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Labeling and enrichment of *Arabidopsis thaliana* matrix metalloproteases using an active-site directed, marimastat-based photoreactive probe

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ABSTRACT

Matrix metalloproteases (MMPs) are secreted or membrane-bound zinc-containing proteases that play diverse roles in development and immunity in plants and in tissue remodeling in animals. We developed a photoreactive probe based on the MMP inhibitor marimastat, conjugated to a 4-azidotetrafluorobenzoyl moiety as photoreactive group and biotin as detection or sorting function. The probe labels At2-MMP, At4-MMP, At5-MMP, and likely other plant MMPs in leaf extracts, as shown by transient At-MMP expression in *Nicotiana benthamiana*, protein blot, and LC–MS/MS analysis. This MMP probe is a valuable tool to study the post-translational status of MMPs during plant immunity and other MMP-regulated processes.

1. Introduction

Matrix metalloproteases (MMPs) are zinc-containing endopeptidases that reside in the extracellular matrix either as soluble, transmembrane or membrane-associated proteins.[1a] The Merops database classifies MMPs in clan MA, family M10.[1b] MMPs are expressed as zymogens and activated by inter- and intra-proteolytic cleavage of the prodomain, thereby displacing the conserved cysteine residue that coordinates the active site zinc.[1c] Human MMPs have important functions in tissue remodeling and an imbalance in their tight post-translational regulation causes various pathological conditions.[1d]

Plant MMPs have been described for soybean,[2a,b] *Arabidopsis thaliana*,[2c] cucumber,[2d] *Medicago trancatula*,[2e] pine,[2f] tobacco,[2g] and *Nicotiana benthamiana*.[2h] Plant MMPs are encoded by intronless genes and consist of a signal peptide, a prodomain, a protease domain carrying the zinc-binding motif HExGHxxGxxH and often a C-terminal transmembrane domain. Plant MMPs play versatile biological roles in growth and development[2d,e,j] as well as in pathogen and symbiont infections.[2g–i] Transcript levels of MMP-encoding genes in soybean, tobacco and *N. benthamiana* are strongly induced during infection with pathogenic bacteria,[2g–i] and silencing one of the MMPs in *N. benthamiana* enhances susceptibility to infections by *Pseudomonas syringae* pv. *tabaci*.[2h] Furthermore, *MtMMPL1* from *M. truncatula* is induced early during nodulation with symbiotic bacteria, and silencing of this gene disturbs the nodulation process. However, this MMP-like protein contains a mutation of an active site residue which may render it proteolytically inactive.[2e]

The *A. thaliana* genome encodes for five MMPs (At1-MMP–At5-MMP),[2c] which have high homology to human MMP-7. At1-, At2-, At3-, and At5-MMPs carry a cleavable signal peptide and a C-terminal transmembrane domain.[2c] At3-MMP has an additional putative GPI anchor motif.[2c] At4-MMP has an uncleavable signal peptide and no transmembrane domain or GPI anchor motif.[2c] Similar to the activation of human MMPs,[1c] incubation of At1-MMP with an organomercury compound leads to the formation of a truncated protein of 27 kDa.[2c] It can be assumed that At2-MMP–At5-MMP also require processing before they gain activity. Once activated, animal MMPs are regulated by endogenous proteinaceous inhibitors,[1b] but no functional inhibitor homolog has been identified in plants yet. These studies[2] make clear that plant MMPs are interesting research subjects involved in diverse biological processes. However, their post-translational regulation hampers a prediction of MMP activity based on transcript or protein levels. A direct readout of MMP activity in complex proteomes would therefore represent a valuable tool to provide functional information on MMPs.

Activity-based protein profiling (ABPP) is an efficient strategy to detect proteins in their active isoforms. This approach is based on the use of fluorescent or biotinylated probes that react covalently

