Chapter 11

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

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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

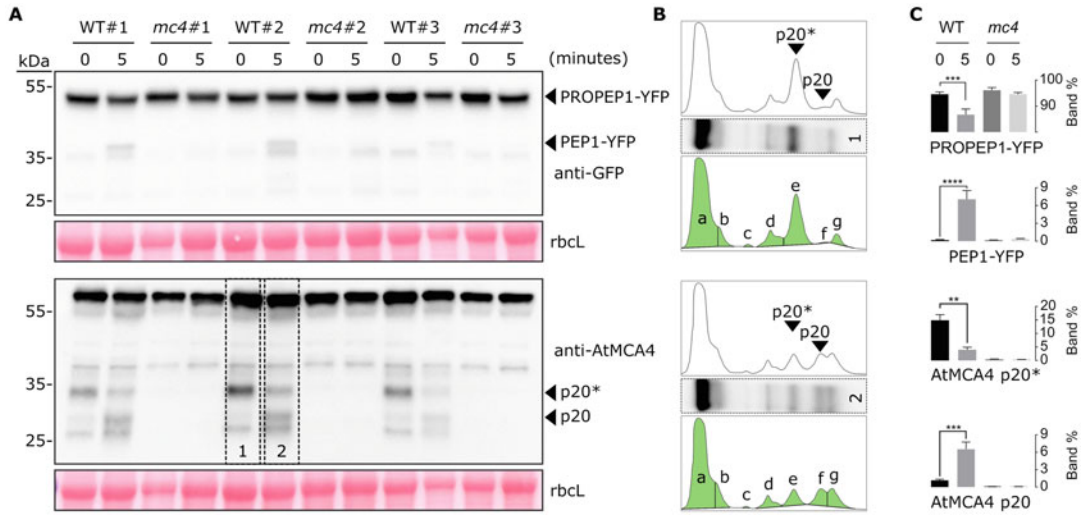

Fig. 1 Western blot of AtMCA4 and a PROPEP1-YFP fusion protein in Arabidopsis seedlings damaged with forceps. (**a**) Western blot of Arabidopsis wild type (WT) and *atmca4* mutant (*mc4*) seedlings expressing a PROPEP1-YFP fusion protein were damaged with forceps and incubated for 5 min at room temperature (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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