

TECHNICAL ADVANCE/RESOURCE

Activity profiling of vacuolar processing enzymes reveals a role for VPE during oomycete infection

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SUMMARY

Vacuolar processing enzymes (VPEs) are important cysteine proteases that are implicated in the maturation of seed storage proteins, and programmed cell death during plant–microbe interactions and development. Here, we introduce a specific, cell-permeable, activity-based probe for VPEs. This probe is highly specific for all four Arabidopsis VPEs, and labeling is activity-dependent, as illustrated by sensitivity for inhibitors, pH and reducing agents. We show that the probe can be used for *in vivo* imaging and displays multiple active isoforms of VPEs in various tissues and in both monocot and dicot plant species. Thus, VPE activity profiling is a robust, simple and powerful tool for plant research for a wide range of applications. Using VPE activity profiling, we discovered that VPE activity is increased during infection with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*). The enhanced VPE activity is host-derived and *EDS1*-independent. Sporulation of *Hpa* is reduced on *vpe* mutant plants, demonstrating a role for VPE during compatible interactions that is presumably independent of programmed cell death. Our data indicate that, as an obligate biotroph, *Hpa* takes advantage of increased VPE activity in the host, e.g. to mediate protein turnover and nutrient release.

Keywords: protease activity profiling, legumain, VPE, aza-epoxide, caspase 1, activity-based protein profiling, technical advance.

INTRODUCTION

Vacuolar processing enzymes (VPEs) are cysteine proteases that are responsible for processing and maturation of vacuolar proteins and are involved in both plant development and immunity. VPEs are also called legumains or asparaginyl endopeptidases, and are classified as members of family C13 in the MEROPS protease database (<http://merops.sanger.ac.uk/>) (Rawlings *et al.*, 2012). The C13 family belongs to the CD clan, which also contains caspases (family C14A) and metacaspases (family C14B). Caspases are the main players in regulation of programmed cell death (PCD) in animals, whereas metacaspases are involved in PCD in plants and fungi (Tsiatsiani *et al.*, 2011). Clan CD proteases contain a His–Cys catalytic dyad, and have strict substrate requirements for the amino acid preceding the cleavable bond (P1 position). For instance, VPEs cleave substrates preferably after Asn residues (hence the

name asparaginyl endopeptidases), whereas caspases cleave substrates specifically after Asp residues (Crawford and Wells, 2011; Tsiatsiani *et al.*, 2011).

VPEs are thought to be evolutionary related to caspases because they share structural homology despite their low sequence similarities (Chen *et al.*, 1998; Hara-Nishimura *et al.*, 2005; Hatsugai *et al.*, 2006). For instance, motifs surrounding the catalytic amino acids are conserved between VPEs and caspases. Furthermore, autocatalytic conversion of the inactive precursor protein into functional VPE resembles the processing and activation of caspase-1 (Hara-Nishimura *et al.*, 2005; Hatsugai *et al.*, 2006). Maturation of Arabidopsis γ VPE occurs in three steps (Kuroyanagi *et al.*, 2002) (Figure 1a). The pre-protein precursor (preproVPE, ppVPE) carries a 22-amino-acid N-terminal signal peptide that is co-translationally removed in the ER to produce

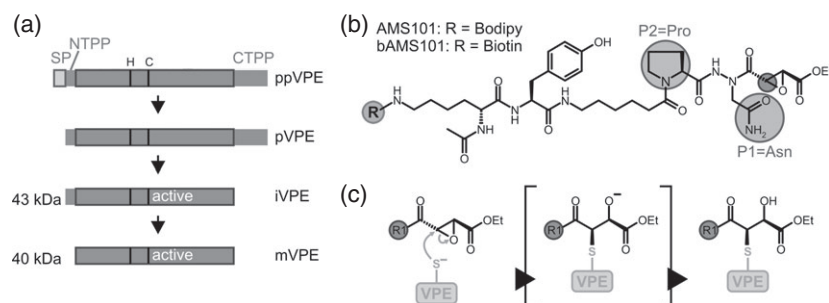


Figure 1. Mechanism of VPE maturation and probes.

(a) Maturation of γ VPE. In the first step, the signal peptide (SP) is co-translationally removed from preproVPE (ppVPE), resulting in proVPE (pVPE). Next, the autoinhibitory C-terminal propeptide (CTPP) is removed, resulting in a 43 kDa active intermediate VPE (iVPE). Finally, the N-terminal pro-peptide (NTPP) is removed, resulting in an active 40 kDa mature VPE (mVPE). The catalytic residues (His and Cys) are indicated. Adapted from Kuroyanagi *et al.*, 2002.

(b) Structure of the aza-epoxide probes used in this study. AMS101 and bAMS101 differ only in the reporter tags: AMS101 carries a BODIPY fluorescent reporter and bAMS101 carries a biotin affinity tag (full structure in Figure S1). Both probes have an asparagine at the P1 position and a proline at the P2 position. The nucleophilic trap in the epoxide is indicated with a dash circle.

(c) Proposed reaction mechanism of aza-epoxide. The sulfur of the catalytic Cys of VPE acts as a nucleophile on the epoxide, resulting in a stable covalent and irreversible sulfoether bond.

proVPE (pVPE). ppVPE and pVPE are both inactive. Transfer to the acidic vacuole causes self-catalytic conversion into the 43 kDa intermediate isoform (iVPE) by removal of the C-terminal inhibitory pro-peptide (CTPP). The subsequent removal of the N-terminal pro-peptide (NTPP) produces the mature 40 kDa isoform (mVPE). Both iVPE and mVPE are active proteases (Kuroyanagi *et al.*, 2002).

The well-known cysteine protease inhibitors E-64 and leupeptin do not inhibit VPE activity because they do not carry an Asn residue (Hatsugai *et al.*, 2004; Rojo *et al.*, 2004). VPEs are specifically inhibited by Ac-ESEN-CHO (Hatsugai *et al.*, 2004), as well as by the general caspase inhibitor VAD-FMK and the caspase-1 inhibitors YVAD-CMK, YVKD-CMK and Ac-YVAD-CHO (Hatsugai *et al.*, 2004; Rojo *et al.*, 2004). Likewise, VPE cleaves the caspase-1 substrate YVAD but not the caspase-3 substrate DEVD, demonstrating that VPEs exhibit caspase-1-like activity (Hatsugai *et al.*, 2004; Rojo *et al.*, 2004). The fact that VPEs also interact with inhibitors and substrates carrying Asp residues at the P1 position has been explained by the acidic vacuolar pH, which causes partial protonation of the Asp side chain and eliminates its negative charge (Kato *et al.*, 2005). Indeed, VPE can also process natural substrates after Asp residues, but with low rates (Hiraiwa *et al.*, 1999).