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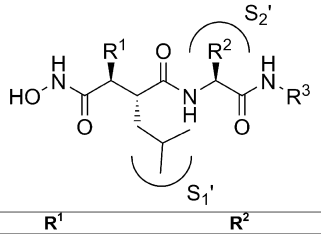
with active site residues of target enzymes in an activity-dependent manner.[3] ABPP has been used in plants to detect activities of papain-like Cys proteases,[4a,b] the proteasome[4c,d] and Ser hydrolases.[4e] Probes for these enzymes are based on irreversible inhibitors that trap the enzyme in the covalent intermediate state. However, similar irreversible inhibitors are not available for metalloproteases since their catalytic mechanism does not involve a covalent intermediate with the substrate. Hence, an additional chemical step, for example, involving a photoreaction, is required. Therefore, probes for metalloproteases are typically based on reversible inhibitors, equipped with a photoreactive group. The feasibility of this concept was proven, for example, for kinases[5] and other protein classes, including metalloproteases.[6] Photoreactive probes that target the active site display the accessibility of the active site, which is a hallmark for enzyme activity.[4f] Capture compounds, the basis of a technology termed Capture Compound Mass Spectrometry (CCMS), are trifunctional small molecules containing a selectivity, photo-reactivity and sorting function in a defined and optimized geometry, with which a covalent bond is formed outside of the binding site of the protein upon irradiation.[7] Proteins that have affinity to the reversible inhibitor are covalently attached upon photo-activation and the cross-linked proteins are isolated using streptavidin-coated magnetic beads. In contrast to ABPP, CCMS is not restricted to (active) enzymes but addresses all kinds of proteins interacting with small molecules, including inhibitors, substrate(analog)s, and drugs.

Hydroxamates are a well-studied group of reversible inhibitors suitable for metalloprotease probes. They are bidendate chelators that bind zinc in the active site and are potent broad-spectrum inhibitors of MMPs. Marimastat and batimastat (Fig. 1) are two important examples of this compound class.[8] Plant MMPs are sensitive to hydroxamate inhibitors since both At1-MMP[2c] and cucumber MMP[2d] are efficiently inhibited by batimastat. Here, we developed high-affinity, marimastat-based photoreactive probes and tested them for labeling plant MMPs.

2. Results and discussion

We employed the marimastat moiety as the targeting group of our probe since it inhibits a wide range of human MMPs in the low nM concentration range and shares similar inhibition profiles with batimastat,[8] which inhibits one of our target proteins, At1-MMP.[2c] For probe synthesis, our previously described marimastat-linker construct[9] was coupled to two different conjugates containing biotin and a photoreactive moiety to yield probes **1** and **2** (Fig. 2, see Supplementary data for synthesis). Our probes differ from previously described hydroxamate-based probes.[6a–e] The majority of published probes are peptidomimetics of succinyl hydroxamate[6b–d] which do not contain the hydroxyl group in position R^1 and often employ different amino acids to target the S_2' pocket. Another group of hydroxamate-based peptide probes carries a C-terminal hydroxamate.[6e] Some hydroxamate-based probes contain the photoreactive group (probe type A,[6a] Fig. 1) or various amino acid side chain residues (probe type B,[6b] Fig. 1) at the R^2 position to target the S_2' pocket. Probes of type B[6b] are similar to our probes **1** and **2**, however, the original marimastat (R^2 = *tert*-butyl) has not been employed for labeling MMPs before.



Compound	R^1	R^2	R^3
Marimastat[6]	OH	*tert*-butyl	methyl
Batimastat[6]	CH_2S-thiophen-2-yl	benzyl	methyl
Probe type A[6a]	OH	reactive group	reporter group
Probe type B[6b]	OH	AA side chains	reactive and reporter groups

Figure 1. General structure of hydroxamate-based MMP inhibitors and probes. S_1' and S_2' refer to the substrate binding pockets that bind amino acids at the P_1' and P_2' positions of the substrate, respectively.

The position[6d] and nature[6e] of the photoreactive group is an important parameter for metalloprotease probes. The most common and best characterized photoreactive groups are aryl azides, fluorinated aryl azides, diazirines and benzophenones. The carbene generated from the trifluoromethyl diazirine shows a high reactivity and good C–H and O–H insertion rates.[10a] However, the increased reactivity and long half life of azo-intermediates can lead to false-positive labeling reactions, especially in biological matrices relevant for MMP investigations.[10a] The slightly less reactive aryl azides may undergo isomerization to 1,2-didehydroazepines. The fluorinated aryl azide derivatives maintain the broader reactivity of the primarily generated nitrene.[10b,c] Triplet benzophenones show a decrease in reactivity compared to nitrenes generated from aryl azides and carbenes from diazirines, but their ability to revert to the singlet ground state and to be reactivated several times[10d,e] is considered beneficial. We therefore chose to incorporate two different photocrosslinkers into our probes; an azidotetrafluorobenzoyl moiety for probe **1** and benzophenone for probe **2** (Fig. 2).

