Host Cell Entry of Powdery Mildew Is Correlated with Endosomal Transport of Antagonistically Acting VvPEN1 and VvMLO to the Papilla

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Challenge by a nonadapted powdery mildew fungal pathogen leads to the formation of a local cell-wall apposition (papilla) beneath the point of attempted penetration. Several plasma membrane (PM) proteins with opposing roles in powdery mildew infection, including *Arabidopsis thaliana* PENETRATION1 (PEN1) and barley (*Hordeum vulgare*) MILDEW RESISTANCE LOCUS O (MLO), are localized to the site of powdery mildew attack. PEN1 contributes to penetration resistance to nonadapted powdery mildews, whereas MLO is a susceptibility factor required by adapted powdery mildew pathogens for host cell entry. Our previous studies have demonstrated that the vesicle and endosomal trafficking inhibitors, brefeldin A and wortmannin, have opposite effects on the penetration rates of adapted and nonadapted powdery mildews on grapevine. These findings prompted us to study the pathogen-induced intracellular trafficking of grapevine variants of MLO and PEN1. We first identified grapevine (*Vitis vinifera*) VvPEN1 and VvMLO orthologs that rescue *Arabidopsis Atpen1* and *Atmlo2 mlo6 mlo12* null mutants, respectively. By using endomembrane trafficking inhibitors in combination with fluorescence microscopy, we demonstrate that VvMLO3/VvMLO4 and VvPEN1 are co-trafficked together from the PM to the site of powdery mildew challenge. This focal accumulation of VvMLO3/VvMLO4 and VvPEN1 to the site of attack seems to be required for their opposing functions during powdery mildew attack, because their subcellular localization is correlated with the outcome of attempted powdery mildew penetration.

The majority of potentially phytopathogenic microbes that plants encounter fail to infect. This also applies to nonadapted powdery mildew species and is the result of plant basal immunity, where the initial recognition of pathogen attack is mediated by pattern recognition receptors that bind conserved pathogen-associated molecular patterns (PAMPs) (Robatzek et al. 2006), such as fungal chitin (Miya et al. 2007). This so-called PAMP-triggered immunity (PTI) induces the polarized secretion of cell wall materials and antimicrobials, which results in the formation of localized cell wall reinforcements (papillae) that

are assumed to prevent further pathogen ingress (Thordal-Christensen 2003). Currently, there are two pathways in the dicotyledonous model plant *Arabidopsis thaliana* that are known to be important for penetration resistance against powdery mildews (Lipka et al. 2005). The first involves PENETRATION2 (AtPEN2) and PENETRATION3 (AtPEN3), which, according to genetic data, function in the same pathway. In the current model, the atypical AtPEN2 myrosinase is involved in the biosynthesis of antimicrobial molecules that are delivered to the site of powdery mildew infection via the ATP-binding cassette (ABC) transporter AtPEN3 (Bednarek et al. 2009; Lipka et al. 2005; Stein et al. 2006). The second pathway requires the plasma membrane (PM)-localized soluble *N*-ethylmaleimide sensitive factor attachment receptor (SNARE), PENETRATION1 (AtPEN1; AtSYP121 in *Arabidopsis*)/REQUIRED FOR MLO-SPECIFIED RESISTANCE2 [HvROR2] in barley for powdery mildew penetration resistance (Collins et al. 2003). SNARE proteins are important for membrane fusion events during endomembrane trafficking, including polarized secretion. Complexes formed between SNARE on both the target membrane and vesicles enable membrane tethering to facilitate fusion and cargo release (Lipka et al. 2007; Pratelli et al. 2004). AtPEN1 forms a ternary SNARE complex with SYNAPTOSOMAL-ASSOCIATED PROTEIN 33 (AtSNAP33) and the VESICLE-ASSOCIATED MEMBRANE PROTEINs AtVAMP722 and AtVAMP721 (Kwaaitaal et al. 2010; Kwon et al. 2008). AtPEN1 re-localizes from the PM to the papilla following powdery mildew challenge, possibly via the formation of multivesicular bodies (MVB) and exosome secretion (Meyer et al. 2009; Nielsen et al. 2012).