Four VPE-encoding genes have been identified in Arabidopsis: α VPE, β VPE, γ VPE and δ VPE (Kinoshita *et al.*, 1999; Gruis *et al.*, 2002). β VPE plays a key role in processing of storage proteins during seed maturation (Gruis *et al.*, 2002; Shimada *et al.*, 2003). δ VPE is highly expressed at early stages in developing seeds (Gruis *et al.*, 2002) and is required for PCD of cell layers during seed coat formation (Nakaune *et al.*, 2005). VPEs are also required for cell death triggered by the fungal toxin fumonisins B1 (Kuroyanagi *et al.*, 2005), and for a successful symbiosis of the fungus *Piriformospora indica* with Arabidopsis that involves cell death (Qiang *et al.*, 2012). Likewise, γ VPE knockout lines

showed increased susceptibility to turnip mosaic virus and the necrotrophic pathogen *Botrytis cinerea* (Rojo *et al.*, 2004). Together, these data show that Arabidopsis VPEs are involved in the regulation of PCD during plant immunity, symbiosis and development.

VPEs also play important roles in other plant species. Experiments with tobacco mosaic virus in *Nicotiana tabacum* demonstrated that virus-induced hypersensitive cell death is blocked by VPE silencing and VPE inhibitors (Hatsugai *et al.*, 2004). In monocots, VPEs are required for processing of glutelin, the dominant seed storage protein in rice (Wang *et al.*, 2009; Kumamaru *et al.*, 2010). A similar role is expected for nucellain, a VPE ortholog in barley seeds (Linnestad *et al.*, 1998). VPEs also process seed storage proteins (albumins, globulins and ricin) in storage vacuoles in seeds of pumpkin and castor bean (Hara-Nishimura *et al.*, 1991, 1993; Shimada *et al.*, 2003). VPE is also thought to mediate the maturation of concanavalin A, the lectin of jackbean seeds, which involves deglycosylation, processing after Asn residue and formation of a *de novo* peptide bond (Abe *et al.*, 1993; Min and Jones, 1994; Sheldon *et al.*, 1996). In addition, VPEs process Asn-Gly bonds in the PV100 protein of pumpkin seeds, producing multiple functional seed proteins (Yamada *et al.*, 1999).

The importance of VPEs, combined with their post-translational control of activity through cystatins, processing and pH, for example, calls for new and simple methods to directly monitor VPE activities in tissues or extracts of various plant species. The activity of enzymes may be monitored by using activity-based probes. Activity-based probes are reporter-tagged inhibitors that react with active site residues of enzymes in a mechanism-dependent manner (Cravatt *et al.*, 2008; Edgington *et al.*, 2011). Labeling reflects enzyme activities because the availability and reactivity of active sites are hallmarks of protein activities

(Kobe and Kemp, 1999). Activity-based protein profiling has been extensively used in the animal field to study diverse protease families (Serim *et al.*, 2012). More recently, activity-based probes have been introduced in plant research for the proteasome (Gu *et al.*, 2010; Kolodziejek *et al.*, 2011), metalloproteases (Lenger *et al.*, 2012), serine hydrolases (Kaschani *et al.*, 2009a, 2012a; Nickel *et al.*, 2012) and glyceraldehyde dehydrogenases (Kaschani *et al.*, 2012b). Activities of papain-like cysteine proteases (family C1A of clan CA) may be displayed using DCG-04, a biotinylated derivative of the broad-range cysteine protease inhibitor E-64 (Greenbaum *et al.*, 2000). Since its introduction into plant science (Van der Hoorn *et al.*, 2004), DCG-04 has been widely used to monitor papain-like cysteine protease activities in Arabidopsis (Van Esse *et al.*, 2008; Wang *et al.*, 2008; Kaschani *et al.*, 2009b; Lampl *et al.*, 2010; Gu *et al.*, 2012; Richau *et al.*, 2012; Shindo *et al.*, 2012), tomato (Rooney *et al.*, 2005; Tian *et al.*, 2007; Shabab *et al.*, 2008; Van Esse *et al.*, 2008; Song *et al.*, 2009; Kaschani *et al.*, 2012; Hörger *et al.*, 2012; Lozano-Torres *et al.*, 2012), tobacco (Gilroy *et al.*, 2007), maize (Van der Linde *et al.*, 2012) and wheat (Martinez *et al.*, 2007). These studies illustrate the wide applicability of activity-based probes in plant science. Although DCG-04 targets cysteine proteases, this probe does not label VPEs because DCG-04 does not carry an Asn or Asp residue at the P1 position.

Here we report the use of an activity-based probe to monitor VPE activities in plants. This probe displays highly specific labeling of mVPE and iVPE that is pH-dependent and is competed for by the caspase-1 inhibitor YVAD-CMK. Additionally, this probe is suitable for subcellular *in vivo* imaging of VPEs. Using this probe, we observed a previously unnoticed up-regulation of γ VPE activity during compatible but not incompatible interactions of Arabidopsis with the biotrophic pathogen *Hyaloperonospora arabidopsidis* (*Hpa*). Further studies demonstrated a role for VPEs during compatible *Hpa* interactions.

RESULTS

Aza-asparagine probes specifically label VPEs

We synthesized and tested an activity-based probe called AMS101 (Figure 1b and Figure S1). AMS101 is similar to an earlier introduced aza-epoxide-based legumain probe (Lee and Boggyo, 2009), but carries a different fluorophore and linker. AMS101 is also distinct from legumain probes based on acyloxymethylketone reactive groups (Sexton *et al.*, 2007). AMS101 contains an aza-asparagine residue (Asn, N) to selectively target VPEs, which require an Asn at the P1 position (Hara-Nishimura *et al.*, 1993; Yamada *et al.*, 1999). To exclude cross-reactivity with papain-like cysteine proteases, AMS101 carries a Pro residue at the P2 position. Pro at P2 is preferred by legumains and but plant papain-

like cysteine proteases have poor affinity for Pro at P2 (Sexton *et al.*, 2007; Richau *et al.*, 2012). The reactive group of AMS101 is an epoxide that reacts with the catalytic cysteine residue of VPE. Reaction of the catalytic cysteine of VPEs with the epoxide results in a stable covalent and irreversible sulfoether bond (Figure 1c). We synthesized AMS101 with two reporter tags: a BODIPY fluorescent tag (AMS101) and a biotin affinity tag (bAMS101).

To assess labeling of VPEs with AMS101, leaf extracts of Arabidopsis wild-type plants (ecotype Col-0), a γ VPE over-expressor line (γ OE), a γ VPE knockout line (γ KO) and a quadruple knockout line (qKO) were incubated with fluorescent AMS101. Labeling of Arabidopsis Col-0 extracts produces fluorescent signals at 43 and 40 kDa, corresponding to the iVPE and mVPE isoforms, respectively (Figure 2a, lane 1). Labeling of γ OE extracts results in stronger signal intensities for iVPE and mVPE and a less intense signal at 32 kDa (Figure 2a, lane 2). These 32, 40 and 43 kDa signals are absent in the qKO line (Figure 2a, lane 4), indicating that VPEs are the major target of AMS101. The majority of the signal is absent in the single γ VPE knockout (γ KO) (Figure 2a, lane 3), indicating that γ VPE is the dominant VPE in leaf extracts. However, a weak 40 kDa signal remains in the γ KO line that is absent in the qKO line, indicating that at least one more VPE is labeled in leaf extracts, in addition to γ VPE (Figure 2a and Figure S2).