The IC_{50} value for the inhibition of active recombinant human MMP-2 was determined for probe **1** to be 4.7 ± 0.9 nM. For marimastat itself we determined an IC_{50} value of 3.2 ± 1.0 nM which compares well with the reported value of 6 nM for human MMP-2.[8] The affinity of probe **1** to human MMP-2 is relatively high compared to previous metalloprotease probes which were reported with IC_{50} values in the range of low nM to high μM.[6a–e] We validated the functionality of probe **1** by capturing human recombinant MMP-2 and showed by SDS–PAGE that active MMP-2 is captured as full-length protein, lacking the pre- and pro-domains (see Supplementary data for details).[16]

We cloned At2-, At4-, and At5-MMP into binary vectors for expression in planta to demonstrate labeling of plant MMPs (see Supplementary data for expressed protein sequences). The cloned MMPs were transiently overexpressed as C-terminally HA-tagged proteins in *N. benthamiana* by means of transient *Agrobacterium tumefaciens*-mediated transformation (agroinfiltration).[11] Protein extracts of leaves transiently overexpressing At-MMPs were analyzed by western blotting with α-HA antibodies. Only At4- and At5-MMP were detected with sizes of 40 and 50 kDa, respectively, consistent with the expected molecular mass of glycosylated full-length proteases (with aglycon masses of 34 and 37 kDa, respectively, Fig. 3B; lanes 1 and 2). MMP overexpression does not affect the protein profile when compared to the p19 control, indicating that MMPs do not degrade abundant proteins and that MMPs do not accumulate to very high levels (data not shown).

Extracts of leaves expressing At4- and At5-MMP were incubated and crosslinked with probe **1** as detailed in Figure 3A. Preincubation with an excess of marimastat prior to labeling with probe **1** was used as a control for labeling specificity. Extracts of leaves not overexpressing At-MMPs served as a control for the detection of endogenously biotinylated proteins. Detection of biotinylated proteins after labeling revealed specific, marimastat-sensitive signals of 25 kDa in At4-MMP and 35 kDa for At5-MMP samples (Fig. 3B, lanes 6 and 8). These signals did not appear in extracts of leaves not overexpressing At-MMPs (Fig. 3B, lane 4). No signals of 25 or 35 kDa appeared on the anti-HA western blot of the labeled proteins (Supplementary data, Fig. S1), indicating that the

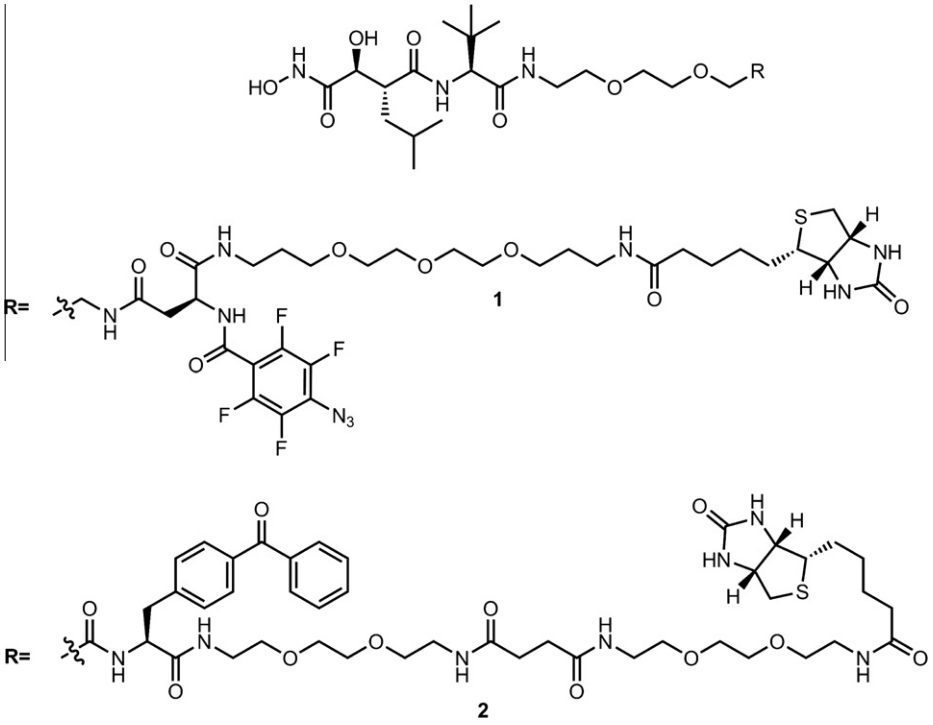


Figure 2. Structures of the marimastat-based probes **1** and **2** as used for labeling experiments of *Arabidopsis thaliana* MMPs.