Some powdery mildew species have adapted to grow on specific plant hosts. These adapted species have found a way to overcome or suppress host-mediated PTI-based penetration resistance. There is a growing body of evidence to suggest that adapted powdery mildew species use specific host proteins to facilitate penetration into the host cell. MILDEW RESISTANCE LOCUS O (MLO) proteins are a family of plant-specific integral membrane proteins with a heptahelical topology (Devoto et al. 1999, 2003). Barley mutants that lack a functional version of HvMLO display broad-spectrum resistance to all known powdery mildew isolates of *Blumeria graminis* f. sp. *hordei*. HvMLO also accumulates at the site of powdery mildew challenge (Bhat et al. 2005). In *Arabidopsis*, there are three functionally partially redundant co-orthologs of HvMLO (*AtMLO2*, *AtMLO6*, and *AtMLO12*), such that only *Atmlo2 mlo6 mlo12* triple mutants display complete immunity to powdery mildew while *mlo2* single mutants exhibit partial resistance. This resistance is abolished in the *Atmlo2 pen1*

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mutant (Consonni et al. 2006), suggesting that the AtPEN1-mediated contribution to defense is antagonized by the presence of *Atmlo2*. In grapevine, three members of the *MLO* family (*VvMLO3*, *VvMLO4*, and *VvMLO17*), which are orthologous to the *AtMLO2*, *AtMLO6*, and *AtMLO12* genes, were found to be specifically induced at the transcriptional level during infection by grapevine powdery mildew (*Erysiphe necator*) (Feechan et al. 2008), suggesting that these *VvMLO* proteins may have a role in modulating antifungal defense responses in grapevine. However, the mechanism by which *MLO* suppresses PTI to facilitate penetration of adapted powdery mildew species on selected plant hosts is still not understood.

Several studies have revealed indirect evidence that secretory pathways of plant host cells are pivotal to the outcome of powdery mildew attack. For example, the depolymerization of the actin cytoskeleton following treatment with the fungal-derived inhibitor cytochalasin E (CE) (Feechan et al. 2011; Kobayashi et al. 1997; Yun et al. 2003) or the overexpression of actin depolymerization factors (*ADFs*) were both found to compromise penetration resistance to nonadapted powdery mildew species (Miklis et al. 2007). The actin cytoskeleton has been proposed to facilitate the delivery of secretory vesicles, which are thought to be involved in basal defense against powdery mildew (Kwon et al. 2008). Furthermore, actin was recently implicated in the trafficking of AtPEN3-green fluorescent protein (GFP) to the site of fungal attack (Underwood and Somerville 2008). In addition, vesicle trafficking inhibitors such as brefeldin A (BFA) and wortmannin (WM) have also been shown to compromise penetration resistance to nonadapted powdery mildew species (Feechan et al. 2011). BFA interferes with ADP ribosylation factor GTP exchange factors (ARF-GEF) (Geldner et al. 2003; Jackson and Casanova 2000), which are important for recruiting protein coats required for vesicle formation and cargo selection (Teh and Moore 2007). In fact, the trafficking of AtPEN1 from the PM to the papilla is dependent on the BFA-sensitive ARF-GEF, GNOM (Nielsen et al. 2012). WM is thought to inhibit endocytosis via phosphatidylinositol 3-kinase (PI3K), an enzyme involved in the production of the endosomal membrane lipid phosphatidylinositol 3-phosphate (Wang et al. 2009).

In the presence of an adapted powdery mildew species, actin-dependent and actin-independent defense pathways are under negative control by HvMLO (Miklis et al. 2007). However, the trafficking components required for *MLO* localization during powdery mildew infection remain unknown. By using transgenic *Arabidopsis* plants and employing the actin inhibitor CE and the trafficking inhibitors WM and BFA, we show that VvPEN1 and VvMLO share the same endocytic trafficking pathway, suggesting that these proteins are recycled from the PM to papillae at the site of attempted powdery mildew ingress. The extent of this ARF-GEF-dependent trafficking to the powdery mildew attack site correlates with the outcome of attempted powdery mildew penetration.

RESULTS

The grapevine SNARE VvPEN1 mediates penetration resistance to powdery mildew in *Arabidopsis*.

Certain members of the SNARE protein family have been shown to play a specific role in penetration resistance against powdery mildew, including the Qa SNARE HvROR2 from barley and AtPEN1/AtSYP121 from *Arabidopsis* (Collins et al. 2003). In order to identify which Qa SNARE is involved in penetration resistance against powdery mildew in grapevine, we performed a phylogenetic analysis of the predicted Qa SNARE translation products from the *Vitis vinifera* PN40024 genome sequence (Jallion et al. 2007) together with the 18

members of the *A. thaliana* Qa SNARE family (Lipka et al. 2007) and the barley Qa SNARE HvROR2. AtPEN1/AtSYP121 and HvROR2 were found to cluster with the translated product of the grapevine sequence, VIT_08s0032g01150 (Supplementary Fig. S1). Primers designed to the VIT_08s0032g01150 genomic sequence were used to amplify a product from *V. vinifera* 'Cabernet Sauvignon' root cDNA encoding a 323-amino-acid protein with high sequence similarity to AtPEN1/AtSYP121 and HvROR2, including a conserved Qa-type SNARE domain. This gene product was designated as VvPEN1.