Mass spectrometric analysis was performed to confirm the identity of AMS101-labeled proteins. Leaf extracts of Arabidopsis Col-0, γ KO and γ OE plants were labeled with bAMS101. Biotinylated proteins were purified and separated on a protein gel (Figure 2b). 40 and 43 kDa signals and corresponding controls were excised from the gels and subjected to in-gel trypsin digestion. Tandem MS analysis identified a total of 13 and 102 spectra in the Col-0 and γ OE samples, respectively, corresponding to γ VPE-derived peptides (Figure 2c, bands 1–4). The number of spectral counts correlates with the signal intensities observed on the gel (Figure 2b, bands 1–4). None of these VPE-derived peptides were found in the no probe control or in samples from the γ KO line (Figure 2c, bands 1–4). Two of the four peptides are unique for γ VPE, whereas the other two are ambiguous with either α VPE or δ VPE. The peptides found only covered 15% of the mVPE protein sequence, and the same peptides were detected for both iVPE and mVPE isoforms (Figure 2d). One explanation for the low coverage is that most of the expected tryptic fragments of γ VPE are too large to be detected with the MS settings used (Figure 2d). No other VPE proteins were detected in this experiment. This is probably due to by the limited sensitivity of detection, combined with the relatively low expression of other VPE genes (Figure S3) (Kuroyanagi *et al.*, 2005).

To test whether VPE inhibitors prevent AMS101 labeling, leaf extracts of Arabidopsis γ OE were pre-incubated with an excess of two inhibitors: the caspase-1 inhibitor YVAD-

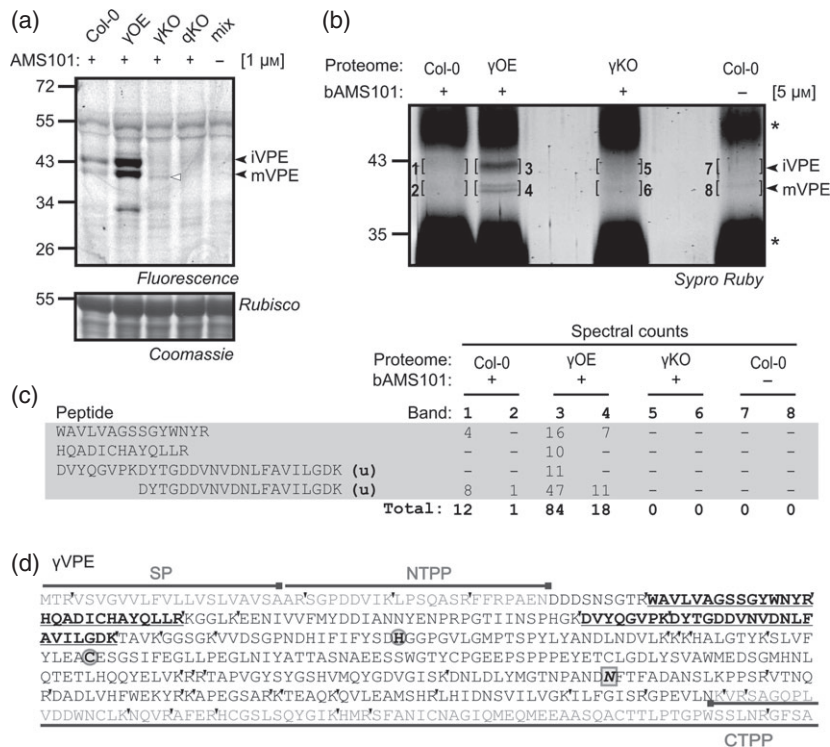


Figure 2. γ VPE is the major target of AMS101 in Arabidopsis leaf extracts. (a) AMS101 labels γ VPE in leaf extracts. Leaf extracts of Arabidopsis wild-type plants (Col-0), the γ VPE over-expressor line (γ OE), the γ VPE knockout line (γ KO) and the quadruple VPE knockout line (qKO) were labeled for 2 h at pH 5.5 with 1 μ M AMS101. A mixture of proteomes was used as a no probe control (mix). Fluorescent proteins were detected by in-gel fluorescent scanning. The white arrowhead indicates a weak 40 kDa signal that is absent in the qKO line. (b) Pull-down of bAMS101-labeled proteins. Leaf extracts of Arabidopsis wild-type plants (Col-0) and the γ VPE knockout (γ KO) and γ VPE over-expressor (γ OE) lines were labeled with 5 μ M bAMS101, and biotinylated proteins were purified. Purified samples were separated on a protein gel, stained with SYPRO® Ruby and visualized by fluorescence scanning. Eight bands (1–8) were excised. Proteins were digested by trypsin and identified by tandem MS analysis. The asterisk indicates neutravidin. (c) Identified peptides. Two ambiguous VPE peptides and two unique peptides (u) from γ VPE were identified. The total spectral counts correlate with the signal intensity in (b). (d) Peptide coverage in the γ VPE protein sequence. Indicated are the mature protease domain (bold letters), catalytic residues (bold in circle), the putative N-glycosylation site (bold in square), putative trypsin cleavage sites (triangles), the signal peptide (SP), the N-terminal pro-peptide (NTPP) and the C-terminal pro-peptide (CTPP). Identified peptides are in bold and underlined.

CMK, which inhibits VPE activity (Hatsugai *et al.*, 2004), and the caspase inhibitor xxAD-AOMK (x = mixture of amino acids), an acyloxymethylketone-based inhibitor that also carries an Asp at the P1 position (Berger *et al.*, 2006). Pre-incubation with both inhibitors suppressed labeling of iVPE and mVPE (Figure 3a), indicating that AMS101 labeling is prevented by VPE inhibitors.