HA tag is not present on the labeled MMP proteins. Meanwhile, the precursor At4-MMP and At5-MMP were detected to similar levels on anti-HA western blots, irrespective of UV treatment or probe presence (Supplementary data, Fig. S1). A 30 kDa signal is detected in all lanes and represents an endogenously biotinylated protein, presumably biotin carboxyl carrier protein, BCCP (Fig. 3B, lanes 3–8). The molecular mass of the labeled proteins is 15 kDa lower than that of the full-length proprotease.

To verify the identity and of the labeled proteins, we enriched biotinylated proteins on streptavidin beads. The samples were digested on-bead with trypsin and eluted peptides were analyzed by LC–MS/MS (Fig. 3A). At4-MMP was unambiguously identified from the At4-MMP-containing sample, with 21 spectral counts covering 48% of the protein sequence (Fig. 3C, Supplementary data, Tables 3–5). From the At2-MMP containing samples, three At2-MMP peptides were identified with moderate scores (Fig. 3C, Supplementary data, Tables 3–5). Two peptides identified from the At5-MMP sample cover 8% of the At5-MMP sequence (Fig. 3C, Supplementary data, Tables 3–5). Importantly, no peptides of At2-MMP, At5-MMP, and only two peptide spectral counts for At4-MMP were found if the samples were preincubated with an excess of marimastat (Fig. 3C and Supplementary data, Tables 3–5), confirming that labeling can be competed by addition of the original MMP inhibitor.

The same labeling workflow (Fig. 3A) was applied to the lysates using probe **2**. However, no At-MMP labeling was detected by streptavidin blotting or LC–MS/MS analysis (data not shown). The inferior labeling performance of the benzophenone-bearing probe **2** is supported by the literature. In a comparative study of the labeling efficiencies of metalloprotease probes with benzophenone and trifluoromethyldiazirine as reactive groups, no labeling of spiked metalloprotease in crude lysates was observed for the benzophenone probe in contrast to a clear signal for the diazirine probe.[6e]

The small size of labeled MMPs is consistent with the fact that plant MMPs are active as 20–35 kDa isoforms. At1-MMP, for example, is active as a 27 kDa protein.[2c] Furthermore, Soybean MMP (SMEP1) was isolated as a 19 kDa active protease[2a]; cucumber MMP is active as 22 and 18 kDa isoforms,[2d] and tobacco MMP is active as 36 and 22 kDa isoforms.[2k] Similarly, the 62 kDa human MMP-2 produces a 43 kDa isoform that lacks part of the protease domain after the Zinc binding domain[12a] and the 82 kDa human MMP-9 produces 35 kDa mature proteases.[12b] The mechanisms of post-translational regulation in MMPs are not completely understood yet and the physiological function of the various active isoforms are still under discussion. C-terminal truncation may alter substrate specificity as the C-terminal domain is important for substrate binding.[1,12] These aspects should be further investigated by time-dependent labeling of lysates and addition of various protease inhibitors to determine when cleavage occurs as well as the class of protease which is responsible for MMP truncation.

The peptide coverage indicates that the labeled At4-MMP retained most of its prodomain. The presence of a prodomain in a labeled MMP implies that it was not inhibiting protease activity during labeling. Otherwise, the active site zinc would be coordinated by the Cys switch motif of the prodomain, and not be available to bind the hydroxamate moiety in probe **1**. The presence of a prodomain in active MMPs is not unprecedented. The active isoform of human MMP-9 also contains the prodomain with the cysteine switch.[13] Furthermore, inhibition of human MMPs by TIMP-1 traps the protease in a prodomain-containing isoform.[1b] Therefore, the presence of part of the prodomain does not exclude MMP labeling.

