Pto*DC3000 and its derived mutants are resistant to kanamycin.
2. Bacterial OD_{600} should be between 0.5 and 0.8 to be sure that bacterial growth is in the exponential phase.
3. The control should be based on the same Agrobacterium strain carrying the same vector and at same final OD_{600}. Use empty vector (EV) or *hp*GFP as controls in overexpression or depletion assays, respectively. Agrobacterium suppresses *P. syringae* growth directly and/or indirectly, so a mock control (i.e., water or buffer infiltration) is not very useful [2].
4. For agroinfiltration, use the youngest, fully expanded leaves of *N. benthamiana*.
5. To infiltrate the bacterial suspension into *N. benthamiana* leaves, expose the lower surface of the leaf with one gloved hand. Use a needle or plastic tip to gently scratch the abaxial side of the leaf with a tiny (<1 mm) scratch on the epidermis only. Fill a 1 mL needleless syringe with the bacterial suspension. Place the syringe tip on the scratch and position the

syringe perpendicular to the leaf surface to ensure even distribution of pressure. Apply slight counter-pressure to the adaxial side of the leaf with your gloved finger. Slowly push the plunger of the syringe to inject the bacterial suspension into the leaf. The infiltrated area should turn dark upon infiltration. To infiltrate an entire leaf, multiple infiltration points might be necessary. If the leaf is only partially infiltrated, mark the infiltrated area with a marker pen. To reduce leaf damage, avoid injection of the vascular system.

6. We recommend analyzing at least 3 (ideally 4–6) plants per condition per experiment. For example, three plants for the control (p19 + EV or *hp*GFP) and three plants for your protein of interest.
7. Protein accumulation in agroinfiltrated leaves may differ between proteins. Likewise, depletion of endogenous proteins by *hp*RNAi depends on the turnover of the target protein and may need time. For instance, 3 days upon agroinfiltration of *hpFLS2* was not enough to deplete endogenous *Nb*FLS2 [2].
8. When possible, the activity of the protein of interest should be detected before *P. syringae* inoculation. For instance, the ROS burst can be monitored upon treatment of the relevant ligand to test PRR functionality or enzymatic assays can be used to detect protein activities as previously described [2]. If there is no functional assay to monitor the activity of your protein candidate, you can show protein accumulation or depletion by western blot. Alternatively, RT-PCR can be used to monitor the absence of the transcript upon RNAi but this might not reflect protein depletion.
9. Agroinfiltration of certain effectors can cause cell death, causing problems for leaf sampling. Therefore, to study the impact of microbial effector on bacterial growth, we recommend using old plants (i.e., 5–6-week-old) and to use a low agrobacterium inoculum (e.g., final $OD_{600} = 0.1$) to prevent tissue collapse prior to leaf sampling.
10. Use personal protective equipment when working with plant pathogens.
11. Spray infect or infiltrate *P. syringae* onto or in the agroinfiltrated area, respectively (Fig. 1a and b).
12. Spray 70% ethanol on laboratory bench surface or laminar flow hood after infecting plants with pathogens to prevent pathogen spread.
13. You can monitor growth of Agrobacterium using gentamicin selection, but only when the used *P. syringae* strain is not gentamicin resistant. Alternatively, *Agrobacterium tumefaciens* can be selected from *P. syringae* infected tissue on LB agar

medium with the selection conferred by the binary plasmid (e.g., 50 μg/mL kanamycin).

14. Harvest leaf samples from the agroinfiltrated and infected leaves.
15. Repeat this process if needed until the tissues are well homogenized and the solution is green due to the chlorophyll release from the leaves.
16. We recommend using a calibrated multichannel pipette, preferably electronic.
17. Processing from most diluted to most concentrated dilution makes it unnecessary to change pipette tips between the dilutions.
18. Metal beads can be reused by washing them at the end of the assay with 70% ethanol for 30–60 s and drying under the laminar flow cabinet.
19. If *P. syringae* growth is low, we encourage to increase the concentration of the inoculum (e.g., from $OD_{600} = 0.001$ to 0.01 and $OD_{600} = 0.1$ to 1 for injection and spray inoculation, respectively).
20. In the agromonas method, the colony count of *P. syringae* on LB agar plates can be generally performed from the homogenate dilution 10^0 to 10^{-3} (i.e., dilution from row A to row D), as the presence of *A. tumefaciens* suppresses *P. syringae* growth [2, 5]. The expected *Pseudomonas* growth in EV expressing leaves is about 10^3 to 10^4 CFU/cm^2 for spray infection and 10^5 to 10^6 CFU/cm^2 for infiltration. The colony count of *A. tumefaciens* on LB agar plates can be generally performed from the homogenate dilution 10^{-4} to 10^{-6} (i.e., dilution from row E to row G) as the expected growth for *A. tumefaciens* carrying EV is about 10^7 to 10^9 CFU/cm^2 [2].
21. Prevent accidental phytopathogen release by autoclaving everything that might have been in contact with infected plant material and the pathogens used in this assay.

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Chapter 21

Analysis of Virus Spread Around the Cell Death Zone at Spatiotemporal Resolution Using Confocal Microscopy

Tjaša Lukan, Anna Coll, Špela Baebler, and Kristina Gruden

Abstract

The role of programmed cell death (PCD) in hypersensitive response (HR)-conferred resistance depends on the type of host–pathogen interaction and therefore has to be studied for each individual pathosystem. Here we present and explain the protocol for studying the role of PCD in HR-conferred resistance in potato plants in the interaction with the viral pathogen. As an experimental system, we use genotype Rywal, where the virus spread is restricted and HR PCD develops 3 days post potato virus Y (PVY) inoculation. As a control of virus multiplication and spread, we include its transgenic counterpart impaired in salicylic acid (SA) accumulation (NahG-Rywal), in which the HR-PCD occurs but the spread of the virus is not restricted. To follow the occurrence of virus-infected cells and/or virus multiplication outside the cell death zone, we use GFP-tagged PVY (PVY-N605(123)-GFP) which can be monitored by confocal microscopy. Any other plant-pathogen system which results in PCD development could be studied using a modified version of this protocol.

Key words HR cell death, Resistance, Potato cv. Rywal, GFP-tagged potato virus Y, Confocal microscopy

1 Introduction

In plants, hypersensitive response (HR)-conferred resistance is an effective defense response which is triggered by the resistance (R) genes. HR is manifested as the formation of necrotic lesions [1]. Nevertheless, the mechanisms of HR-conferred resistance are not yet fully deciphered. Following pathogen recognition by the R protein, programmed cell death (PCD) is induced and the pathogen is restricted to the site of inoculation [2]. The question if the PCD restricts pathogen proliferation or if it is merely collateral damage of the resistance mechanisms has long been discussed [3]. In general, it is more likely that cell death contributes to pathogen arrest of obligate pathogens that require living cells in order to replicate [4], as it was suggested for the oomycete [5, 6]. However, the restriction of the pathogen spread was also

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shown to be uncoupled from the HR cell death in some (hemi)-biotrophic plant pathosystems, involving bacteria [7], oomycetes [8], and viruses [9]. It thus seems that the role of cell death in resistance depends on the type of host–pathogen interaction and therefore has to be determined for each individual pathosystem. We here explain the protocol for studying the dynamics of virus-infected cells occurrence outside the cell death zone. Our experimental system is potato cv. Rywal, which develops HR cell death 3 days after potato virus Y (PVY) inoculation. Since the virus is in cv. Rywal restricted to the border of the cell death zone, as a control of virus multiplication we include transgenic counterpart of Rywal impaired in SA accumulation (NahG-Rywal), where the lesions occur but the spread of the virus is not restricted. To follow the occurrence of virus-infected cells and/or virus multiplication outside the cell death zone by confocal microscopy, we use GFP-tagged PVY (PVY N605(123)-GFP) (Lukan et al., 2021; in a process of submission). In this infective clone, based on PVY-N605(123) (Bukovinszki et al., 2007), GFP was inserted between NIb and CP coding regions and was flanked by protease recognition sites, allowing excision from the polyprotein and release into the cytoplasm after translation, which can be followed as GFP fluorescence. Any other pathosystem which results in PCD development could be studied using this protocol with some modifications.

2 Materials

2.1 Plant Propagation Material

1. Potato (*Solanum tuberosum* L.) cv. Rywal plants and plants expressing NahG transgene (NahG-Rywal; [10]) in tissue culture (*see* **Note 1**).
2. Sterile plastic boxes with lids for growing plants in tissue culture (e.g., Duchefa).
3. Murashige and Skoog basal medium including vitamins and saccharose (MS30): mix 5 g Murashige and Skoog medium including vitamins (Duchefa), 30 g saccharose, 8 g agar and add highly pure water (ddH_20) up to 1000 mL. Adjust pH to 5.8 and sterilize by autoclaving (*see* **Note 2**).
4. Sterile paper.
5. Sterile tweezers.
6. Sterile scalpel knife.
7. 95% ethanol.
8. Burner (such as Bunsen).
9. Approximately 20 seeds of *Nicotiana clevelandii* A. Grey tobacco.

10. 1 mM Gibberellic acid (GA3): dissolve 17 mg of GA3 in 50 mL ddH_2O.
11. 69 pots with soil.

2.2 Tobacco and Potato Inoculation

1. Solution A: dissolve 31.2 g $NaH_2PO_4 \times 2H_2O$ in 1000 mL ddH_2O (*see* **Note 3**).
2. Solution B: dissolve 28.4 g Na_2HPO_4 in 1000 mL ddH_2O (*see* **Note 3**).
3. 20 mM phosphate buffer for tobacco inoculation: mix 1.3 mL of solution A and 8.7 mL of solution B and add ddH_2O to 100 mL. Adjust pH to 7.6 using concentrated HCl or NaOH (*see* **Note 4**).
4. 20 mM phosphate buffer with DIECA for potato inoculation: mix 1.3 mL solution A, 8.7 mL solution B, and 0.225 g DIECA and add ddH_2O to 100 mL. Adjust pH to 7.6 using concentrated HCl or NaOH (*see* **Note 5**).
5. Frozen inoculum containing PVY-N605(123)-GFP virus (*see* **Note 6**).
6. Extraction plastic bag (e.g., Bioreba).
7. Automated or hand homogenizer (e.g., Bioreba).
8. Carborundum powder (0.037 mm).

2.3 Confocal Microscopy

1. Sampling tool for the excision of leaf disks with diameter 4 mm (e.g., Miltex TM biopsy punch, Ted Pella Australia).
2. Tweezers.
3. Cover glasses for microscopy.
4. Oil for microscopy.
5. Confocal microscope.