To determine whether AMS101 labels all four Arabidopsis VPEs, we transiently over-expressed α VPE, β VPE, γ VPE and δ VPE by agro-infiltration of *Nicotiana benthamiana*. Proteins were extracted and labeled with AMS101. All four VPEs were labeled with AMS101, and labeling causes slightly different isoform profiles (Figure 3b,c). α VPE labeling results in two major signals at 43 and 46 kDa and a weaker signal at 30 kDa (Figure 3b, lane 1). β VPE labeling results in a weak signal at 40 kDa and two strong signals at 26 and 28 kDa (Figure 3b, lane 2). γ VPE labeling results in three strong signals at 43, 40 and 32 kDa (Figure 3b, lane 3), consistent with the labeling

pattern of Arabidopsis leaf extracts (Figure 2a). Labeling of δ VPE shows 2 weak signals at 40 and 43 kDa (Figure 3b, lane 4). However, the upper 43 kDa signal is also detected in the empty vector control, suggesting that this represents an endogenous VPE from *N. benthamiana* (Figure 3c). In contrast, the 40 kDa signal only appears upon δ VPE expression, indicating that this represents δ VPE (Figure 3c). The relatively weak δ VPE signal may be caused by the low solubility of this protein (Nakaune *et al.*, 2005). In conclusion, AMS101 is a suitable probe for all four Arabidopsis VPEs.

Characterization of labeling conditions

To characterize γ VPE activity further, leaf extracts were incubated with AMS101, and samples were analyzed at various time points. Fluorescent signals were quantified from protein gels and plotted against time. Labeling of extracts occurs within minutes, and maximum labeling is reached in 2 h. iVPE and mVPE show the same pattern of

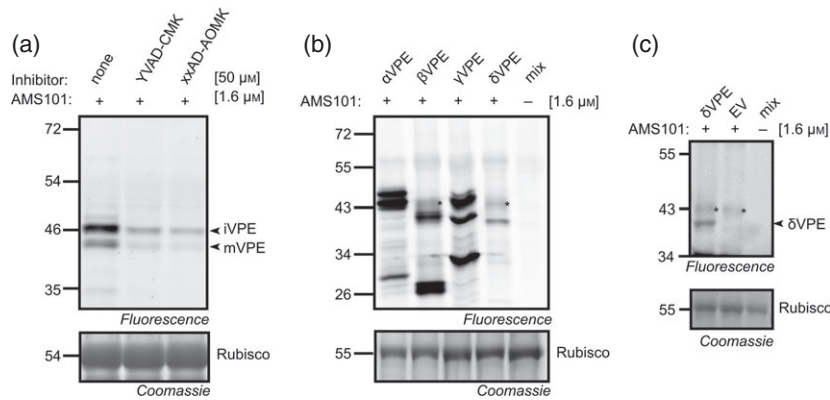


Figure 3. Competition of labeling and detection of all four Arabidopsis VPEs.

(a) Caspase-1 inhibitors suppress AMS101 labeling. Leaf extracts of Arabidopsis γ OE plants were pre-incubated for 30 min with 50 μ M of the caspase-1 inhibitor YVAD-CMK or xxAD-AOMK (x = mixture of amino acids), followed by 2 h labeling with 1.6 μ M AMS101. (b) AMS101 labels all four Arabidopsis VPEs. α VPE, β VPE, γ VPE and δ VPE from Arabidopsis were over-expressed in *Nicotiana benthamiana* by agro-infiltration. Protein extracts were labeled at pH 5.5 with 1.6 μ M AMS101 for 2 h. A mixture of proteomes was used as a no probe control (mix). Samples were analyzed on a protein gel with fluorescence scanning and Coomassie staining. The asterisks indicate endogenous VPE. (c) AMS101 labels Arabidopsis δ VPE and an endogenous VPE from *N. benthamiana*. Long exposure of the labeled sample containing δ VPE compared to the empty vector (EV) control and the no probe control (mix), showing δ VPE and an endogenous VPE (asterisks).

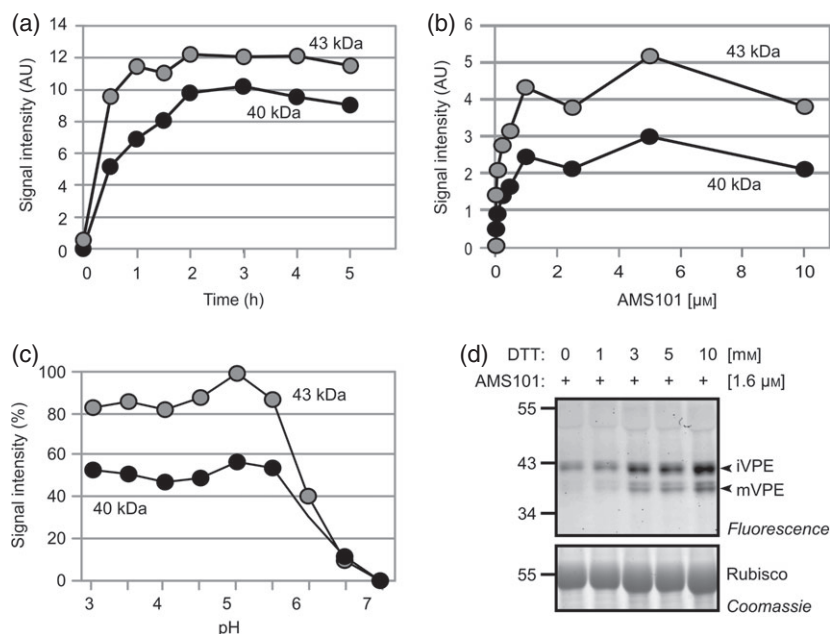


Figure 4. Labeling reflects characteristics of VPE activity.

(a) Maximum labeling is reached in 2 h. Leaf extracts of Arabidopsis γ OE plants were incubated at pH 5.5 with 1 μ M AMS101, and samples were collected at various time points. (b) VPE labeling may be saturated. Leaf extracts of Arabidopsis γ OE plants were labeled with various probe concentrations for 2 h at pH 5.5. (c) VPE labeling occurs only at acidic pH. Leaf extracts of Arabidopsis γ OE plants were incubated at various pH and labeled for 2 h with AMS101. (d) The presence of a reducing agent increases VPE labeling. Leaf extracts of Arabidopsis wild-type plants were labeled at pH 5.5 for 2 h with 1.6 μ M AMS101 at various DTT concentrations. Fluorescent proteins were detected by in-gel fluorescent scanning, and quantified from the protein gels, and plotted against time, probe concentration and pH, respectively (a–c).

labeling, indicating that AMS101 labels both isoforms simultaneously without preference (Figure 4a).

To investigate whether labeling may be saturated, leaf extracts were incubated with increasing AMS101 concentrations. Both iVPE and mVPE reached saturation at 1 μ M

AMS101 (Figure 4b), indicating that active VPE concentrations are limiting and that both isoforms have a similar affinity for AMS101.

To study the pH dependency of labeling, leaf extracts were incubated with AMS101 at various pH. Maximum

γ VPE labeling occurs at pH 5–5.5, and γ VPE is hardly labeled at neutral pH (Figure 4c). These data are consistent with the expected activity of VPE in the vacuole, and correlate with a maximum VPE proteolytic activity of VPE at pH 5.5 (Kuroyanagi *et al.*, 2002).