Arabidopsis Atpen1 mutants are compromised in penetration resistance and, therefore, allow enhanced entry levels of nonadapted powdery mildew species (Collins et al. 2003). To determine whether VvPEN1 could contribute to penetration resistance, we generated transgenic *Arabidopsis* lines expressing this gene in the *Atpen1* null mutant background (Fig. 1B). The heterologous expression of VvPEN1 was found to enhance penetration resistance in the *Atpen1* mutant because penetration rates of the nonadapted powdery mildew species *E. necator* into epidermal cells of transgenic lines *Atpen1-1VvPEN1-1* and *Atpen1VvPEN1-2* were reduced to levels approaching that observed in wild-type (Col-0) plants (Fig. 1A).

GFP-AtPEN1 has been previously found to accumulate in papillae following challenge by adapted and nonadapted powdery mildew species (Assaad et al. 2004; Bhat et al. 2005). To find out whether the protein signatures required for this pathogen-triggered relocalization are conserved in VvPEN1, we transformed a construct encoding an N-terminal GFP fusion into the *Atpen1* mutant and examined the subcellular localization of GFP-VvPEN1 by fluorescence microscopy. In the absence of the pathogen, GFP-VvPEN1 was localized predominantly to the cell periphery, presumably the PM (Fig. 1C), as shown previously for AtPEN1 and ROR2 (Assaad et al. 2004; Collins et al. 2003). However, following challenge with either a nonadapted powdery mildew, *E. necator* (Fig. 1D and E) or the adapted powdery mildew species *E. cichoracearum* (Fig. 1F and G), VvPEN1 was often (in approximately one-third of the cases; for quantification, discussed below) found to accumulate in the papilla formed beneath the penetration peg.

VvMLO3 and VvMLO4 are susceptibility factors to powdery mildew in transgenic *Arabidopsis*.

We previously characterized the *VvMLO* gene family in grapevine and identified three candidates (*VvMLO3*, *VvMLO4*, and *VvMLO17*) that may be involved in the susceptibility of grapevine to *E. necator*. This prediction was based on the extent of sequence similarity to the *Arabidopsis* genes involved in powdery mildew susceptibility (*AtMLO2*, *AtMLO6*, and *AtMLO12*) (Consonni et al. 2006), the presence of a characteristic C-terminal sequence motif (Panstruga 2005), and the observation that these three *VvMLO* genes are transcriptionally up-regulated in response to powdery mildew infection (Feechan et al. 2008). To obtain further evidence that these grapevine *VvMLO* susceptibility candidates have a role in powdery mildew penetration, we transformed the *Arabidopsis Atmlo2 mlo6 mlo12* triple mutant, which is resistant to *E. cichoracearum* infection (Consonni et al. 2006), with either *VvMLO3*, *VvMLO4*, or *VvMLO17* under the control of the constitutive *Cauliflower mosaic virus* 35S promoter. As a control, the *Atmlo2 mlo6 mlo12* mutant was also transformed with *VvMLO6*, which was previously shown to be transcriptionally unresponsive to powdery mildew infection (Feechan et al. 2008) and which lacks key conserved amino acid sequence motifs found in *VvMLO3*, *VvMLO4*, and *VvMLO17* (and the *AtMLO* susceptibility proteins). Transcript accumulation of the different *VvMLO* genes in the transgenic *Atmlo2 mlo6 mlo12* mutant lines was confirmed using semiquantitative

Fig. 1. *VvPEN1* rescues the *Atpen1* mutant phenotype. **A**, Penetration efficiency of the nonadapted powdery mildew pathogen *Erysiphe necator* on *Arabidopsis* wild type (Col-0), the *Atpen1* mutant, and five independent transgenic *Atpen1* lines expressing *VvPEN1*. Each data point represents the mean of three independent experiments ($\pm$ standard deviation). In each experiment, a minimum of 100 germinated conidia were scored on each of three leaves for each line. Asterisk indicates a statistically significant difference from *Atpen1* (** indicates $P < 0.005$; Student's t test). **B**, Semiquantitative reverse-transcription- polymerase chain reaction analysis of *VvPEN1* expression in transgenic *Atpen1* lines 1 to 5. *AtEF1A* was used as an internal control. Results shown are representative of two replicate experiments. **C–G**, *Atpen1* lines expressing a green fluorescent protein (GFP)-*VvPEN1* fusion construct were examined for subcellular GFP localization **C**, in unchallenged plants; following challenge with **D** and **E**, the nonadapted powdery mildew species *E. necator* or **F** and **G**, the adapted powdery mildew species *E. cichoracearum*, fungal structures on the epidermal cell surface were visualized with propidium iodide (**E** and **G**). **D** to **G**, Arrowheads mark the sites of attempted fungal entry. Images were taken at 24 h after pathogen inoculation and are representative of the results obtained in three independent experiments. Scale bar = 30 μ M.