3 Methods

3.1 Growing Potato Plants

1. Grow healthy potato plants in stem node tissue culture.
2. After 6–8 weeks, cut 30 explants containing nodes from plants of each genotype on the sterile paper under sterile conditions using sterile tweezers and scalpel knife.
3. Grow them in plastic boxes with MS30 medium under controlled environmental conditions (22 ± 2 °C in the light and 19 ± 2 °C in the dark with 70–90 μmol/m^2/s^2 radiation (OSRAM L 58 W/77 FLUORA lamps, Germany) and a 16-h photoperiod).
4. Two weeks after node segmentation, transfer them to soil.

5. Grow the plants in a growth chamber under controlled environmental conditions (21 ± 2 °C in the light and 18 ± 1 °C in the dark, at a relative humidity of 75% ± 2%, with at least 90 μmol/m^2/s^2 radiation (L36W/77 lamps, Osram, Germany) and a 16-h photoperiod).
6. After 3–4 weeks, plants are ready to be inoculated.

3.2 Inoculum Preparation and Potato Infection

Inoculate 15 plants of each genotype with PVY-N605(123)-GFP and 5 plants of each genotype with mock inoculum as specified below.

1. Soak approximately 20 seeds of tobacco plants in 1 mM gibberellic acid for at least 4 h.
2. Rinse them three times in tap water and grow in soil under controlled environmental conditions as specified above (*see* Subheading 3.1, **step 5**).
3. After 2 weeks, transfer 8 tobacco plants to separate pots with soil and grow them for additional 3 weeks.
4. Unfreeze the inoculum containing PVY-N605(123)-GFP infectious clone and dilute it in the 20 mM phosphate buffer for tobacco inoculation in 1:1 buffer to plant material ratio (add 1 mL of the buffer to 1 mL of the inoculum).
5. Dust the first three fully developed bottom leaves of 3 weeks old tobacco plants with carborundum powder and rub them with the inoculum (~60 μL per leaf).
6. After 10 min, wash the leaves with tap water.
7. After the virus had spread to tobacco systemic leaves (3–4 weeks), confirm its presence under confocal microscope by detecting GFP fluorescence (*see* Subheading 3.3 and **Notes** 7 and **8**).
8. Weigh the selected areas of upper non-inoculated leaves of tobacco plants and ground them in phosphate buffer supplemented with DIECA in the 1:4 plant material to buffer ratio (*see* **Note 9**).
9. For mock-inoculation, use leaves from non-inoculated tobacco plants.
10. Dust the first three fully developed bottom leaves of 3–4 weeks old potato plants (from Subheading 3.1, **step 6**) with carborundum powder.
11. Rub the leaves with the inoculum (~60 μL per leaf).
12. After 10 min, wash the leaves with tap water.

3.3 Following Virus Spread by Confocal Microscopy

Presence of the virus is confirmed indirectly by the GFP accumulation using confocal microscopy. To follow the occurrence of virus multiplication and/or its spread outside the cell death zone in

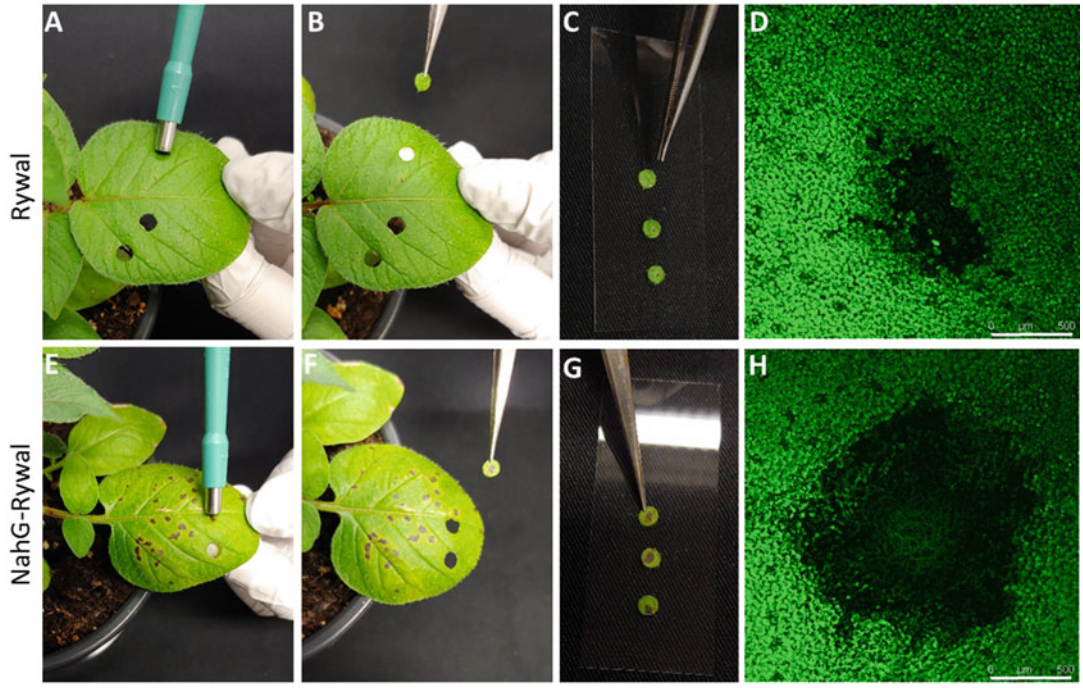

Fig. 1 Sampling lesions and surrounding tissue for confocal imaging. Tissue sections were excised at 7 days post inoculation from the second inoculated leaf from plants of genotypes Rywal (**a**, **b**) and NahG-Rywal (**e**, **f**). Tissue sections were placed on the cover glass and covered (**c**, **g**). The lesions were located by following chlorophyll fluorescence using confocal microscope (**d**, **h**). Black: lesion, green: chlorophyll fluorescence. The scale is 500 μm

potato plants, the GFP fluorescence should be followed in the tissue adjacent to cell death zone from 3 to 7 days post inoculation by confocal microscopy (*see* **Note 10**).

1. On each day, excise five lesions with a surrounding tissue from the second inoculated leaf from three plants of each genotype (*see* **Note 11** and Fig. 1a, e).
2. Place them on the cover glass, add a drop of water, and cover with another cover glass (Fig. 1b, c, f, g).
3. Using confocal microscope, scan the adaxial surface of first leaf section with 488 nm laser and collect the emission in the channel for chlorophyll fluorescence under low magnification to locate the lesion in the center of the field of view (hereafter region of interest) (*see* **Note 12** and Fig. 1d, h).
4. Zoom in the region on the edge of cell death zone in the same channel (*see* **Note 13**).
5. Generate a z-stack by collecting fluorescence emissions sequentially through two channels (GFP and chlorophyll fluorescence) after excitation with 488 nm laser (Fig. 2a, c) (*see* **Notes 14** and **15**).

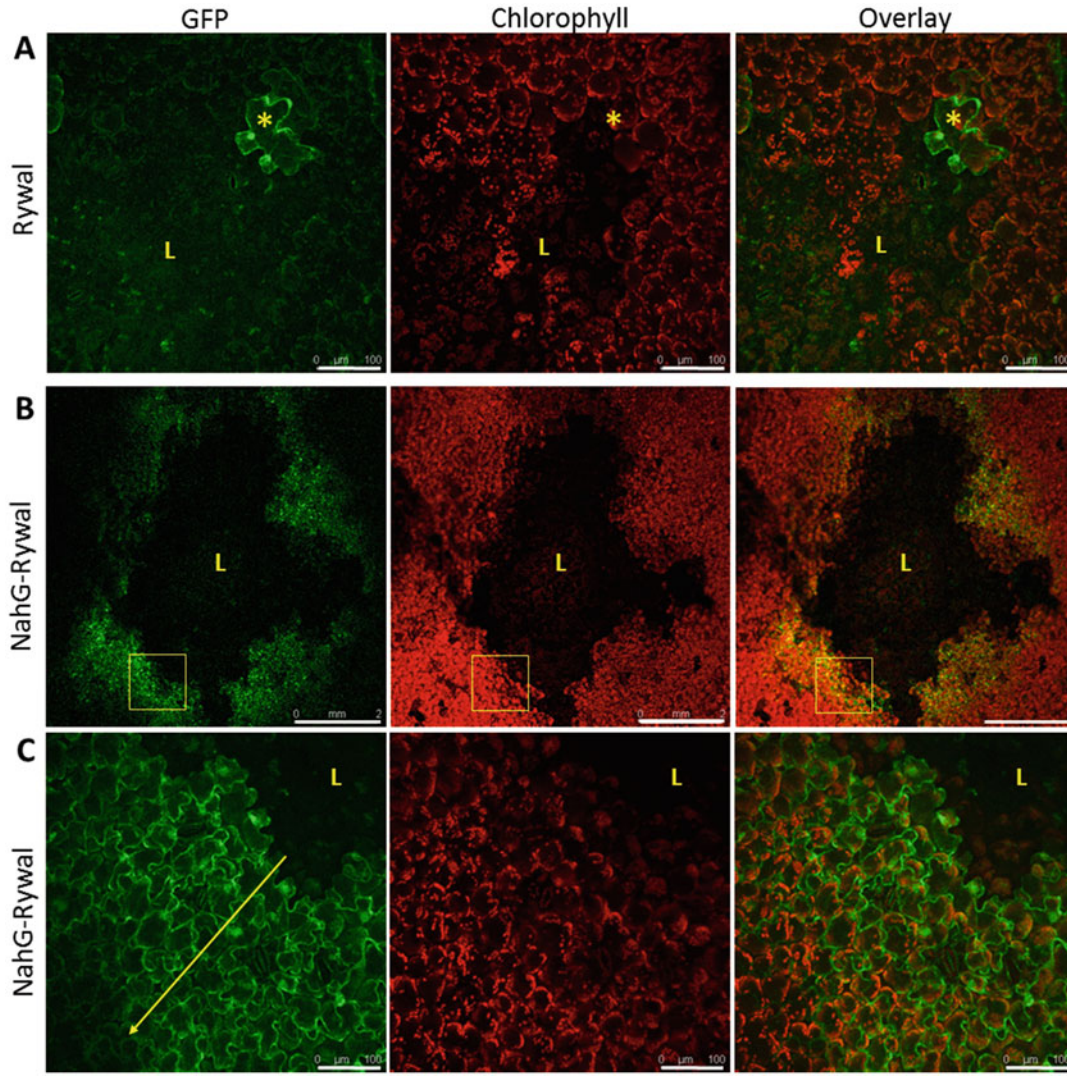

Fig. 2 Occurrence of virus-infected cells and/or virus multiplication outside the cell death zone. We followed GFP fluorescence in Rywal (**a**) and NahG-Rywal (**b**, **c**) 7 days post inoculation with GFP-tagged potato virus Y (PVY-N605(123)-GFP). The area marked with a yellow square is presented in (**c**). From left to right: PVY-N605 (123)-GFP accumulation (green), chlorophyll fluorescence (red), overlay of chlorophyll fluorescence and PVY-N605(123)-GFP accumulation. Asterisk indicates the infected cell located outside the cell death zone. L: lesion. Direction of the virus spread is presented by the arrow. Virus has spread to the fifth line of epidermal cells if counting from the edge of the lesion. The scale is 100 μm (**a** and **c**) or 400 μm (**b**)

In the control genotype (NahG-Rywal in our case), select three regions of interest according to the GFP fluorescence that you observe under low magnification (Fig. 2b, one selected region is marked by yellow square). In the studied genotype (cv. Rywal in our case), the GFP fluorescence is not visible under low magnification due to fewer cells with virus multiplication and, consequently, lower amount of GFP. Therefore, three regions of interest should be selected randomly.