To determine whether reducing conditions promote VPE labeling, leaf extracts of Arabidopsis Col-0 plants were labeled with AMS101 at various dithiothreitol (DTT) concentrations. DTT concentrations above 3 mM significantly increase VPE labeling (Figure 4d), demonstrating that reducing agents improve labeling of γ VPE in leaf extracts.

AMS101 labels VPE-like proteins in various plant species

To investigate whether AMS101 labels legumains of other plant species, leaf extracts of various dicot and monocot plants were pre-incubated with and without an excess of the caspase-1 inhibitor YVAD-CMK and labeled with AMS101. All seven tested proteomes showed labeling with AMS101 that was suppressed by YVAD-CMK (Figure 5), demonstrating that AMS101 labels VPE-like proteins in leaf extracts of both monocot and dicot plants. Signal intensities vary between plant species, indicating that different species have different levels of VPE activities. AMS101-labeled proteins migrate at between 30 and 50 kDa, consistent with that various sizes expected for active VPEs (Hatsugai *et al.*, 2006). Taken together, these results show that AMS101 is a specific probe to study the activity of VPE-like proteins in various plant species.

Live imaging of VPEs

We next used AMS101 to image VPE labeling of Arabidopsis cell cultures by confocal microscopy. Imaging of

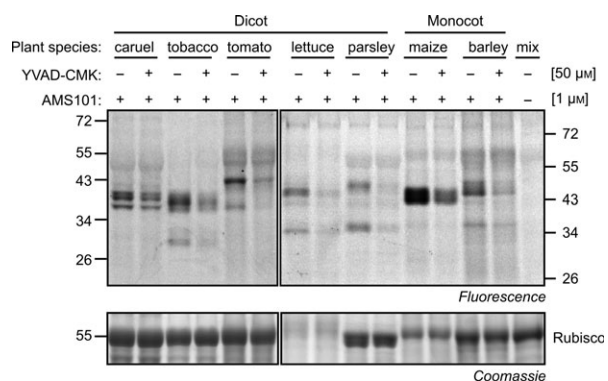


Figure 5. AMS101 labels VPE-like proteins in various plant species. Leaf extracts (2.5 μ g ml protein⁻¹) from various plant species were pre-incubated for 30 min with 50 μ M YVAD-CMK and labeled with 1 μ M AMS101 for 2 h. A mixture of proteomes was used as a no probe control (mix). Samples were separated on a protein gel and analyzed by fluorescent scanning and Coomassie staining. The plant species were caruel (*Brassica drepanensis*), tobacco (*Nicotiana benthamiana*), tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa* var. *capitata*), parsley (*Petroselinum hortense*), maize (*Zea mays*) and barley (*Hordeum vulgare*).

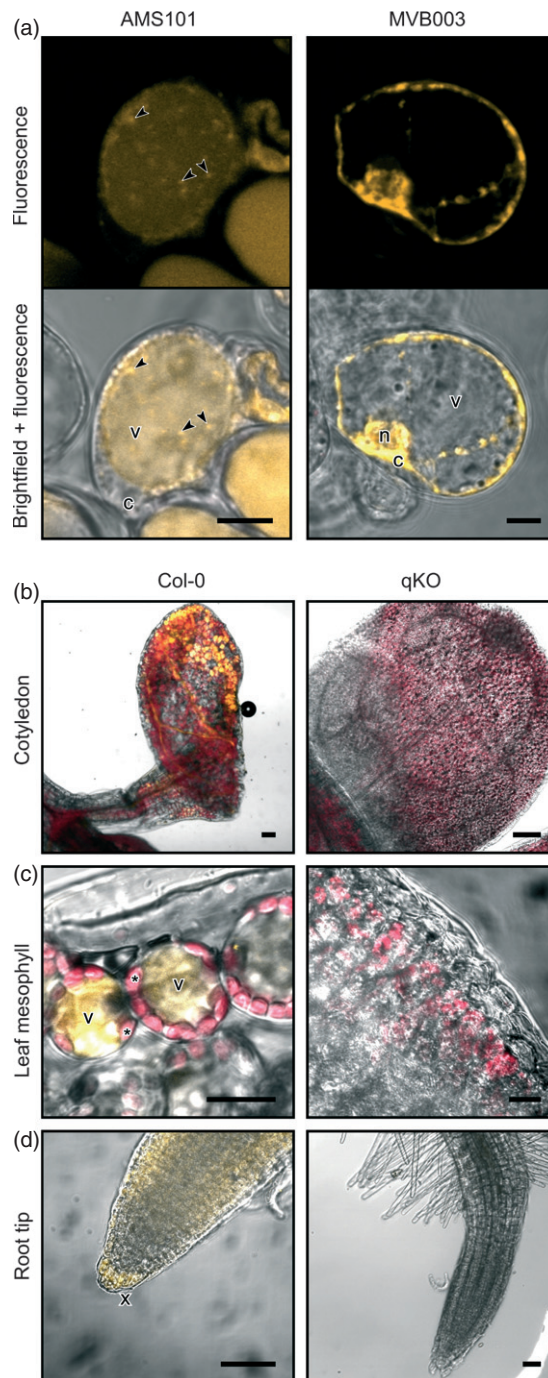


Figure 6. Live imaging of VPE labeling.

(a) AMS101 accumulates in the vacuole. Arabidopsis cell cultures were incubated with 2 μ M AMS101 or the proteasome probe MVB003 for 1.5 h. Cells were washed and imaged by confocal microscopy. Mobile fluorescent spots are located inside the vacuole (arrowheads). v, vacuole; c, cytoplasm; n, nucleus. Scale bars, 10 μ m.

(b–d) VPE labeling of cotyledons (b), mesophyll cells (c) and roots (d). Seven-day-old Arabidopsis wild-type (Col-0) or *vpe* knockout (qKO) seedlings were incubated for 5 h (b, c) or 20 h (d) with 2 μ M AMS101. Seedlings were washed and imaged by confocal microscopy. Merged pictures (bright-field + fluorescence + chlorophyll) are shown. Red, chlorophyll; yellow, AMS101. v, vacuole; *, chloroplast; x, root cap. Scale bars = 100 μ m (b), 20 μ m (c) and 50 μ m (d).

AMS101 fluorescence was compared with that for MVB003, a fluorescent probe for the proteasome (Kolodziejek *et al.*, 2011). AMS101 labeling causes strong fluorescence in the vacuole but not in the nucleus or cytoplasm (Figure 6a, left). Mobile fluorescent speckles were observed inside the vacuole (Figure 6a, left, arrowheads). By contrast, MVB003 labels specifically the nucleus and cytoplasm but not the vacuole (Figure 6a, right). These fluorescent signals are consistent with the subcellular localization of VPE (vacuole) and the proteasome (nucleus and cytoplasm).