reverse-transcription-polymerase chain reaction (RT-PCR) (Fig. 2B). Both *VvMLO3* and *VvMLO4* but not *VvMLO17* or *VvMLO6* were found to significantly increase *E. cichoracearum* penetration in the *Atmlo2 mlo6 mlo12* mutant (Fig. 2A).

The heterologous expression of *VvMLO3* was also found to rescue the early leaf senescence phenotype of the *Atmlo2 mlo6 mlo12* mutant (Consonni et al. 2006, 2010) (Fig. 2C and D). This data supports the hypothesis that at least *VvMLO3* and

Fig. 2. *VvMLO3* and *VvMLO4* partially rescues the *Atmlo2 mlo6 mlo12* triple mutant. **A**, Penetration efficiency of the adapted powdery mildew *Erysiphe cichoracearum* on *Arabidopsis* wild type (Col-0), the *Atmlo2 mlo6 mlo12* mutant, and independent transgenic *Atmlo2 mlo6 mlo12* lines expressing either *VvMLO3*, *VvMLO4*, *VvMLO17*, or *VvMLO6* (four independent lines per transgene). Each data point is the mean of three independent experiments ($\pm$ standard deviation). In each experiment, a minimum of 100 germinated conidia were scored on each of three leaves for each line. Asterisks indicate a statistically significant difference from *Atmlo2 mlo6 mlo12* (** and * indicate $P < 0.005$ and 0.05 , respectively; Student's *t* test). **B**, Semiquantitative reverse-transcription-polymerase chain reaction analysis of *VvMLO3*, *VvMLO4*, *VvMLO6*, or *VvMLO17* expression in transgenic *Atmlo2 mlo6 mlo12* mutant lines 1 to 4 and a control *Atmlo2 mlo6 mlo12* mutant line. *AtEF1A* was used as an internal control. Results shown are representative of two replicate experiments. **C** and **D**, *VvMLO3* rescues the early senescence phenotype of the *Atmlo2 mlo6 mlo12*. Pictures were taken of 6-week-old flowering plants of *Col-0*, *Atmlo2 mlo6 mlo12*, and *Atmlo2 mlo6 mlo12* lines expressing *VvMLO3*, *VvMLO4*, *VvMLO6*, and *VvMLO17* in pots (**C**) and close-ups of individual plants from each pot (**D**).

VvMLO4 act as powdery mildew susceptibility factors in grapevine and suggests that these two genes may show functional redundancy with respect to powdery mildew susceptibility but likely adopt separate roles with regard to leaf senescence.

VvMLO accumulation at attack sites correlates with powdery mildew penetration.

Next, we investigated the localization of the VvMLO proteins in the *Atmlo2 mlo6 mlo12* mutant using constructs expressing C-terminal GFP fusions of each of the VvMLO proteins. VvMLO4-GFP, VvMLO17-GFP (Fig. 3A and B), VvMLO3-GFP, and VvMLO6-GFP (Supplementary Fig. S2B and C) were all found to be localized to the cell periphery (presumably the PM) when expressed in *Atmlo2 mlo6 mlo12* mutant plants compared with free (cytoplasmic) GFP, which was found throughout the cell. Following inoculation with *E. cichoracearum*, both VvMLO4-GFP (Fig. 3B and C) and VvMLO3-GFP localized to the site of powdery mildew appressoria formation, which is in agreement with previous localization studies on HvMLO (Bhat et al. 2005). However, VvMLO17-GFP was rarely found at the site of powdery mildew attack (Fig. 3E and F). To investigate whether the increased ability of VvMLO4 to rescue the *Atmlo2 mlo6 mlo12* triple mutant compared with VvMLO17 (Fig. 2) might be correlated to the frequency of focal accumulation of these proteins under the site of infection, the number of VvMLO4-GFP and VvMLO17-GFP localizations under appressoria were scored

in the transgenic *Atmlo2 mlo6 mlo12* background following *E. cichoracearum* infection. VvMLO4-GFP was found to be present under $30.9 \pm 6.9\%$ of appressoria, whereas VvMLO17-GFP was only present under $7.7 \pm 2.2\%$ of *E. cichoracearum* appressoria (Fig. 3G). This observation suggests that the ability of VvMLO4 but not VvMLO17 to enhance powdery mildew penetration in the *Atmlo2 mlo6 mlo12* mutant (Fig. 2) might be related to the frequency of VvMLO4 localization at the site of powdery mildew attack.