3.4 Calculating the Percentage of Positive Lesions and Evaluating Virus Spread

1. Once you generate z-stacks for all regions of interest (*see* **Note 16**), export them as maximum projections of single and overlayed channels (Fig. 2) (*see* **Note 17**).
2. Define the lesions for which you detect GFP in the cells outside the death zone as positive lesions (*see* **Note 18**).
3. Calculate the percentage of positive lesions for each time point by dividing the number of lesions in which the PVY-N605 (123)-GFP accumulation was detected in at least one cell outside the cell death zone by the number of all analyzed lesions and present the results on the graph (Fig. 3).
4. To evaluate the virus spread, determine the number of the GFP-containing cells in the analyzed regions around the positive lesions. In case of uncountable GFP-containing cells due to extensive virus spread (e.g., NahG-Rywal at later time points), define the number of the GFP-containing cells as uncountable and only determine the number of infected cells in one line stretching perpendicular to the cell death zone (as an example, 5 rows of epidermal cells surrounding the cell death zone are presented by an arrow in Fig. 2c).

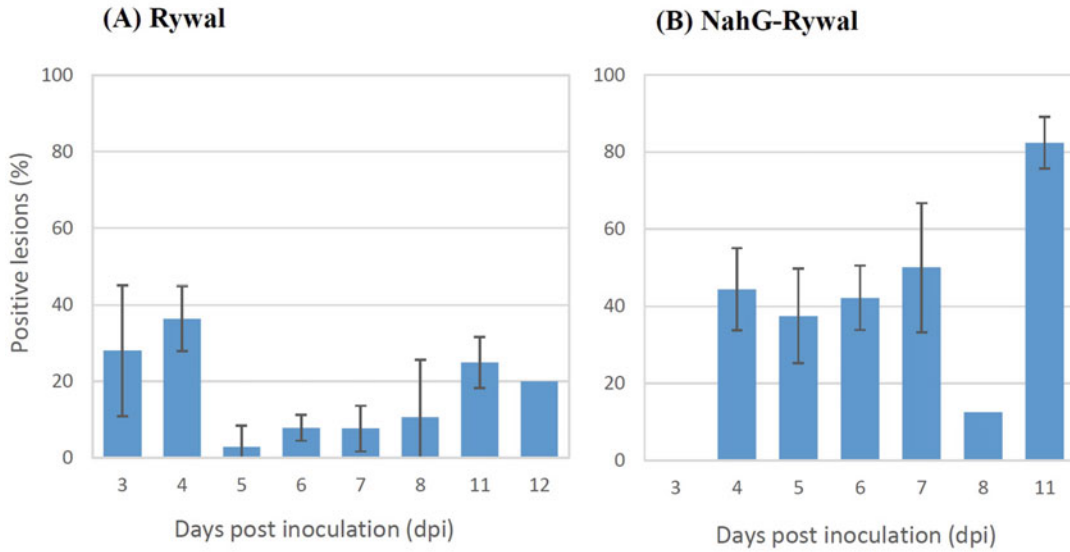

Fig. 3 Percentage of lesions with detected PVY-N605(123)-GFP accumulation outside the cell death zone. The percentage of positive lesions was calculated as the number of lesions with GFP detected in at least one cell outside the cell death zone, divided by the number of all analyzed lesions in Rywal (**a**) and NahG-Rywal (**b**) at different days post inoculation. Error bars represent the standard errors. Individual data are available in [9]. The Figure is republished from Cell death is not sufficient for the restriction of potato virus Y spread in hypersensitive response-conferred resistance in potato, Lukan et al., 2018, Frontiers in Plant Science, 9, 168. https:/doi.org/10.3389/fpls.2018.00168, according to Creative Common Attribution ("CC BY") licence

4 Notes

1. For other potato genotypes or other plant species, modifications of some parameters such as environmental conditions for plant growing, inoculum preparation, confocal parameters settings might be required.
2. MS30 medium can be stored at 4 °C for a few months.
3. Solutions A and B can be prepared in advance and stored at −20 °C.
4. The phosphate buffer for tobacco inoculation can be stored at 4 °C for 1 month.
5. The phosphate buffer with DIECA for potato inoculation can be stored at 4 °C for 1 month.
6. Inoculum consists of homogenized leaves of tobacco (*N. clevelandii*) plants containing PVY-N605(123)-GFP virus in 20 mM phosphate buffer for tobacco inoculation. Plant material to buffer ratio in the inoculum is 1:1 (1 mL of phosphate buffer per each gram of plant material).
7. Excite GFP with 488 nm laser and follow emission between 505 and 530 nm. For inoculum preparation use only the infected areas of the leaves with comparable intensity of GFP fluorescence.
8. To maintain PVY-N605(123)-GFP virus, inoculate 8 tobacco plants every 2 weeks. Weigh the selected areas of upper non-inoculated leaves of tobacco plants, ground them in phosphate buffer for tobacco inoculation in the 1:4 plant material to buffer ratio (4 mL of phosphate buffer per each gram of plant material) and use for tobacco inoculation. Tobacco plants should be inoculated with a new aliquot of frozen inoculum after 12 passages (every 6 months). To store the inoculum at −80 °C, weigh the selected areas of upper non-inoculated leaves of *Nicotiana clevelandii* plants and ground them in phosphate buffer for tobacco inoculation in the 1:1 plant material to buffer ratio (1 mL of phosphate buffer per each gram of plant material).
9. Use BIOREBA extraction plastic bag with a small amount of buffer and grind with automatic or manual device. When homogenized, add the remaining buffer. To inoculate 10 plants, 1 g of plant material is needed.
10. Samples could also be analyzed at additional time points. In this case, the number of the inoculated plants should be adjusted accordingly. Avoid late time points (after 7 dpi), since the senescence results in higher autofluorescence.

11. For the excision of plant tissue, use the tool for sampling. Each plant should be discarded after sampling and a new plant should be used on the next day.
12. Use 5× objective. Optical and digital zoom should be set according to the lesion size (higher at early time points). Laser: 488 nm. Emission window: 505–530 nm.
13. Zoom in by increasing optical zoom.
14. To reduce background noise, each region of interest should be scanned twice (line average 2 in the software settings will take the sum of pixel values from each of two scans and use arithmetic mean as the final value in the image).
15. The size of a z-stack depends on each sample and should cover the whole epidermis and at least 20 μm of the mesophyll.
16. Z-stacks should be obtained for three regions of interest for each of 5 lesions on the second leaf of three plants for each genotype.
17. Export images in a tiff format.
18. The cell is considered to contain the PVY N605-GFP, when the GFP fluorescence is observed in the cytoplasm.

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Chapter 22

A User Guide to Validation, Annotation, and Evaluation of N-Terminome Datasets with MANTI

Fatih Demir and Pitter F. Huesgen

Abstract

A large variety of enrichment procedures for protein N-termini have been developed to trace protease activity and determine precise cleavage sites, as well as other N-terminal protein modifications. Typically, enriched N-terminal peptides are identified by tandem mass spectrometry using standard database search engines, in many cases the popular MaxQuant software package. **M**axQuant **A**dvanced **N**-**t**ermini **I**nterpreter (MANTI) is a software package that helps to validate, annotate, and visualize peptide identifications in N-termini datasets in a rapid and straightforward manner. Usage of MANTI and especially its graphical interface Yoğurtlu MANTI in detail are described to enable users to take full advantage of the software package and the multitude of options it has to offer.

Key words N-termini, N-terminomics, Proteolysis, Positional proteomics, Bioinformatics, MANTI, Analysis

1 Introduction

Protein N-termini define distinct proteoforms derived from proteolytic processing, protein maturation, use of alternative translation initiation sites or alternative splicing [1]. Therefore, N-terminal peptides generated in shotgun proteome digests can be used as a proxy to identify such proteoforms on a proteome-wide scale. Similar to other post-translational modifications, detection of N-terminal peptides in complex proteome background is challenging but facilitated by specific enrichment. A large variety of different protocols have been developed to enable enrichment, identification, and relative quantification of protein N-terminal peptides across multiple conditions [2, 3]. All result in samples that are highly enriched in protein N-terminal peptides, which are subsequently analyzed by nano LC-MS/MS using standard bottom-up proteomics methods. Next, software packages such as MaxQuant [4], OpenMS [5], Proteome Discoverer [6], and TPP [7] are used

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to match acquired tandem MS spectra to peptide sequences in protein sequence databases using "semi-specific" searches. In specific searches, the search engine is looking for the cleavage specificity of the protease used for proteome digestion on both sides of the identified peptides (e.g., Arg and Lys for tryptic digests). In contrast, semi-specific free N-terminal searches are considering peptides that are only delimited C-terminally by the digestion protease, but are variable on the N-terminal side (e.g., are not required to be after Arg or Lys for tryptic digests). Applying the semi-specific search strategy enables high-throughput identification of N-terminal peptide sequences.

The interpretation of the resulting datasets requires a high degree of experience with N-termini dataset handling and can be challenging using standard protein-centric tools. To overcome this hurdle, we developed MANTI: MaxQuant Advanced N-termini Interpreter [8]. MANTI is a user-friendly software package to semi-automatically validate and evaluate protein N-termini datasets queried with MaxQuant (*see* **Note 1**). The identified N-termini are annotated and classified by a variety of sources with respect to known processing events such as signal-, transit-, or propeptide cleavages (canonical cleavages) and other position-specific features. This clearly separates the canonical cleavages from yet unknown N-termini including neo-N termini arising from proteolytic cleavages (noncanonical cleavages). The primary source for this information is UniProt [9] when UniProt FASTA protein databases are used during the database search with MaxQuant. However, MANTI can also handle custom databases and associated annotation information, including predictions of subcellular localization and protein maturation sites from tools like LOCALIZER [10] or TargetP2.0 [11]. For human and murine data, the TopFINDer and PathFINDer tools incorporated in the TopFIND database enable additional annotation of proteases known to cut at or near the identified N-termini and the activity of proteolytic cascades [12]. MANTI is able to handle datasets from MS1-based quantification as determined by MaxQuant, including stable isotope labeling by reductive dimethylation [2] and, correspondingly, other stable isotope labeling techniques like SILAC or acetylation/butyrylation (*see* **Note 2**) as well as quantification by MS2 reporter ion intensity from, e.g., TMT [13] labeling. Overall, MANTI features a high degree of automation in the processing of N-termini results. With the current version 5.0 of MANTI, a graphical interface named Yoğurtlu MANTI accompanies the MANTI software package, which offers a convenient interface and enables the user to take advantage of the many features and options of MANTI.