The labeling procedure can be further improved to expand the range of MMPs detected by our approach. We detected overexpressed At4-MMP, At5-MMP, and At2-MMP from total leaf extracts. Since most MMPs are transmembrane proteins, their signals can be increased, simply by isolating membrane proteins before or after labeling. Further adjustments of the extraction procedure are needed to detect the other MMPs more efficiently.

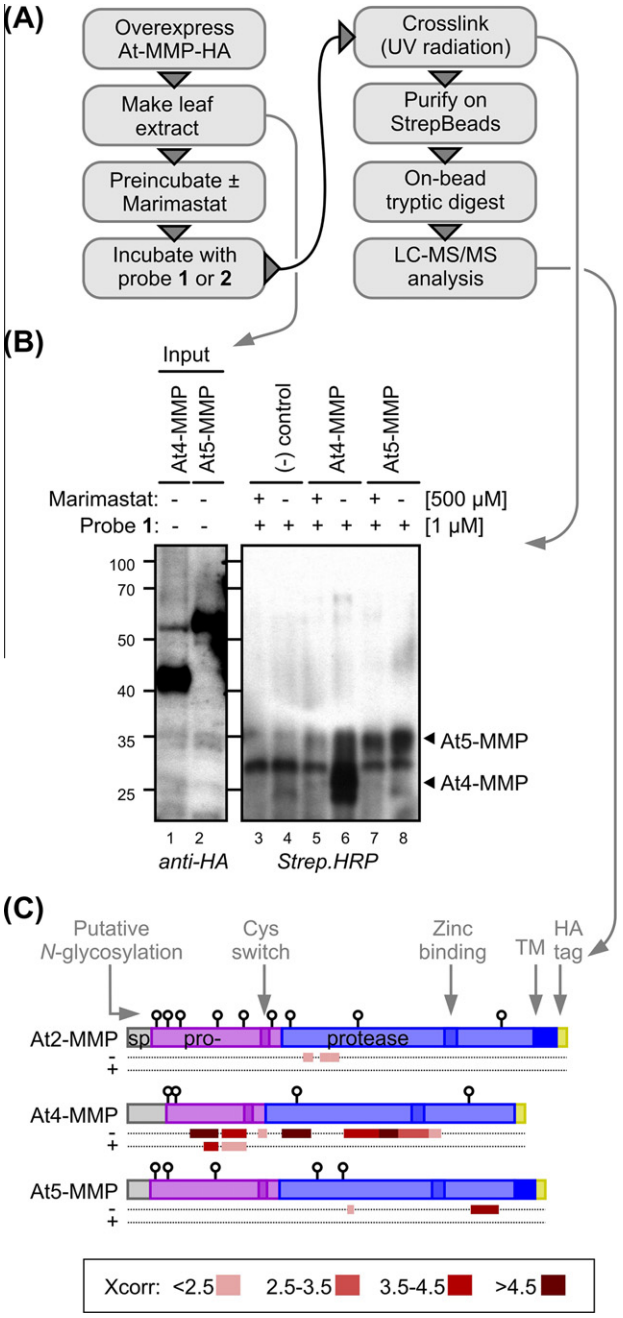


Figure 3. Labeling of leaf extracts overexpressing At-MMP. (A) Workflow of the labeling experiment. (B) Detection of (labeled) MMPs in extracts of leaves transiently overexpressing At4-MMP or At5-MMP together with silencing inhibitor p19. The (−)control is from leaves agroinfiltrated with p19 alone. Proteins were extracted from agroinfiltrated leaves and detected on protein blots using α-HA antibody, or labeled with probe **1** and then detected with streptavidin-HRP (right). (C) Identified peptides (red) covering the MMP prodomain (purple) and protease domain (blue). Labeled proteins were purified from At-MMP containing extracts, digested with trypsin and analyzed by LC–MS/MS. Characteristics of the MMP protein sequences are indicated on top. Identified peptides are shown on dashed lines with Xcorr scores indicated as red scales summarized on the bottom. +/−, with or without marimastat competition, respectively. TM, transmembrane domain.

3. Conclusion

Marimastat-based probe **1** is a high-affinity photoreactive probe for MMPs. We have demonstrated by detecting labeled proteins on streptavidin-HRP blots and by LC–MS/MS analysis that probe **1** can label At4-MMPs and At5-MMP and likely several other MMPs in a marimastat-sensitive manner. Further optimization and application of this methodology will contribute to the elucidation of the role and post-translational regulation of plant MMPs during plant–pathogen interactions and other biological processes.