Endosomal trafficking of VvPEN1 and VvMLO3/4.

AtPEN1 is secreted to and incorporated into papillae following powdery mildew challenge (Meyer et al. 2009). Recent evidence suggests that AtPEN1 is recycled from the PM to papillae, most likely via MVB, and that the reorganization of this membrane material is dependent on the ARF-GEF, GNOM (Nielsen et al. 2012). To determine the cellular machinery required for the intracellular trafficking of VvPEN1 and VvMLO4, the actin cytoskeleton depolymerizing drug CE, the endocytosis inhibitor WM, and the ARF-GEF vesicle-trafficking inhibitor BFA were employed. These inhibitors were infiltrated into the abaxial side of leaves of *pen1 GFP-VvPEN1* lines at concentrations previously found to be effective in *Arabidopsis* (Feechan et al. 2011). CE (10 μ M) had no effect on the localization and distribution of GFP-VvPEN1 at the PM compared with the control (0.2% dimethyl sulfoxide [DMSO] only) (Fig. 4A and B). However, BFA (50 μ M) and

Fig. 3. Focal accumulation of VvMLO at the site of powdery mildew penetration is required for partial rescue of the *Atmlo2 mlo6 mlo12* triple mutant. **A**, VvMLO4-green fluorescent protein (GFP) is localized to the cell periphery. **B**, VvMLO4-GFP accumulation at the site of powdery mildew infection in the *Atmlo2 mlo6 mlo12* mutant expressing VvMLO4-GFP. **C**, Powdery mildew structures on the epidermal cell surface were visualized using propidium iodide. The arrow indicates focal accumulation of VvMLO4-GFP. **D**, VvMLO17-GFP is localized to the cell periphery. **E**, VvMLO17-GFP localization following powdery mildew infection in the *Atmlo2 mlo6 mlo12* mutant expressing VvMLO17-GFP. **F**, Powdery mildew structures on the epidermal cell surface were visualized using propidium iodide. **G**, VvMLO4-GFP and VvMLO17-GFP accumulation at the site of attempted powdery mildew penetration in the *Atmlo2 mlo6 mlo12* background scored at 24 h following *Erysiphe cichoracearum* challenge. Each data point is the mean of two independent experiments ($\pm$ standard deviation). In each experiment, a minimum of 50 germinated conidia were scored on each of three leaves for each line. Asterisk indicates a statistically significant difference between the lines (* indicates $P < 0.05$; Students *t*-test). Scale bar = 30 μ m.

Fig. 4. Inhibitors of vesicle trafficking prevent accumulation of green fluorescent protein (GFP)-VvPEN1 and VvMLO4-GFP at the site of powdery mildew attack. Leaves of an *Atpen1* GFP-VvPEN1 line were treated with **A**, 0.2% dimethyl sulfoxide (DMSO) and inhibitors **B**, 25 μ M cytochalasin E (CE); **C**, 50 μ M brefeldin A (BFA); and **D**, 40 μ M wortmannin (WM) for 2 h. *Atpen1*GFP-VvPEN1 inoculated with *Erysiphe necator* following infiltration with **E**, 0.2% DMSO and 50 μ M BFA or **F**, 40 μ M WM and scored for the presence of GFP-VvPEN1 in papillae. Leaves of *Atmlo2 mlo6 mlo12* VvMLO4-GFP treated with **G**, 0.2% DMSO and inhibitors **H**, 25 μ M CE; **I**, 50 μ M BFA; and **J**, 40 μ M WM for 2 h. *Atmlo2 mlo6 mlo12* VvMLO4-GFP inoculated with *E. cichoracearum* following infiltration with **K**, 0.2% DMSO and 50 μ M BFA or **L**, 40 μ M WM and scored for the presence of VvMLO4-GFP beneath *E. cichoracearum* appressoria. Data shown are the mean of three independent experiments ($\pm$ standard deviation). In each experiment, a minimum of 50 germinated conidia were scored on each of three leaves for each line. Arrows indicate the location of membrane aggregations. Insets D and J show enlarged images ($\times 3$) of the membrane aggregations in the area indicated by the unboxed white arrow. Asterisk indicates a statistically significant difference from control (DMSO treatment; * indicates $P < 0.05$; Student's *t* test). Scale bar = 30 μ M.