2 Materials

1. MANTI is a Perl software package, open source and freely usable under the Perl Artistic License 2.0 (https://www.perlfoundation.org/artistic-license-20.html). Obtain corresponding packages, manuals, and auxiliary annotation data from MANTI's project homepage at https://MANTI.sourceforge.io. The core MANTI.pl program only requires a Perl interpreter installed on the local computer, which are already installed on most Linux platforms. Users on other platforms can obtain Perl distributions like Strawberry Perl (http://strawberryperl.com/, for Windows) and ActiveState Perl (https://www.activestate.com/products/perl, available for a variety of operating systems).
2. The graphical interface for MANTI, Yoğurtlu MANTI, is written in Perl/Tk and thus requires the Perl/Tk bindings to be installed on the computer. Without Perl/Tk, you can still take full advantage of the core command line MANTI program but will not be able to use the graphical interface. To overcome this hurdle, a ready-to-use Windows 10 ×64-platform version of Yoğurtlu MANTI is distributed with each MANTI version and requires no Perl installation on the user's computer.
3. In addition to the core functionality of MANTI, take advantage of two supplied R scripts to produce a set of versatile visualizations, namely polvops.R for volcano plot generation and bufipretty.R for the generation of general data processing and evaluation plots. Those auxiliary R scripts require a set of R packages to be installed, all of those can be obtained via CRAN: GGally, UpSetR, ggcorrplot, ggpubr, ggrepel, stringr, svglite. Install missing R packages in one run with the helper R script "MANTI_R_InstallPackages.R," which comes with MANTI v5.0+.
4. MANTI requires several files of the MaxQuant database search result ("txt") folder to operate: (1) summary.txt, (2) parameters.txt (optional), (3) peptides.txt, (4) modificationSpecificPeptides.txt, and (5) proteinGroups.txt.

3 Method

3.1 Standard MANTI Workflow with Yoğurtlu MANTI Interface

1. Start the Yoğurtlu MANTI interface by double-clicking the corresponding .exe file under Windows systems or the corresponding .pl file containing the source code under other operating systems.
2. In the MANTI GUI, define the location of the MaxQuant search results by selecting the "txt/summary.txt" file and the

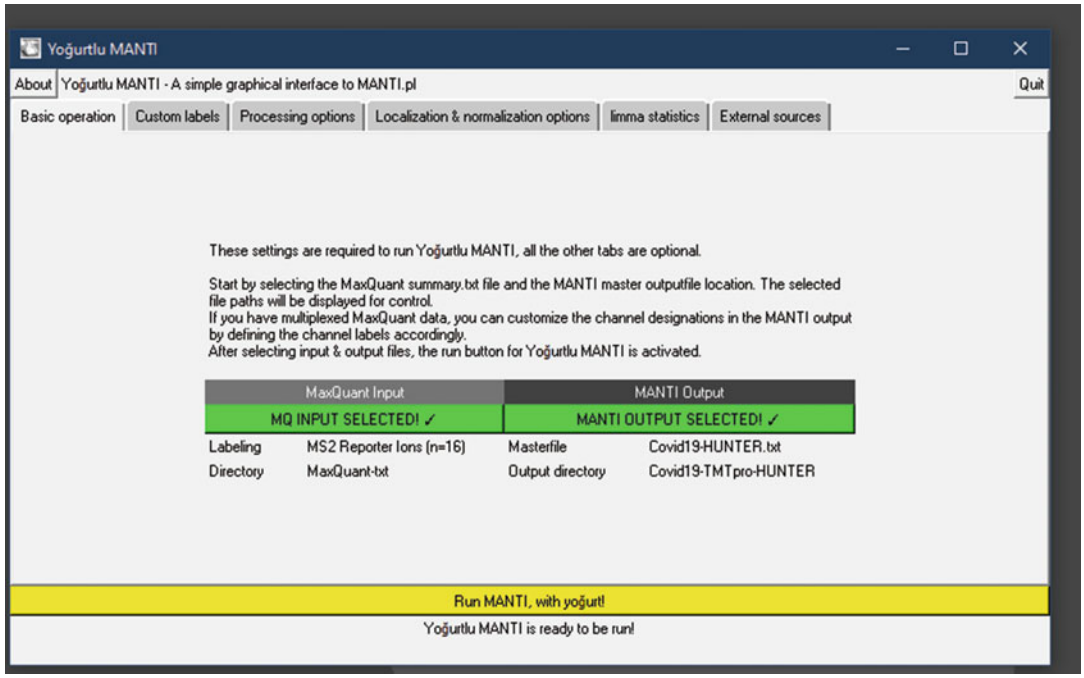

Fig. 1 Main window for the Yoğurtlu MANTI graphical interface to MANTI. Define all parameters required for MANTI operation in the first tab of the graphical interface. Select the location of the MaxQuant "txt" folder and define the output file/folder location and start the MANTI processing with default settings

folder for the MANTI output "masterfile." After the selection of the input and output files, MANTI will display the automatically detected labeling strategy (Fig. 1) and allow the user to directly start the MANTI processing with default settings (on the command line this can be triggered by calling MANTI with the single option **–y**):

(a) Fetch UniProt annotation (--**annotate**).

(b) Unmatched feature annotations are not displayed (--**blind-feature**).

(c) Do not report feature details in distance fields (--**quiet-dist**).

(d) Report direction of distance to annotated feature (--**with-sign**).

(e) Use alternative, stability-centric amino acid map order (--**z-aa-map**).

3. Look up the results in the defined "Masterfile" folder location. MANTI automatically parses the corresponding files from the given MaxQuant "txt/" result folder, starting with the "modificationSpecificPeptides.txt" file and saves the analysis results in different files and directories relative to the defined "Masterfile" (Fig. 2).

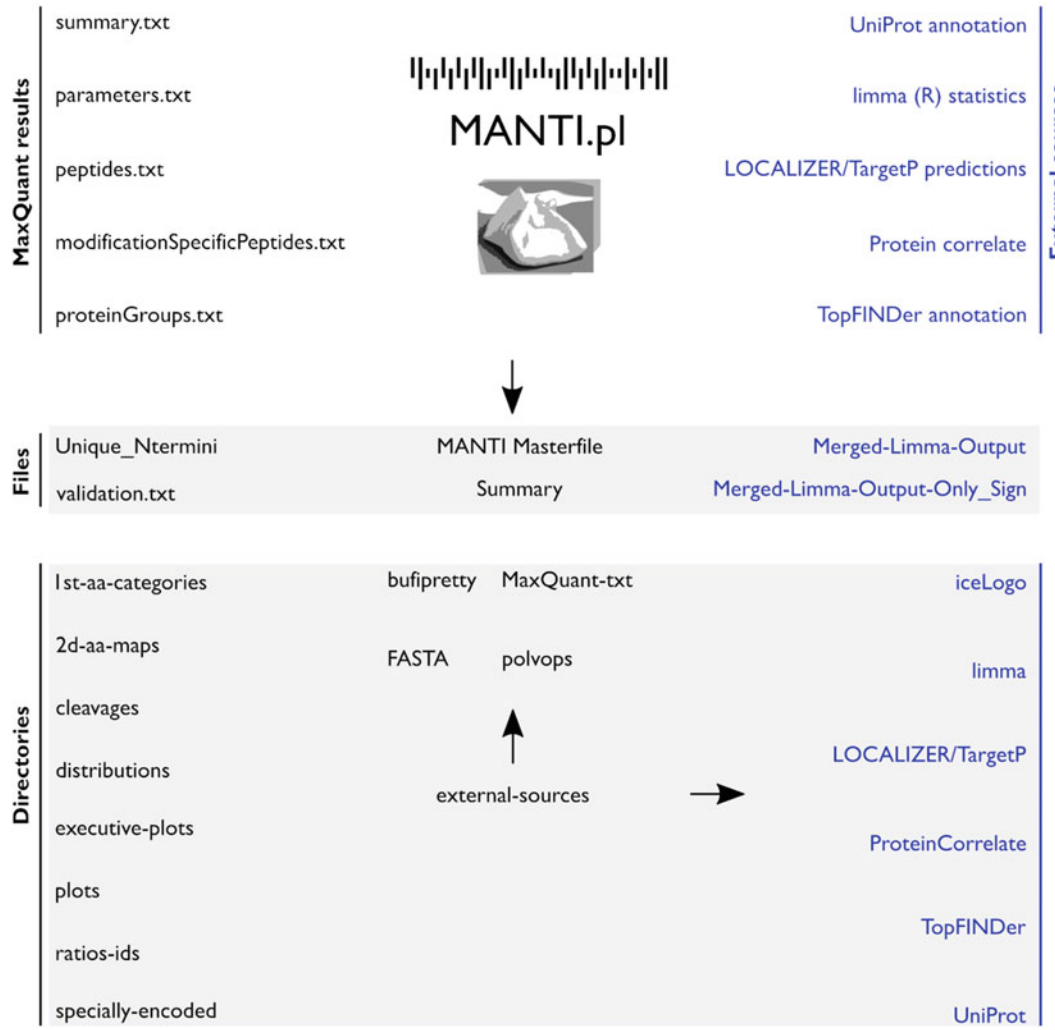

Fig. 2 MANTI sources and output files and directories. The MANTI Masterfile is the central data assembly where most information is gathered. All incorporated external raw files (blue) are copied into the external-sources folder in addition to the MaxQuant and MANTI supplied folders to allow a complete recapitulation of the MANTI workflow. Supply the parameters.txt file during MANTI runtime to gain detailed information in the MANTI summary about the MaxQuant query parameters and the used FASTA database

4. MANTI lists all validated N-terminal peptides in the MANTI masterfile, whereas all details about the MANTI processing are stored in the MANTI summary file (*see* **Note 3**). Additionally, general characteristics like the number of significantly up-/downregulated canonical/noncanonical N-termini as well as the "grouped experiment" name is given in the summary file (*see* **Note 4**) for MS1-quantified data (*see* **Note 5**).
5. Check the validation.txt file for the results of the N-terminal peptide validation process to exclude systematic errors (*see* **Note 6**).