To confirm that fluorescent signals detected in the vacuole correspond to VPE labeling, seedlings of Col-0 and qKO lines were labeled *in vivo* with AMS101. Fluorescent signals were detected in vacuoles of cotyledons (Figure 6b), leaf mesophyll cells (Figure 6c) and the root tip (Figure 6d) of Col-0 but not qKO seedlings. These data demonstrate that VPE is the main target of AMS101 *in vivo* and that AMS101 is suitable for *in vivo* imaging.

γ VPE activity is up-regulated during *Hpa* infection

To investigate VPE activity during immune responses and plant-pathogen interactions, we screened proteomes of infected plants for differential AMS101 labeling. We discovered that VPE labeling increases during infection with the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) (Figure 7a). Activation of VPE is gradual and occurs for both Col-0 and γ OE plants during compatible interactions (Figure 7a, left). By contrast, increased VPE activity does not occur during the incompatible interaction with the avirulent isolate Cala2 (Figure 7a, right).

Signals are absent upon infection of qKO plants, demonstrating that the fluorescent signals observed are not caused by VPE-like proteases from *Hpa* but are caused by labeling of Arabidopsis VPEs (Figure 7b, lanes 7 and 8). The signal is strongly reduced in the γ KO line (Figure 7b, lanes 5 and 6), indicating that the majority of the signal is caused by γ VPE. However, a weak 40 kDa signal remains in the γ KO line compared to the qKO line, indicating that at least one additional VPE is labeled (Figure 7b, lane 6, and Figure S2). This additional VPE activity increases upon *Hpa* infection (Figure 7b, lane 6, and Figure S2). Increased VPE labeling during *Hpa* infection occurs in both Col-0 and γ OE plants, as well as the *eds1-2* mutant (Falk *et al.*, 1999), indicating that VPE up-regulation is independent of *EDS1* (Figure 7b).

We next measured VPE transcript levels to determine whether increased VPE activity correlates with transcript levels. γ VPE transcript levels are up-regulated during infection of both Col-0 and γ OE plants (Figure 7c), consistent with increased γ VPE activity (Figure 7a,b). The increased γ VPE expression in γ OE plants upon *Hpa* infection is not caused by endogenous γ VPE as the γ OE line is derived from a γ KO line transformed with a 35S:: γ VPE construct (Rojo

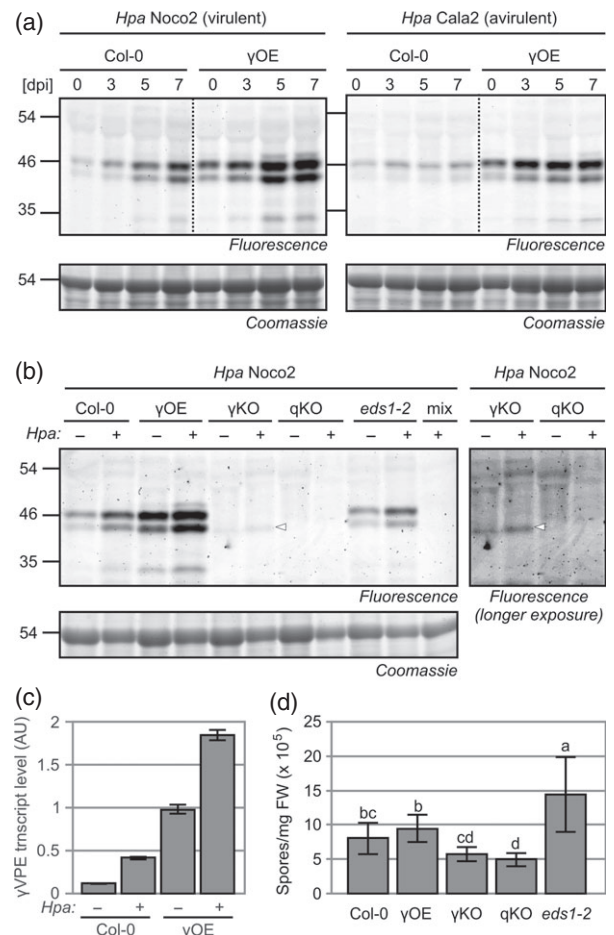


Figure 7. Role of γ VPE activity during *Hyaloperonospora arabidopsidis* (*Hpa*) infection.

(a) γ VPE activity increases during compatible but not incompatible interactions. Col-0 and γ OE plants were spray-inoculated with 4×10^4 spores ml^{-1} of *Hpa* Noco2 (virulent) or Cala2 (avirulent). Leaves were harvested at 0, 3, 5 and 7 dpi, and protein extracts were labeled with $1.6 \mu\text{M}$ AMS101 at pH 5.5 for 2 h. (b) γ VPE labeling increases during infection. Arabidopsis Col-0, γ OE, γ KO, qKO and *eds1-2* plants were spray-inoculated with water (–) or 4×10^4 spores ml^{-1} of *Hpa* Noco2 (+). Leaves were harvested at 5 dpi, and leaf extracts were labeled at pH 5.5 with AMS101 for 2 h. A mixture of extracts was used as a no probe control (mix). The white arrowhead indicates a weak 40 kDa signal that is absent in the qKO line. The lanes for the γ KO and qKO samples are shown on the right with a longer exposure. In (a) and (b), protein samples were separated on protein gels and analyzed by fluorescent scanning and Coomassie blue staining. (c) γ VPE is transcriptionally up-regulated during infection. Arabidopsis Col-0 and γ OE plants were treated with water (–) or 4×10^4 spores ml^{-1} of *Hpa* Noco2 (+). Samples were collected at 7 dpi for quantitative RT-PCR on isolated RNA. Error bars indicate the standard deviation of three technical replicates. The experiment was repeated once with similar results. (d) Reduced sporulation of *Hpa* on *vpe* knockout plants. Arabidopsis mutants were spray-inoculated with the virulent pathogen *Hpa* Noco2. Spores were counted at 7 dpi in triplicate. Values are means \pm SEM for four independent biological experiments. Different letters indicate significant differences between genotypes ($\alpha = 0.05$, Fisher's least statistical difference test, Figure S4).

et al., 2004). The increased VPE expression in the γ OE line is probably due to the presence of the pathogen-inducible *as-1* element in the 35S promoter (Redman *et al.*, 2002).

To determine the role of VPE in *Hpa* infection, sporulation of *Hpa* isolate Noco2 was measured on Col-0, γ OE, γ KO and qKO lines and on the hyper-susceptible *eds1-2* mutant. The absence of all VPEs in the qKO line causes a significant reduction in the spore count (Figure 7d), indicating that VPEs are required to promote *Hpa* virulence. γ KO plants show an intermediate phenotype, suggesting that, in addition to γ VPE, other VPEs also contribute to *Hpa* virulence, consistent with detection of an extra VPE-derived signal in the γ KO line. By contrast, *Hpa* sporulation on γ OE plants does not differ significantly from that on Col-0 control plants. Overall, these data demonstrate a role for γ VPE in promoting *Hpa* infection.