WM (40 μ M) both caused the accumulation of GFP-VvPEN1 in intracellular vesicular membrane aggregations. BFA treatment produced fewer and larger GFP-VvPEN1-marked aggregations (Fig. 4C), referred to as BFA bodies (Satiat-Jeunemaitre et al. 1996), whereas WM treatment resulted in numerous small GFP-VvPEN1-labeled aggregations (Fig. 4D). GFP-VvPEN1 also frequently accumulates in papillae under invading powdery mildew (*E. necator* and *E. cichoracearum*) sporelings (Fig. 1D to G). Therefore, to investigate the effect of BFA and WM on the endosomal trafficking of GFP-VvPEN1 to papillae, BFA and WM were infiltrated into leaves of *pen1* GFP-VvPEN1 lines prior to challenge with the nonadapted powdery mildew species *E. necator*. Both BFA and WM were found to significantly reduce the frequency of GFP-VvPEN1 papillae from 30.8 ± 4.2 to $13.4 \pm 2.2\%$ (Fig. 4E) and 31.2 ± 3.6 to $14.8 \pm 5.3\%$, respectively (Fig. 4F). Similar results were found using the same cellular machinery inhibitors with *Atmlo2 mlo6 mlo12* lines expressing *VvMLO4-GFP* (Fig. 4G to J). CE had no effect on the distribution of VvMLO4-GFP compared with the DMSO control (Fig. 4G and H). However, BFA (50 μ M) and WM (40 μ M) both caused the accumulation of VvMLO4-GFP in intracellular membrane bodies (Fig. 4I and J). As observed for GFP-VvPEN1 (discussed above and Fig. 4C and D), the endomembrane bodies observed in the BFA-treated cells were fewer but much larger in size than those in the WM-treated cells (Fig. 4I and J). Both BFA and WM were also found to significantly reduce the frequency of VvMLO4-GFP accumulation under appressoria from 21.2 ± 2.8 to $5.2 \pm 2.7\%$ and (Fig. 4K) from 31 ± 3.6 to $14.8 \pm 5.3\%$ (Fig. 4L), respectively.

VvPEN1 and VvMLO3/4 are trafficked via the same endocytic vesicles.

The similar behavior of VvPEN1 and VvMLO3/VvMLO4 in response to the tested endomembrane trafficking inhibitors BFA and WM suggests that VvPEN1 and VvMLO3/VvMLO4 may co-traffic via the same vesicular compartments in the same endocytic pathway. In order to investigate this possibility further by co-localization studies, we carried out co-bombardment experiments of cyan fluorescent protein (CFP)-VvPEN1 and VvMLO3-YFP (yellow fluorescent protein) expression constructs into onion epidermal cells followed by incubation in 10 μ M CE, 50 μ M BFA, or 40 μ M WM.

As found for *Arabidopsis* leaf cells (Fig. 4), CE (10 μ M) had no effect on the distribution of VvPEN1 or VvMLO3 fusion proteins in onion cells (Fig. 5D to F and M) compared with the DMSO-treated control cells (Fig. 5A to C and M). In contrast, BFA (50 μ M) induced the formation of BFA bodies in $85.9 \pm 2.3\%$ of transformed cells (Fig. 5G to I and M) and WM (40 μ M) induced smaller endomembrane compartments in $34.8 \pm 6.3\%$ of transformed cells (Fig. 5J to L and M). Furthermore, the merged images (Fig. 4I and L) demonstrate that VvMLO3-GFP and CFP-VvPEN1 are co-localized within the same membrane bodies, further corroborating the co-trafficking of these proteins. Similar results were obtained when comparing the trafficking of VvMLO4-YFP and CFP-VvPEN1 in onion cells (data not shown).

VvPEN1 and VvMLO3/VvMLO4 trafficking is correlated with the outcome of powdery mildew challenge.