6. For graphical visualization, generate data plots using the two supplied R scripts: (1) polvops.R for limma-based significance testing and volcano plot generation, and (2) bufipretty.R for a large set of auxiliary visualizations of the N-termini dataset. Run the R scripts in this specific order, as bufipretty.R incorporates statistical tests for significant changes in abundance from the polvops.R run: first run polvops.R, then reprocess the data with MANTI in order to incorporate the generated statistical test information, and finally, generate the corresponding auxiliary visualizations with bufipretty.R including the significance information from the previous polvops.R run.
7. As an alternative, select the "TERMINATOR" option in the GUI (Fig. 3) for automatic execution of polvops.R to perform

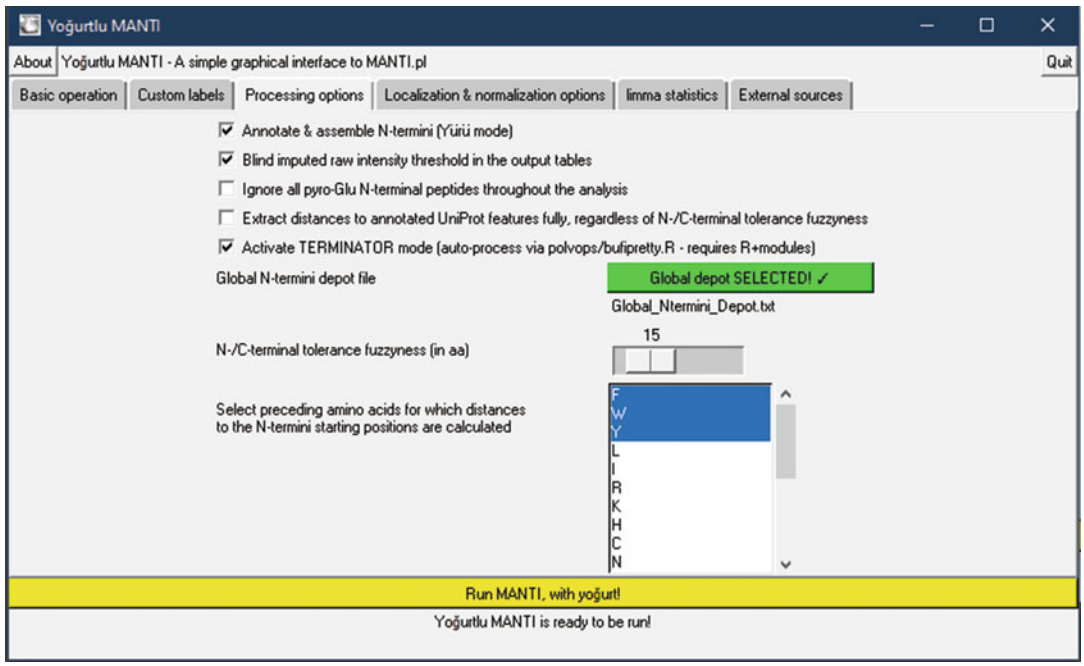

Fig. 3 Set custom processing options for MANTI. Set the main processing settings in the tab "Processing options," where the annotation of N-termini by UniProt data is per default enabled (first option) and imputed raw intensities can be set to 0 instead of the imputed value (second option) for the intensity-based ratios (MaxQuant ratios will not be altered, *see* **Note 8**). Additionally, all pyro-Glu N-terminal peptides can be removed from the output (third option), and the distances to the annotated UniProt features can be printed in full distance (fourth option) regardless of the settings from other distance options. Activate the automated TERMINATOR mode (fifth option) to perform polvops.R and bufipretty.R processing in one run (for dimethylated or SILAC-labeled samples). Select a global N-termini depot file (sixth option) to populate a common text file with the N-termini from this analysis—an existing global depot file will be extended by new N-termini. Adjust the tolerance for annotation feature matching with a "fuzzyness" in amino acids (seventh option), which is the uncertainty of the exact feature location in the protein sequence which is still allowed to match (*see* **Note 9**). Select the amino acids for which a preceding amino acid analysis should be performed (eighth option); correspondingly, the distance of the N-terminal peptide start position to the given preceding amino acid(s) is available as an additional column in the MANTI Masterfile and bufipretty.R generates plots with the distances

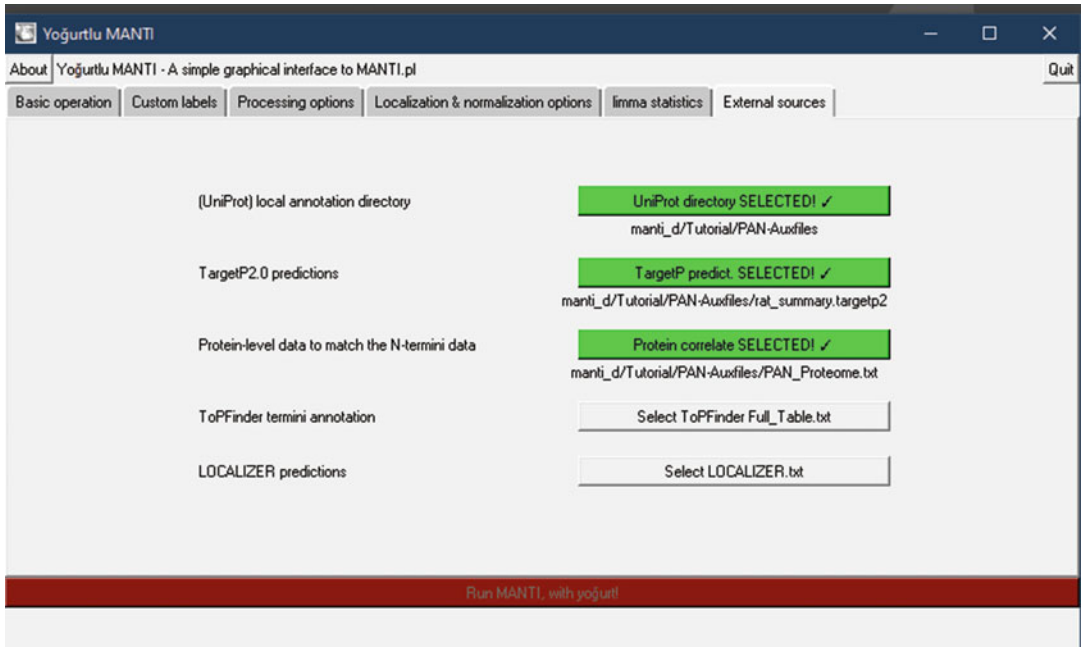

Fig. 4 Select external sources for MANTI annotation. Integrate additional annotation from external sources like TargetP2.0, proteome-level information (e.g., shotgun proteome data), ToPFinder, and LOCALIZER. MANTI will incorporate the annotation for the N-terminal peptides according to the UniProt ID. Select an existing offline UniProt data directory in this dialogue to avoid *on the fly*, online UniProt queries. Download the corresponding UniProt annotation for offline processing from MANTI's project homepage

statistical tests, reprocess the MANTI analysis results, and visualize the data with bufipretty.R in the correct order.

3.2 Annotate Protein N-Terminal Peptides

1. Integrate multiple external sources into MANTI to enrich the N-termini analysis with multiple additional annotation sources (Fig. 2, in blue).
2. For data analyzed with UniProt databases, cleavage-specific information from TopFinder database queries and signal-/transit peptide predictions from LOCALIZER/TargetP2.0 are integrated *on the fly* during MANTI analysis based on the UniProt accession number (Fig. 4) and require no interaction from the user.
3. Supply UniProt or any other annotation data offline to avoid network traffic and to speed up analysis times for MANTI. Corresponding UniProt annotation data packages for common organism are available for download on the MANTI project webpage: https://sourceforge.net/projects/manti/files/UniProt-Annotation/.

3.3 Correlate and Visualize N-Terminal Peptide Abundance with Protein Abundance

1. Integrate data on protein abundance automatically with MANTI. MANTI refers to the corresponding protein-level information as the "protein correlate." Provide the protein correlate as results of a quantitative proteome analysis of the same sample, e.g., shotgun proteome or corresponding pre-TAILS [14] or preHUNTER [2] samples before N-termini enrichment. MANTI will directly display protein ratios as well as existing statistical t-test results (*see* **Note 7**) for protein abundance data in the MANTI Masterfile and the corresponding column names will have a prefix "PROT:".
2. Alternatively, import the corresponding data from Perseus-exported text files [15]. Columns required for the imported proteome-level data are log2FCs (*see* **Note 7**), Protein IDs, Majority protein IDs, Peptides, and Unique Peptides which are by default already included in a Perseus export.
3. Integrate proteome-level data to improve the determination of the preferred protein ID: if on proteome-level a clear, distinct majority protein ID is assigned for the corresponding protein IDs (e.g., due to unique razor peptide assignments), then this distinct majority protein ID is also applied as the preferred protein ID for corresponding N-termini during MANTI analysis.
4. Visualize the correlation of changes in protein and N-terminal peptide abundance in graphs, generated for each data series (Fig. 5). Determine alternatively processed proteins where proteolysis regulates the abundance with the help of those graphs.

3.4 Analyze Using Custom Databases

1. Use custom databases as an alternative to UniProt for specific organisms, which are not (yet) well annotated in UniProt. Supply such local databases as a custom (UniProt) annotation in the GUI (Fig. 4).
2. Store the custom, local annotation within one directory and place a .fasta file with protein sequences in standard FASTA annotation. Optionally, supply a feature mapping as a .gff file and additional feature annotations as a .tab file. MANTI downloads those files automatically from UniProt during the annotation process for UniProt protein IDs. Corresponding UniProt output files are shipped as reference with each MANTI version under the "Tutorial\PAN-Auxfiles" folder. Any user can prepare these files with local existing annotation information: these files are all plain text files. If only sequence information is available, simply put no .gff and .tab files into the local annotation directory.
3. Identification of signal- or transit peptide cleavage sites and classification of canonical cleavages will not be possible without