4. Experimental

4.1. Protein expression

At-MMPs were amplified from cDNA isolated from different developmental stages of *A. thaliana* Col0 by RT-PCR and cloned into pFK26.[14] Cloning details and primer sequences are given in Supplementary Tables 1 and 2, respectively. The reverse primer introduces a HA epitope tag at the C-terminus. Clones were verified by sequencing and correct inserts were shuttled into binary vector pTP5 using EcoRI and HindIII restriction sites. The binary plasmids were transformed into *A. tumefaciens* GV3101 by electroporation. Agroinfiltration in the presence of silencing inhibitor p19 was done as described previously.[14]

4.2. At-MMP labeling and western blot analysis

N. benthamiana leaf lysates were prepared by grinding leaves in 0.4 M borate buffer (pH 7.6) [6.2 g boric acid in 250 mL water, adjusted to pH 7.6 using 10 M NaOH].[16] The extract was cleared by centrifugation and diluted with cross-linking buffer (25 mM Tris pH 7.5, 60 µM $ZnCl_2$, final concentrations) to give a protein concentration of 1 mg/mL. 100 µL of the samples were transferred to a 96-well plate and pretreated with marimastat in DMSO (500 µM final concentration) or DMSO for 10 min. After addition of probe **1** (1 µM final concentration), crosslinking was started by placing a hand-held UV lamp (254 nm (max) and 365 nm (max)) on top of the 96-well plate. The samples were irradiated for 20–30 min on ice. Afterwards, the reaction was stopped by adding 25 µL 4× SDS–PAGE gel loading buffer and heating samples to 90 °C for 5 min. 10–15 µL sample was separated by SDS–PAGE and blotted onto PVDF membrane (Millipore).

Protein blots were incubated with antibodies or streptavidin-horseradish peroxidase (Strep-HRP) in the presence of Tris-buffered saline (50 mM Tris–HCl, pH 7.5; 150 mM NaCl) containing 0.1% Tween-20 and 4% BSA. HA-tagged proteins were detected using mouse α-HA antibody (Sigma) at 8:10000 and rabbit α-mouse antibody conjugated with HRP (Pierce) at 6:10000. Biotinylated proteins were detected using Ultrasensitive Strep-HRP conjugates (Sigma) at 3:10000. Membranes were washed extensively prior to detection of signals with Enhanced Chemiluminescence (ECL, Pierce).

4.3. At-MMP labeling, enrichment and LC–MS/MS analysis

For enrichment of proteins labeled with probe **1**, At-MMP-expressing *N. benthamiana* leaf lysates in 50 mM Tris, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$ were incubated with 1 µM probe **1** in the presence or absence of 500 µM marimastat in a final volume of 100 µL for 5 min on ice. The samples were UV-irradiated within open tubes of a 200 µL-PCR tube strip (recommended 0.2 mL Thermo-Strip, Thermo Scientific, AB-1114) using the caproBox (caprotec bioanalytics GmbH, Berlin) for 4 min at 2 °C. 25 µL 5× wash buffer (250 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 M NaCl, 42.5 µM octyl-β-D-glucopyranoside) was added to each of the samples. After homogenization, 20 µL 10 mg/mL streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin C1, Invitrogen Dynal) were added and the samples were incubated for 3 h at 4 °C keeping the beads in suspension by rotation. The beads were collected using the caproMag magnetic device

(caprotec bioanalytics GmbH, Berlin), the supernatant was discarded. The beads were washed six times with 1× wash buffer (diluted from the 5× wash buffer), once with water, six times with 80% acetonitrile (ACN) and once with water (200 μL, respectively, for each wash step). On-bead tryptic digestion was performed overnight at room temperature under vigorous shaking (>2000 rpm) using 0.5 μg Trypsin (sequencing grade, Roche) in 10 μL 50 mM ammonium bicarbonate. The beads were magnetically collected at the side of the tube's inner wall, the supernatant containing the peptides was transferred into a new tube, dried in a vacuum centrifuge, and stored at −20 °C.

The peptides were analyzed by LC–MS/MS using the LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany). The MS/MS data were analyzed by SEQUEST and X!Tandem by searching a combined database containing tobacco and *N. benthamiana* proteins, supplemented with the HA-tagged Arabidopsis MMPs. Further details on the MS analysis are given in the Supplementary data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.068.

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