We have shown that VvPEN1 and VvMLO3/VvMLO4 are trafficked via the same endosomal pathway and that this BFA- and WM-sensitive vesicle trafficking is required for the pathogen-triggered localization of these proteins to the site of powdery mildew ingress. Previously, we reported that BFA and WM have opposite effects on the penetration rates of adapted and nonadapted powdery mildew species on grapevine (Feechan

et al. 2011). Therefore, to investigate how the inhibition of VvMLO and VvPEN1 trafficking affects powdery mildew infection, the penetration rates of the nonadapted powdery mildew species *E. necator* and the adapted powdery mildew species *E. cichoracearum* were investigated in *Atpen1 VvPEN1* and *Atmlo2 mlo6 mlo12 VvMLO3/VvMLO4* lines following treatment with either 50 μ M BFA or 40 μ M WM. As previously observed in grapevine, both BFA and WM significantly increased the percentage of *Arabidopsis* cells penetrated by the nonadapted powdery mildew species *E. necator* (Fig. 6A and B) but significantly decreased the penetration efficiency of the adapted powdery mildew *E. cichoracearum* (Fig. 6C and D). In fact, it can be seen that, in the presence of these endosomal trafficking inhibitors, the resulting penetration rates of the adapted and nonadapted powdery mildew species are similar. Both BFA (Fig. 6A) and WM (Fig. 6B) significantly affected penetration resistance in *Atpen1*, mediated by VvPEN1, increasing penetration rates, whereas there was no effect on the penetration frequency of the *Atpen1* mutant (Fig. 6A and B). These results suggest that the endosomal trafficking of AtPEN1 in wild-type *Arabidopsis* and the introduced VvPEN1 in *Atpen1* mutants is required for efficient penetration resistance.

Similarly, rescue of the *Atmlo2 mlo6 mlo12* mutant by VvMLO3 or VvMLO4 was found to be significantly inhibited by BFA (Fig. 6C) and WM (Fig. 6D), decreasing fungal penetration rates. However, these inhibitors had no effect on the frequency of penetration observed in the *Atmlo2 mlo6 mlo12* mutant (Fig. 6C and D). Together, these results suggest that the trafficking of the endogenous AtMLO2 in wild-type *Arabidopsis* and of VvMLO3 and VvMLO4 in *Atmlo2 mlo6 mlo12*-rescued mutants is required for powdery mildew susceptibility.

DISCUSSION

Using a phylogenetic approach, we have successfully identified VvPEN1, a grapevine homolog of AtPEN1/AtSYP121 and HvROR2, which rescues the *Atpen1* mutant by restoring penetration resistance against a nonadapted powdery mildew species (*E. necator*) (Fig. 1). Although the two closely related *Arabidopsis* Qa SNARE proteins AtPEN1/AtSYP121 and AtSYP122 share overlapping functions in plant growth and development, only AtPEN1 has a role in powdery mildew resistance (Assaad et al. 2004). Therefore, it is interesting to speculate as to which amino acid sequence differences between these two proteins are responsible for the functional specialization associated with powdery mildew resistance. Pajonk and associates (2008) investigated the N-terminal serine residues of AtSYP121/AtPEN1 and AtSYP122, which are differentially phosphorylated in response to elicitation with the bacterial-derived peptide flg22, at positions S6 and S8 of SYP122 and S7 of AtPEN1 (Benschop et al. 2007; Nühse et al. 2003). The S7 residue is conserved in both AtPEN1 and HvROR2 but this phosphorylation site is dispensable for disease resistance-conferring activity. Our VvPEN1 results support this conclusion, given that there is a glycine at position 7 (as found in AtSYP122) rather than a serine and VvPEN1 still displays disease resistance activity (Fig. 1). The existence of several PEN1 sequences from different species now provides further insight into which amino acids are critical for this functional specialization.

Based on sequence homology, the presence of a characteristic sequence motif, and expression kinetics following powdery mildew infection, we previously identified three *VvMLO* genes (*VvMLO3*, *VvMLO4*, and *VvMLO17*) that may have a role in modulating defense responses against powdery mildew (Feechan et al. 2008). Results of studies with the *Atmlo2 mlo6 mlo12* triple mutant provide further evidence that at least

VvMLO3 and *VvMLO4* act as powdery mildew susceptibility genes in grapevine. The fact that only partial rescue was observed (Fig. 2A) may be due to differences in the amino acid sequence compared with the authentic *AtMLO2* polypeptide, which may affect protein folding or stability in the heterolo-

gous environment. In barley, similarly heterologous expression of rice *OsMlo2* only partially complemented the *mlo* mutant phenotype (Elliott et al. 2002). Notably, only *VvMLO3* was found to rescue the early leaf senescence phenotype of *Atmlo2 mlo6 mlo12* (Consonni et al. 2006, 2010) (Fig. 2C and D).