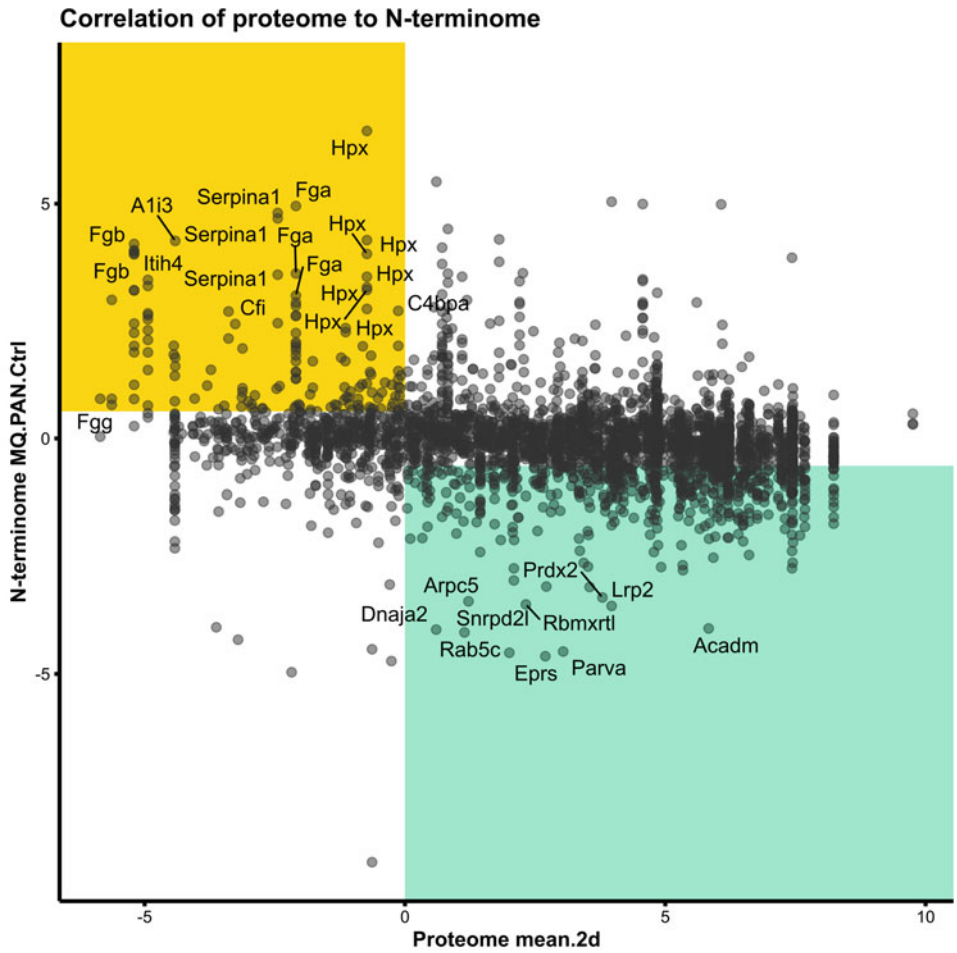

Fig. 5 N-terminome and proteome regulation for the PAN tutorial dataset. Abundance of the N-terminal peptides plotted against the abundance of the corresponding proteins for the PAN reference dataset contained in the MANTI package. N-terminal peptides, which are at least 50% up- or downregulated (log2FC $\pm$ 0.58) in opposite direction to the protein abundance from the corresponding proteome are highlighted in the upper yellow and lower green shaded area of the plot. This plot allows identification of site-specific proteolysis with or without coupled bulk protein degradation

UniProt or local annotation data resources. For non-UniProt databases we encourage the user strongly to predict such processing sites with tools like LOCALIZER [10] or TargetP2.0 [11]. Use TargetP2.0 online at https://services.healthtech.dtu.dk/service.php?TargetP-2.0 (current maximum of 5000 protein sequences per query) or offline as a stand-alone tool under Linux to prepare the predictions for a custom protein sequence database. Present the predictions as a local data resource in MANTI to allow annotation of the signal-/transit peptides, and correspondingly classify the N-termini as

canonical or noncanonical N-termini. This facilitates interpretation of N-termini data greatly for non-standard organisms with poor or no annotations in UniProt.

4 Notes

1. MaxQuant [16] is currently one of the most common software packages for MS1- and MS2-based quantitation. MaxQuant does not designate stable isotope labels as modifications; hence, all non-Met-oxidized peptides are classified as "Unmodified" in the MaxQuant output files, but are labeled correspondingly.
2. MANTI relies on the assignment of the set labels in MaxQuant for the stable isotope labeling detection; the user can choose SILAC-labeled amino acids [17] or acetylation/butyrylation, like for COFRADIC [18], as alternatives to the reductive dimethylation [19]. For the functionality of MANTI, this does not pose any restrictions.
3. The MaxQuant query specifications (variable modifications, labels, experiment names) are stated in the summary file to allow proper validation and interpretation of the MANTI analysis. In addition, the distribution of raw intensities, MaxQuant ratios and their separation among experiments as well as optionally limma statistics (if limma statistics were integrated via the "--join-lm" switch or automatically by the TERMINA TOR mode) are written into the MANTI summary file.
4. Labeling and mean-calculation is performed for systematically labeled experiments like "Control R1," "Control R2," etc. but cannot detect all enumeration/labeling schemes which the user might choose. The matching of the column is performed by regular expressions matching any names with a lower- or upper-case R + digits; the experimental names can be separated by the following characters: "-", "_", "#", and ".". Constant experimental names ending with a "R[NUMBER]" are recognized automatically, and correspondingly grouped as a "pure experimental name" which is used for limma analysis by polvops.R and the generation of all corresponding plots by bufipretty.R. Thus, a constant and consistent naming scheme is strongly encouraged.
5. Take into consideration that MANTI tries to guess experimental designs for dimethylated/SILAC-labeled data, but cannot perform such automated processing for MS2 reporter ion quantifications like iTRAQ/TMT or label-free experiments ("simplex") which are only labeled within one channel.

6. Follow the validation chain of MANTI in the validation.txt file, which lists all non-valid peptide identifications. This also includes N-terminally acetylated peptides in multiplex MS1 experiments without any lysine or arginine in the sequence, where sequences are retained but the corresponding quantitative information blinded in the MANTI Masterfile as the intensities cannot be attributed to a certain channel. Reverse and contaminant protein entries as well as non-valid pyro-Glu peptides are automatically filtered out during this process. All entries in the validation.txt file feature the protein IDs, the corresponding peptide sequence, and the rationale for the non-validation.
7. The corresponding columns of the proteome data file are matched case-insensitive, so upper- or lower-case designations will be ignored during matching. Names for the log2 fold changes have to be "log2FC" or "N: log2FC" (in case of a Perseus-exported dataset, the columns will have the according prefixes) and the columns for the statistical t-test results have to be named "t-test Difference," "t-test Significant," and "-Log t-test p-value" as defined by default in Perseus. Multiple t-test results from a Perseus-exported analysis are also imported automatically. If you manually performed your statistical analysis, we advise to take over the Perseus labeling scheme for the corresponding columns to allow an easy integration of the results within MANTI.
8. Select this option to enable direct discrimination of non-quantified experiments for a peptide. MANTI performs automatically an imputation of the raw intensities (missing values are imputed by the lowest quantified peptide intensity—1) and builds custom, MANTI-specific "log2 Int" ratios of the raw intensities including imputed intensities. Without the option, the user cannot easily discriminate the imputed values from real measurement values—thus, setting this option allows to "blind out" the imputed raw intensities in the output tables.
9. The standard fuzzyness in MANTI is set to five amino acids; if cleavage of the signal peptide is predicted to be at position 75 of the protein but the identified N-terminal peptide starts with amino acid 79, it is not an exact match of the N-terminal peptide to the cleaved signal peptide position. As the distance of the N-terminal peptide start position and the predicted signal peptide cleavage in UniProt or LOCALIZER/TargetP2.0 is below the fuzzyness threshold of five amino acids, this N-terminal peptide will be classified as a canonical cleavage. If an N-terminal peptide would start at position 82, it would be classified as a noncanonical N-terminal peptide, as the distance to the signal peptide cleavage is larger than the set fuzzyness

threshold of five amino acids. One can observe a one or two amino acid deviation for the N-terminal peptide start position from the predicted signal or transit peptide cleavage and the fuzzyness distance option allows for compensation of this uncertainty in signal or transit peptide predictions. Setting the fuzzyness threshold to zero amino acids (Fig. 3, seventh option) allows the user to accept only exact matches of the predicted cleavage positions and the N-terminal peptide start position.

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Chapter 23

Exploring Posttranslational Modifications with the Plant PTM Viewer

Patrick Willems

Abstract

An increasing numbed and diversity of protein posttranslational modifications are mapped by proteomics. Knowledge of modified protein sites can be of direct relevance to steer downstream functional studies, but such information can be difficult to distill from the numerous individual studies that often report modified sites in supplementary data files. In this chapter, we provide basic instructions and use cases to browse and collect plant protein modifications from over 100 studies in a user-friendly manner with the Plant PTM Viewer. The Plant PTM Viewer (https://www.psb.ugent.be/PlantPTMViewer) is a central resource compiling currently 24 modifications types for ten thousands of plant proteins. Next to viewing modifications for a protein of interest, we demonstrate how evolutionary conserved modifications can be retrieved using PTM Blast. In addition, modifications themselves located in a motif or sequence context of interest can be retrieved by PTM search. Altogether, these operations can be performed, are intuitive, and open for non-bioinformatic experts.

Key words Posttranslational modifications, Plant PTM viewer, Proteomics, PTM, Phosphorylation

1 Introduction

Posttranslational modifications (PTMs) give rise to different chemical protein forms, or proteoforms, that are encoded from a single gene. Protein modifications can be enzymatically added, removed, or recognized and can alter protein function in diverse ways. The importance of PTMs is highlighted by the proportion of protein modifying enzymes encoded in the genome, for instance, in *Arabidopsis thaliana* (Arabidopsis), 1000 protein kinases [1] and 570 peptidases [2] together comprise ~6% of the genome. Given the importance of PTMs in protein regulation and physiological processes, proteome-wide PTM discovery technologies have been actively developed during the last decades. Advances in mass spectrometry (MS) and PTM enrichment techniques now routinely allow for the identification of hundreds or even thousands of PTMs in single experiments. For instance, a total of 43,903

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phosphorylation sites were identified and quantified in multiple tissues in an exhaustive Arabidopsis proteome atlas study [3]. Evidently, databases that compile and present protein PTMs of interest have become an indispensable tool for researchers. We developed the Plant PTM Viewer that currently integrates 24 different plant PTM types mapped on ten thousands of proteins by 122 different publications for five plant species (https://www.psb.ugent.be/PlantPTMViewer) [4]. In this chapter, we provide instructions for the various interactive tools offered by the Plant PTM Viewer. Besides browsing PTMs for a protein of interest, this also includes searching for PTMs aligning in homologous protein regions and searching for PTMs in user-defined amino acid sequence motifs of interest.

2 Materials

Browsing of the Plant PTM Viewer can be performed on any computer, smartphone, or tablet with internet connection and is compatible to any web browser. Hence, no specialized equipment is required and plant PTMs are available to everyone without restrictions. The Plant PTM Viewer is PHP-based and uses a MySQL relational database to store PTM data and associated meta-data. Currently, data from 122 publications is available for 24 PTM types. This number will increase in the coming years, as users can submit new datasets to the Plant PTM Viewer (see "Submit" page, https://www.psb.ugent.be/webtools/ptm-viewer/submit.php). PTM data submissions can be kept in private modus with an encrypted link for reviewer access during manuscript submissions.