DISCUSSION

We present a simple, robust and versatile method to monitor VPE activity *in vitro* and *in vivo* in various plant species. Further characterization of VPE activities revealed an unexpected up-regulation of γ VPE activity during compatible but not incompatible interactions with *Hpa*. The reduced sporulation on *vpe* knockout plants indicates that VPEs promote infection in compatible interactions with *Hpa*, independent of programmed cell death.

AMS101 is a highly specific probe for plant VPEs

Four different approaches confirmed that VPEs are the exclusive target of AMS101 in Arabidopsis leaf extracts. First, AMS101 labeling of the γ KO line showed that γ VPE is the main target of this probe. Comparison of the γ KO line with the qKO line showed that another minor VPE is also labeled by AMS101. Second, mass spectrometric analysis of leaf proteomes labeled with bAMS101 confirmed γ VPE labeling. Third, labeling of extracts of *N. benthamiana* leaves transiently over-expressing Arabidopsis VPEs demonstrated that AMS101 labels all four Arabidopsis VPEs. Finally, VPE labeling is competed with the previously described VPE inhibitor Ac-YVAD-CMK (Rojo *et al.*, 2004). The fact that γ VPE is predominantly labeled in leaf extracts is expected as γ VPE is the main VPE expressed in Arabidopsis leaves (Figure S3) (Kinoshita *et al.*, 1999). We speculate that the weak 40 kDa signal detected in the γ KO line (Figures 2a and 7b) is caused by β VPE, because this is the VPE with the second highest transcript levels in leaves (Figure S3), and transient over-expression of β VPE produces a 40 kDa signal (Figure 3b). The low level of β VPE explains why this protein is not detected during MS analysis. The weak labeling in leaf extracts correlates with the fact that VPE genes are not highly transcribed under normal conditions (Figure S3) (Kinoshita *et al.*, 1999).

The labeling experiments also demonstrated that AMS101 labeling reflects VPE activity rather than protein abundance. For instance, AMS101 labels VPE at acidic pH, consistent with maximum proteolytic activity at pH 5.5 (Kuroyanagi *et al.*, 2002). Reducing conditions further

enhance labeling, consistent with the importance of a free thiol at the catalytic site. Finally, VPE cannot be labeled when VPE inhibitors are present. In each of these cases, VPEs are only labeled under conditions where VPEs are active.

An interesting aspect of activity-based labeling of VPEs is that labeling displays different VPE isoforms. In addition to the previously described 43 and 40 kDa isoforms of γ VPE, we detected a 32 kDa isoform. This isoform was detected upon agro-infiltration and in the γ OE line, but also upon infection of wild-type plants with *Hpa*. Similar VPE isoforms at approximately 30 kDa were detected for other Arabidopsis VPEs and in leaves of other plant species, suggesting that this isoform is common for plant VPEs. Interestingly, cleavage sites may be created by deglycosylation of Asn residues (Manoury *et al.*, 1998), and the molecular weights of the small isoforms (Figure 3b) correlate with the position of putative N-glycosylation sites (Figure S5). This may suggest that the small isoform is caused by VPE-mediated cleavage of non-glycosylated or deglycosylated VPEs. The small isoform may have resulted from cleavage after labeling or from labeling of a cleaved isoform. Internal cleavage of a protease into two chains may inactivate the protease or maintain the resulting chains in an active protease complex. For example, human cathepsin F is cleaved but the two-chain protease remains active (Lennon-Dumenil *et al.*, 2001).

We also describe three more applications of AMS101. First, AMS101 labels VPEs in both monocot and dicot plant species, indicating that AMS101 can detect VPEs in all plant species. Second, AMS101 is suitable for imaging of living cells and tissues. AMS101 fluorescence accumulates in the vacuole only in the presence of VPEs. Interestingly, small fluorescent speckles were observed after AMS101 labeling, indicating focal accumulation of VPEs within the vacuole or VPE-dependent sub-compartmentalization of the probe. These speckles may represent autophagic bodies, which result from fusion of autophagosomes with the vacuolar membrane (Bassham, 2007). Moreover, AMS101 labels VPEs in cell cultures, roots, cotyledons and leaves. These findings show that AMS101 is a versatile probe that can be used under various conditions, and in various tissues and plants, both *in vitro* and *in vivo*.

γ VPE promotes *Hpa* compatible interactions

We discovered that γ VPE activity increases during infection with the biotrophic pathogen *Hyaloperonospora arabidopsidis*. The increased γ VPE activity is independent of EDS1, and correlates with up-regulated γ VPE transcript levels. The absence of all VPEs decreases *Hpa* sporulation, indicating that VPEs are beneficial for *Hpa* pathogenicity. The enhanced resistance is only partial in the γ KO mutant, suggesting that another VPE, in addition to γ VPE, contributes to *Hpa* sporulation. Indeed, we detected a weak, *Hpa*-inducible VPE signal in the γ KO line, which may represent

β VPE because of its 40 kDa size and relatively high transcript level compared to α VPE and δ VPE in leaves (Figure S3). Our data are consistent with the observation that γ VPE is the major, but not the only, VPE involved in fumonisin B1-induced cell death in Arabidopsis leaves (Kuroyanagi *et al.*, 2005).

Our observations on the role of VPEs in *Hpa* infection are surprising for two reasons. First, *Hpa* infection is not thought to involve PCD, indicating that the role of VPE in *Hpa* infection is independent of PCD. Second, in contrast to the promotion of *Hpa* growth by VPE activity, VPEs suppress the growth of turnip mosaic virus and *Botrytis cinerea* (Rojo *et al.*, 2004). VPEs also promote symbiosis with *Piriformospora indica* (Qiang *et al.*, 2012). In both these interactions, VPEs are thought to regulate PCD.

As an obligate biotroph, *Hpa* has lost metabolic pathways and hydrolytic enzymes during co-evolution with its host, and relies on the host machinery to obtain nutrients (Baxter *et al.*, 2010). Consistent with this principle, perturbations in the accumulation of homoserine and threonine make the plant an unsuitable host for *Hpa* (Van Damme *et al.*, 2009; Stuttmann *et al.*, 2011). *Hpa* may benefit from up-regulated VPE activity in the host by increased nutrient release or degradation of otherwise harmful proteins.

In conclusion, we present a simple, versatile and powerful method to monitor the activities of VPE isoforms *in vivo* and *in vitro* and in various tissues of various plant species. We used this method to discover an unexpected increase in γ VPE activity during *Hpa* infections, which led to the discovery of a role for VPEs during *Hpa* infections. AMS101 is a potent tool for further studies on the role and regulation of this important class of Cys proteases in plants.

EXPERIMENTAL PROCEDURES

Synthesis of AMS101

The synthesis of AMS101 is described in Data S1. Aliquots are available upon request.