3 Methods

3.1 Browsing and Functional Interpretation of PTMs for a Protein of Interest

For individual protein studies, knowledge of identified PTMs is of special interest and can lead to novel biological hypotheses and inspire downstream studies. For instance, Cys oxidation sites can pinpoint redox-sensitive or active sites of proteins, whereas N-terminal processing sites can hint at degradation sites or stable N-termini , *e.g.* chloroplastic or mitochondrial proteoforms where the N-terminal transit peptide has been truncated. After retrieving a protein of interest, PTMs and associated meta-data (identified peptides, quantification, confidence scores, ...) can be viewed in a tabular format next to an annotated protein sequence highlighting the respective PTM positions. In addition, protein domains and active sites can be highlighted and consulted interactively. Taken together, this not only enables the "site-seeing" of PTMs, but also an initial functional assessment.

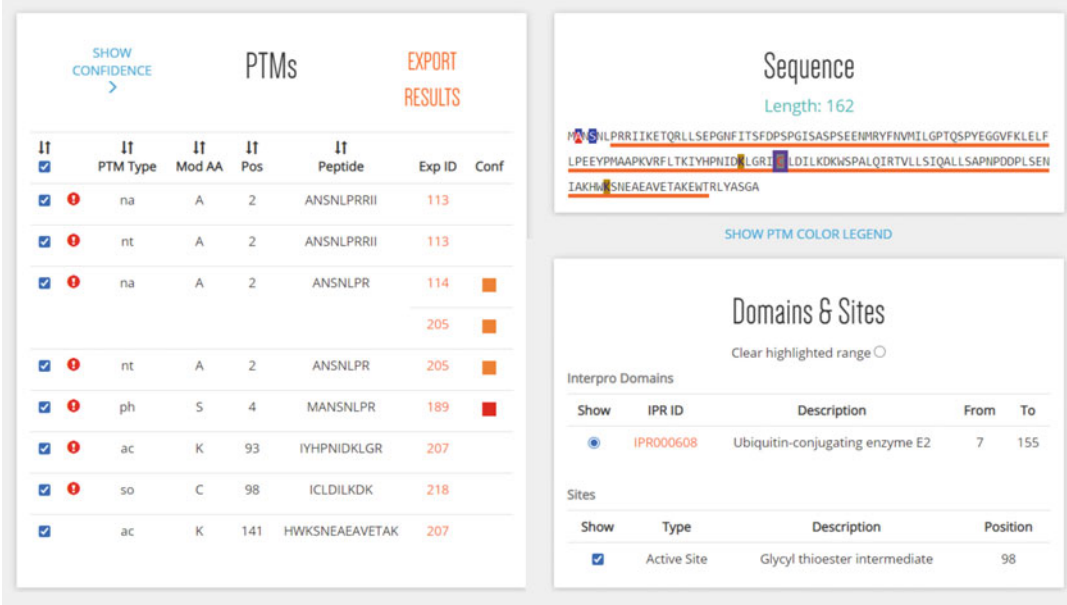

Fig. 1 Protein PTM overview of UBIQUITIN-CONJUGATING ENZYME 36 (UBC36, AT1G16890.3). All PTMs, protein domains, and functional sites were selected for visualization in the PTM Sequence overview. This overview can be accessed by https:/www.psb.ugent.be/webtools/ptm-viewer/protein.php?id=AT1G16890.3

3.1.1 Query a Protein of Interest

1. Navigate to https://www.psb.ugent.be/PlantPTMViewer.
2. Click the "Protein Search" tab.
3. Query your protein of interest using either (a) a gene accession "Search by identifier" tab sheet (*see* **Note 1**), (b) an amino acid sequence of minimal five residues that exactly matches within the protein sequence in the "Search by sequence" tab sheet, or (c) a name/description in the "Search by description" tab sheet. Click the search button.
4. A search results box will appear listing all protein isoforms (*see* **Note 2**) with their respective species, number of PTMs and different PTM types, and cross-references to PLAZA [5] and UniProtKB [6] identifiers.
5. Click the protein isoform of interest to open the protein overview. As example, we will view the protein overview of the UBIQUITIN-CONJUGATING ENZYME 36 (UBC36; AT1G16890.3 representative protein form) (Fig. 1).

3.1.2 Inspect PTMs and Their Confidence

All PTMs identified by proteomics studies are displayed in the PTM table on the left side. Each row in the table indicates a modified site and is catalogued per identified peptide sequence by MS. If a peptide matches proteins encoded from different gene loci, a red exclamation mark symbol is displayed (*see* **Note 3**). The respective study (or studies) where the peptide was identified is displayed by

an experimental identifier and can be clicked to view further experimental information. An intuitive color scale provides an impression of the identification score quality by the MS/MS search algorithm (hover over the color scale for more information). In addition, localization probabilities provided by algorithms, such as the PTM Score algorithm [7] used by Andromeda/MaxQuant, indicates the probability (ranging from 0 to 1) that a PTM is located at a respective position within the peptide. All table headers are sortable alphanumerically by clicking the double arrows. More confidence scores can be viewed by clicking the "Show confidence button" (*see* **Note 4**). In some studies, a quantitative comparison of PTM levels between conditions (*e.g.* stress versus control) is described and the log2 fold change value can be viewed in the table as well as a corresponding P- or Q-value significance, if provided. In case of our example protein, UBC36, 6 different PTM sites can be discerned for 5 PTM types. Some PTM sites are stronger supported than others. For instance, N-terminal acetylation of the second amino acid, exposed after N-terminal methionine excision, is reported by two peptides "ANSNLPR" and " ANSNLPRRII " identified in three independent MS studies. Other PTMs such as the phosphorylation of Ser4 is only identified once by a single study by a peptide with a low MASCOT score of 23. As such, this is certainly lower confident than the N-terminal acetylation site described earlier. Notably, all peptides also match the homologous protein UBC35 (AT1G78870)—and thus both or only one of these homologs could be modified by these PTMs.

3.1.3 View Modified Residues Within the Protein Sequence

The PTM Sequence overview on the top right aims to intuitively view PTMs in the protein sequence, highlighting a residue according to PTM type. To know the respective PTM type associated with the color, either consult the PTM color legend by clicking "Show PTM color legend" or move the mouse over the residue to view the two- or three-letter abbreviation. Each of the currently 24 PTMs is represented by a two- or three-letter abbreviation that can be consulted in the PTM color legend. PTMs of interest can be interactively displayed by the checkboxes present in the PTM Table. In case of UBC36, five modified positions are highlighted. Note that N-terminal acetylation (abbreviated as "na") and N-terminal proteolysis ("nt") coincide on the second residue, indicated by red/blue diagonal coloring, as first the N-terminal methionine is cleaved after which N-acetyltransferases modify the newly exposed N-terminal residue.

3.1.4 Relate PTMs to Functional Protein Domains or Sites

The "Domains & Sites" table, at the right bottom side, allows to consult and visualize annotated protein domains from the InterPro database [8] and active sites annotated in the UniProtKB database [8] (*see* **Note 5**). Both functional protein domains and sites are

interesting to inspect alongside PTMs as it enables to hypothesize on possible functional consequences of PTMs. In case of UBC36, the Ubiquitin-conjugating enzyme E2 domain (IPR000608) encompasses nearly the whole protein (position 7–155, orange underlining). Of special interest, the active site forming the glycyl thioester intermediate with ubiquitin (position 98, highlighted rectangle) is identified to be S-sulfenylated [9]. As such, Cys oxidation might impact the ubiquitin conjugation capacity and thus oxidative stress might inactivate E2 ubiquitin conjugating enzymes, as reported in yeast for instance [10].

3.2 Retrieve PTMs Within Sequence Contexts of Interest with PTM Search

It can be of biological interest to retrieve PTMs within specific sequence contexts. For instance, if for a given protease a preferred substrate cleavage specificity has been determined experimentally, potential protein substrate cleavage sites matching this specificity could be searched in the proteome. Alternatively, automated searching for PTMs residing in a specific protein domain or matching active sites could be of interest. Lastly, searching for PTMs in close proximity to other PTMs is instrumental to study PTM crosstalk. To address such questions, and more, the PTM Search is an excellent tool.

3.2.1 Querying PTMs in User-Defined Sequence Contexts

1. Click the "PTM Search" tab.
2. In the "Search expression" field, enter a protein sequence with residues and PTMs to be searched. Regular expression operators can be used for amino acid residues (see Table 1) and PTMs (see Table 2). We will later describe two specific examples of PTM Searches in a biological context (Subheadings 3.2.2 and 3.2.3).
3. After clicking "Show advanced options," diverse result filters can be chosen. By default, only hits to representative proteins (*see* **Note 2**) of genes are in the result list. In addition, it can be

Table 1
Residue regular expression (regex) operators that can be used in a PTM Search

Regex operator	Description	Query example
[ ]	Match multiple residues	MA[ER]T → MAET and MART
X or .	Match any residue	MAXT or MA.T → MAAT, MACT, MADT, etc.
?	Zero or one occurrence	MAE?T → MAET and MAT
*	Zero or more occurrence	MAE*T → MAT, MAET, MAEET, etc.
{ }	Specify a range of occurrence	MAE[19]T → MAEEET MAE{2,4}T → MAET, MAEET, MAEEET MAE{4,}T → MAEEEET, MAEEEEET, etc. MAE{,3}T → MAT, MAET, MAEET, and MAEEET

Table 2
PTM regular expression (regex) operators that can be used in a PTM Search. For PTM two- or three-letter code abbreviations—see https://www.psb.ugent.be/webtools/ptm-viewer/ptm_info.php

Regex operator	Description	Query example
xx	Any PTM type	MC(xx)T → MC(so)T, MC(ro)T, MC(no)T, etc.
|	Modified any of these PTMs	MC(so|ro)T → MC(so)T or MC(ro)T
&	Modified both PTMs	MC(so&ro)T → both MC(so)T and MC(ro)T

opted to solely return PTMs supported by peptides matching uniquely to proteins of a single gene, PTMs of a specific plant species, or PTMs residing in a specific InterPro protein domain or matching functional sites (*see* **Note 5**) of proteins.

4. After entering the search expression, and possibly selection of advanced search parameters, click "Search."
5. The duration of the search depends on the ambiguity of the search expression. If specifying very tolerant or wide search expression, the search can take longer, in contrast to very specific searches.
6. When finished, a result table will appear below with the protein identifier, description, species, and respective PTM position and neighboring residues of the searched PTM. By default, the 10 flanking amino acids are displayed (can be altered in the advanced options). If more than one PTM is searched, the position of the first and last PTM is displayed.

3.2.2 Searching Enzymatic Specificities: Protease Substrates

Protein modifying enzymes can have a certain substrate specificity, recognizing specific residue motifs. For instance, the substrate cleavage specificities were determined biochemically for five Arabidopsis matrix metalloproteinases (At-MMPs) [11]. All of these five At-MMPs cleave before Leu and/or Ile, with often a preference for Ala two residues after the N-terminally exposed residue (P3' position). Translating this into a PTM search expression results in "[LI](Nt)XA," i.e., a N-terminally exposed Leu or Ile due to proteolytic cleavage followed by any residue and Ala. Performing this query results in 22 different proteins that might be At-MMP substrates—although to be further verified by follow-up experiments.

3.2.3 PTM Crosstalk

Diverse PTMs can exert crosstalk and influence the presence of each other. For instance, different PTM types can compete for the same amino acid residue, or a given PTM can stimulate or inhibit the presence of another PTM in its proximity by affecting the recognition by protein modifying enzymes. The PTM Search can investigate PTM crosstalk instances as multiple PTMs can be searched in specific patterns. For instance, addition of β-N-acetylglucosamine

(O-GlcNAcylation, "og" in Plant PTM Viewer) and phosphorylation can both modify Ser and Thr residues. While it has been suggested that they directly compete for the same residues, it has been shown that phosphorylation in the sequence motif [ST](ph) P [TVA] [ST](og) can negatively affect O-GlcNAcylation [12]. Here, we assessed the co-occurrence of both modifications and test whether such modified motifs can be found in Arabidopsis. To view coinciding phosphorylation and O-GlcNAcylation sites we search "[ST](ph&og)," thus searching Ser or Thr modified by both PTMs. This results in 61 different proteins harboring one or more of such Ser/Thr sites sensitive to phosphorylation and O-GlcNAcylation. When searching the crosstalk motif "[ST](ph)P[TVA][ST](og)" [12], solely two proteins matching such modified motifs are found. While these results do not provide any functional proof of crosstalk between both modifications, they can provide a good starting point to suggest candidates for downstream experiments.

3.2.4 Searching PTMs in Functional Domains

Next to searching PTMs within specific (modified) protein sequence motifs, querying PTMs that match functional sites of proteins or reside within functional protein domains can be of interest. A protein modification often associated with protein activity is the oxidation of Cys residues, which are intrinsically reactive and often located in protein active sites, for example, in cysteine proteases. To test this, we searched for any Cys modification, "C(xx)," that matches a functional protein site by specifying all sites ("Site," "Active site," "Binding site," and "Metal Ion-binding Site"—*see* **Note 5**) in the advanced parameter options. This delivers 111 different Cys-modified proteins, of which the majority of Cys are reversibly oxidized ("ro"), S-sulfenylated ("so"), or S-nitrosylated ("no"). Besides searching active sites, PTM Search can also be confined to specific protein domains. For instance, Cys S-sulfenylation was frequently observed in RNA-binding domains such as the RNA-recognition motif (InterPro domain IPR000504) in Arabidopsis [13]. To search how many RNA-binding proteins with this motif are oxidation-sensitive, we searched "C(so|no|ro)" (thus S-sulfenylation, S-nitrosylation, or reversible oxidation of Cys) in the InterPro domain IPR000504. This resulted in 25 different proteins with such domains. This might be of interest to their function, as RNA-binding capacity in this motif has been shown to be affected by oxidation-sensitive Cys [14].

3.3 Assess PTM Conservation Homologous Proteins Using PTM BLAST

The occurrence of PTMs at similar aligned positions in homologous proteins within or between species can suggest the PTM to be conserved in multiple proteins and species, possibly even associated with a similar functional role. Such conserved PTMs can be intuitively tracked down in the Plant PTM Viewer by using the PTM BLAST function. Below we describe two ways to run PTM BLAST,

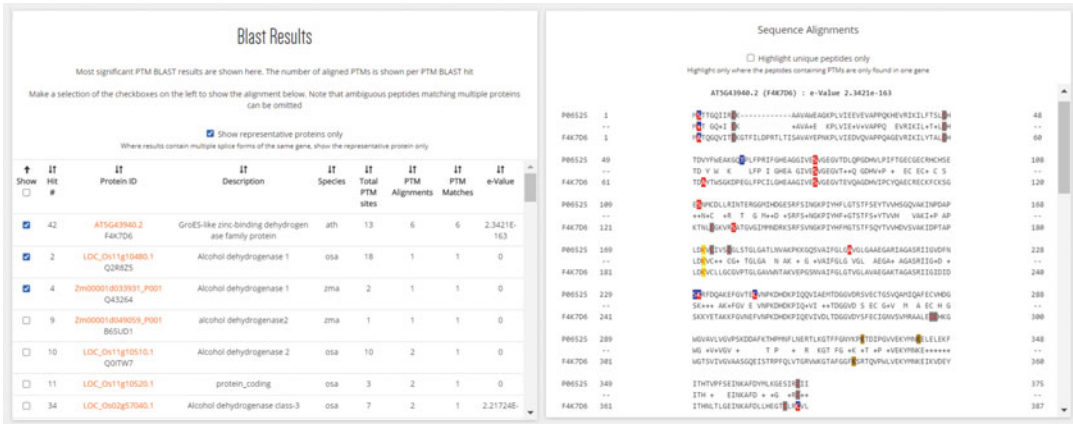

Fig. 2 PTM BLAST results for ALCOHOL DEHYDROGENASE 1 (ADH1; AT1G77120.1). The PTM BLAST result table is displayed on the left side, whereas the PTM Sequence alignments are on the right side

either querying a plant protein or by inputting a user-specified modified protein sequence.

3.3.1 *PTM BLAST for Plant Proteins*

1. Access the protein PTM overview of a plant protein to be queried (see Subheading 3.1). As a test example, we will perform PTM BLAST for ALCOHOL DEHYRDOGENASE 1 (ADH1; AT1G77120.1).
2. Scroll down to the "BLAST" area. By default the whole protein sequence is selected and can be queried by PTM BLAST by clicking "BLAST selected." If desired, a certain subregion of the protein can be searched (*see* **Note 6**).
3. Upon clicking, it will display "BLAST in progress" and, typically within 30 s, a "Display BLAST results" link will appear to view the results in a pop-up screen.
4. The BLAST results are sorted in a table (Fig. 2—left) according to the number of PTM matches, and subsequently by the BLAST E-value. A 'PTM match' is defined as an aligned residue modified by the same PTM type, whereas an aligned PTM carrying different PTM types is designated a 'PTM alignment'.
5. Sequence PTM alignments (Fig. 2—right) can be viewed by scrolling down. By default, the top three PTM BLAST hits are displayed. To display any other selection of hits, click the respective checkboxes. Within the BLAST aligned regions, PTMs are color-coded as in the PTM Sequence overview described before. In this case, the query protein ADH1 shows strong alignment (BLAST E-value 2.3421e-163) to AT5G43940.2, a glutathione dehydrogenase. In total, 6 PTM sites are identical and matched by gene-specific peptides (*see* **Note 7**). For instance, three S-sulfenylation sites were identified in both dehydrogenases at aligned positions—

indicating potential conserved oxidation-sensitive hotspots within these hydrogenases.

6. PTM BLAST queries can also be instrumental to view conserved PTMs across species. In this case, the second and third top PTM Blast hit shows conserved phosphorylation of ADH1 T60 in both rice (*Oryza sativa*) and maize (*Zea mays*). Note that in these species no cysteine oxidation studies are included in the Plant PTM Viewer, and analysis of evolutionary PTM conservation thus logically also depends on the PTM profiling studies per species.

3.3.2 PTM BLAST for User-Specified Modified Protein Sequences

1. Click the "PTM Blast" tab.
2. Enter a protein sequence of minimal 50 residues. Modifications can be specified within the protein sequence using the same PTM syntax as described earlier in the PTM Search option (Table 2). In addition, a FASTA header description (e.g., ">Your description") can be entered if desired.
3. PTM BLAST results can be opened and viewed similar as described above in Subheading 3.3.1. This option can be of interest to specify for instance modified protein sequences from other organisms not present in the Plant PTM Viewer.

4 Notes

1. For fast access to protein overviews for specific accessions, hyperlinks can be used in the following format: https://www.psb.ugent.be/webtools/ptm-viewer/protein.php?id=<YOUR ACCESSION> (for instance, replace <YOUR ACCESSION> with AT5G04200.1). The respective annotations and protein accessions used for the five plant species can be viewed in the "Resources" tab sheet (https://www.psb.ugent.be/webtools/ptm-viewer/resources.php).
2. The Plant PTM Viewer stores all protein sequences from a gene locus shaped by alternative splicing events. For instance, in case of the Araport11 annotation [15] for *Arabidopsis thaliana*, this comes down to 27,655 protein-coding genes that encode 48,359 transcripts encoding different protein forms (with for instance .1, .2 accession suffixes). This can be important in cases where specific peptides/PTMs can only be assigned to specific protein isoforms (and possibly actively contribute to the function of that specific isoform). However, in tools such as PTM Search and PTM Blast, result tables show by default the representative protein isoform, defined as the isoform with the longest amino acid sequence for a gene locus, in order to reduce the complexity. However, a checkbox can be de-selected in order to display all protein isoforms in the results.

3. Some peptide sequences can match multiple proteins encoded from different gene loci. As such, there is no certainty to conclude the modified peptide, and thus PTM site, to match this protein. This might occur frequently for strongly homologous proteins due to gene duplication events.
4. Scrutinization of modified peptide confidence scores is important as false positives are unavoidable amongst peptide identifications. Cases where a PTM site is identified by multiple peptide sequences, e.g., by trypsin missed cleavages, or multiple studies are logically more confident than PTM sites identified by a single study with low scoring peptide identification. In the PTM table overview, an intuitive color scheme is given based on fixed thresholds for the widely used cross-correlation (XCorr) score [16], MaxQuant score [17], or MASCOT score [18]. More details can be obtained by clicking the "Show confidence" button above the PTM table, which will display the respective identification score, posterior error probability (PEP), and ppm precursor deviation. For further inspection, the respective study and/or proteomics data should be consulted.
5. The UniProtKB database provides experimental and electronic annotation for protein residues important to protein function. We extracted four different functional types: "Active Site," "Binding Site," "Metal Ion-binding Site," and "Site." More information on these assigned functional annotations can be consulted in the UniProtKB help-section https://www.uniprot.org/help/function_section.
6. By specifying an amino acid range within the protein, a subsequence of the protein (minimal 50 amino acids) can be queried with PTM BLAST instead of the full protein sequence. This could be of interest to restrict the PTM BLAST to, for instance, a functional protein domain with PTMs of interest. Such PTM BLAST searches can deliver different matches, as in the end the alignment, and thus length, of the protein sequence is a major determinant in the PTM BLAST tool.
7. An important option within the PTM BLAST display is to only show peptides that uniquely match to proteins encoded from a single gene locus (*also see* **Note 3**). Strongly homologous proteins are logically retrieved by BLAST, often resulting in the display of PTMs identified by peptides matching ambiguously to both aligned proteins. In such case, we are dealing with an ambiguous peptide-to-protein assignment and can thus not conclude the occurence of a conserved PTM. In contrast, if a PTM is identified in homologous proteins with peptides matching uniquely to each homologous protein, one can deduce a conserved PTM.

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