Plant material and pathogen infections

Arabidopsis thaliana ecotype Columbia plants (Col-0) were grown in a growth chamber at 24°C (day)/20°C (night) under a 12 h light regime. The γ VPE knockout mutant (γ KO) (Rojo *et al.*, 2003), the γ VPE over-expressor line (γ OE) (Rojo *et al.*, 2003), the *vpe* quadruple knockout mutant (qKO) lacking all four VPEs (Gruis *et al.*, 2004), and the *eds1-2* mutant (Falk *et al.*, 1999) have been described previously. Cell cultures (ecotype Landsberg) were sub-cultured weekly in medium containing 3% w/v sucrose, 0.5 mg l⁻¹ 1-naphthaleneacetic acid, 0.05 mg l⁻¹ 6-benzylaminopurine and 4.4 g of Murashige and Skoog Gamborg B5 vitamins (Duchefa, www.duchefa-biochemie.nl), pH 5.7. Infection with *Hyaloperonospora arabidopsidis* (*Hpa*) isolates Noco2 or Cala2 was performed on 2-week-old seedlings by spray inoculation with water (control) or 4 × 10⁴ spores ml⁻¹. Plants were incubated at 20°C (day)/22°C (night) at 60% relative humidity, and leaves were harvested at various time points.

Sample preparation and labeling

Proteins were extracted by grinding rosette leaves in an Eppendorf tube using blue sticks (Sigma, www.sigmaldrich.com). The extract was mixed with 0.5 ml water and cleared by centrifugation for 1 min at 16 000 g. Labeling was usually performed by incubating approximately 100 µg protein in 60 µl buffer containing 70 mM sodium acetate (NaOAc) pH 5.5, 10 mM DTT and 1–1.6 µM AMS101 for 2 h at room temperature (22–25°C) in the dark. Equal volumes of dimethylsulfoxide were added to the no probe controls. The labeling reaction was stopped by adding 4× SDS-PAGE loading buffer containing β-mercaptoethanol, and the reaction mixture was separated on 12% SDS gels. Labeled proteins were visualized by in-gel fluorescence scanning using a Typhoon 9000 scanner (GE Healthcare, http://www.gelifsciences.com) with excitation and emission at 532 and 580 nm, respectively. Fluorescent signals were quantified by ImageQuant 5.2 (GE Healthcare). Inhibition assays were performed by pre-incubating protein extracts with 50 µM Ac-YVAD-CMK (Calbiochem, www.millipore.com/calbiochem) or xxAD-AOMK (Berger *et al.*, 2006) for 30 min before labeling with AMS101. For *in vivo* labeling, cell cultures and 2-week-old seedlings were incubated at room temperature in the dark under gentle shaking in the presence or absence of AMS101. Labeled cell cultures and seedlings were washed twice with culture medium and three times with water before analysis. Fluorescent signals of AMS101 (BOPIDY; excitation 532 nm/emission 580 nm) were detected using a Zeiss LSM 510 confocal microscope (www.zeiss.com). Confocal microscopy was performed using an HeNe1 laser (excitation 534 nm) and a UV laser. Zeiss LSM Image Examiner software was used for confocal image processing.

Agro-infiltration of Arabidopsis VPEs

The open reading frames of Arabidopsis VPEs were amplified by RT-PCR using the primers listed in Table S1. Amplified fragments were cloned into pFK26 (Shabab *et al.*, 2008) using the restriction enzymes listed in Table S1. Expression cassettes were verified by sequencing and inserted into binary vector pTP5 (Shabab *et al.*, 2008). *Agrobacterium tumefaciens* strain GV3101 was transformed with the binary vectors and used for agro-infiltration as described previously (Van der Hoorn *et al.*, 2000). *Agrobacterium* cultures containing binary protease expression vectors were mixed with *Agrobacterium* cultures containing a binary expression vector for silencing inhibitor p19 (Van der Hoorn *et al.*, 2003; Voinnet *et al.*, 2003). Cultures were infiltrated into 5-week-old *N. benthamiana* plants using a syringe without a needle.

Affinity purification and identification of labeled proteins

Leaf extracts were labeled with 5 µM biotinylated AMS101 at pH 5.5 for 2 h. The labeled leaf extracts were applied to PD-10 size-exclusion columns (Bio-Rad, www.bio-rad.com) to remove excess probe. Desalted samples were incubated with 100 µl high-capacity neutravidin agarose resin (Thermo Scientific, www.thermoscientific.com) for 1 h at room temperature under gentle agitation. Neutravidin agarose beads were collected by centrifuging for 10 min at 3000 g. Beads were washed (each 1 min) twice with 0.1% SDS, twice with 6 M urea, once with 50 mM Tris (pH 8) containing 1% Triton X-100, once with 1% Triton X-100, and once with water, then boiled in 30 µl of 2× SDS-PAGE loading buffer containing β-mercaptoethanol. Affinity-purified proteins were separated on a 12% one-dimensional SDS gel, and stained using SYPRO® Ruby (www.bio-rad.com). Specific bands were excised from the gel and subjected to in-gel tryptic digestion and subsequent MS analysis.

LC-MS/MS analyses were performed on an LTQ Velos mass spectrometer (Thermo Scientific, www.thermoscientific.com) coupled to an Proxeon Easy nano liquid chromatograph. The resulting spectra were then processed using Raw2MSM (Olsen *et al.*, 2005), selecting only the six strongest daughter ions per 100 Da. The resulting mgf files were searched against a database containing Arabidopsis Information Resource sequences and common artifacts (TAIRS database, www.arabidopsis.org) using Mascot 2.3 (www.matrixscience.com). The peptide spectra were counted as matches if they exceeded the 95% certainty cut-off.

RNA isolation and RT-PCR

Plant RNA was extracted using an RNeasy plant mini kit (Qiagen, www.qiagen.com) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg total RNA using SuperScript II reverse transcriptase (Invitrogen, www.invitrogen.com). γ VPE transcript levels were quantified by quantitative PCR (Roche, www.roche.com) using *pex4* (At5G25760) as a control. All quantitative real-time PCRs were performed with at least three independent RNA samples. The primers used were 5'-TTACG AAGGCGTGTTTTTC-3' and 5'-GGCGAGGCGTGATACATTT-3' for *pex4*, and 5'-AGTGGGAAGTTGTGGATAG-3' and 5'-CTCCAGGGC AATAGGTACC-3' for γ VPE.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Structures of the VPE probes.

Figure S2. A second VPE signal in leaf extracts is induced upon Hpa infection.

Figure S3. Absolute and relative transcript levels of VPEs in Arabidopsis leaves.

Figure S4. Disease assays with *Hyaloperonospora arabidopsidis*.

Figure S5. Alignment of Arabidopsis VPEs protein sequences showing putative N-glycosylation sites.

Table S1. Cloning procedure of Arabidopsis VPEs genes for agro-infiltration.

Data S1. Synthesis of AMS101.

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