Fig. 5. *VvMLO3* and *VvPEN1* are co-trafficked in the same vesicle bodies. Onion epidermal cells were co-bombarded with *VvMLO3-YFP* and *CFP-VvPEN1* constructs and incubated for 24 h with **A** to **C**, 0.4% dimethyl sulfoxide (DMSO); **D** to **F**, 10 μ M cytochalasin E (CE); **G** to **I**, 50 μ M brefeldin A (BFA); or **J** to **L**, 40 μ M wortmannin (WM) for 2 h. White arrowheads indicate membrane aggregations or BFA bodies. Data shown are representative of three independent experiments. **M**, Percentage of transformed onion cells transiently expressing *VvMLO3-YFP* and *CFP-VvPEN1* that contain membrane aggregations following treatment with 0.2% DMSO, 25 μ M CE, 50 μ M BFA, or 40 μ M WM for 2 h. Values are the mean of three independent experiments ($\pm$ standard deviation). In each experiment, a minimum of 40 transformed onion cells were scored. Asterisk indicates a statistically significant difference from the control (** indicates $P < 0.005$; Student's *t*-test). Scale bar = 55 μ M.

This finding suggests that VvMLO3 has an independent function in terms of senescence despite its shared role with VvMLO4 in powdery mildew susceptibility. However, we also cannot exclude the possibility that different protein accumulation levels of VvMLO3 and VvMLO4 cause the observed difference; whereas lower levels might be sufficient to rescue the powdery mildew phenotype, higher levels (possibly only

achieved with VvMLO3) may be necessary to rescue the early leaf senescence phenotype. Because restoration of wild-type-like leaf senescence by VvMLO3 was seemingly complete, the requirements for complementation of this phenotype appear to be less stringent compared with the incompletely reinstated disease susceptibility phenotype. VvMLO17 failed to rescue the *Atmlo2 mlo6 mlo12* mutant with regard to both powdery

Fig. 6. Endosomal trafficking inhibitors have opposing effects on penetration resistance to nonadapted and adapted powdery mildew fungi. Penetration rates of the nonadapted pathogen *Erysiphe necator* on wild type (Col-0) and *Atpen1* and *Atpen1* lines expressing VvPEN1 following treatment with **A**, 0.2% dimethyl sulfoxide (DMSO) and 25 μ M brefeldin A (BFA) or **B**, 40 μ M wortmannin (WM). Penetration rates of the adapted powdery mildew *E. cichoracearum* on wild type (Col-0), *Atmlo2 mlo6 mlo12*, and *Atmlo2 mlo6 mlo12* lines expressing VvMLO3 or VvMLO4 following treatment with **C**, 0.2% DMSO and 25 μ M BFA or **D**, 40 μ M WM treatment. Leaves were infiltrated with inhibitors immediately before inoculation and penetration events were scored at 48 h after pathogen challenge. Data shown are representative of the results obtained in at least two independent experiments. In each experiment, a minimum of 100 germinated conidia were scored on at least three leaves of each line. Values represent the mean $\pm$ standard deviation. Asterisks indicate a statistically significant difference from control (Col-0 treated with DMSO; ** and * indicate $P < 0.005$ and 0.05 , respectively; Student's t test).

mildew susceptibility and early leaf senescence, which could be due to either functional diversification or insufficient protein accumulation in the transgenic lines. Thus, it is still possible that VvMLO17 acts as a powdery mildew susceptibility factor in its source plant species, grapevine. This is now being examined by the generation of grapevine transgenics expressing RNAi constructs targeting each of these VvMLO genes to assess their role in suppressing defense responses to *E. necator* (A. Feechan, *unpublished*).

It has previously been shown that membrane proteins involved in defense-related processes against powdery mildew penetration are preferentially incorporated into papillae. Both HvMLO and HvROR were found to undergo focal accumulation after challenge with powdery mildew, whereas other PM proteins including aquaporins were not (Bhat et al. 2005). Indeed, the most closely related syntaxin to AtPEN1, AtSYP122, which is also PM localized, is not incorporated into papillae (Nielsen et al. 2012). This preferential integration of only a subset of membrane proteins has also been demonstrated for powdery mildew haustorial encasements where the SNARE proteins AtPEN1 and AtSNAP33 were incorporated into haustorial encasements more frequently than other SNARE (Meyer et al. 2009). Thus, the focal accumulation of VvPEN1 and VvMLO3/VvMLO4 beneath sites of attempted fungal penetration is in agreement with previous studies. The BFA-mediated inhibition of GFP-VvPEN1 trafficking and a concomitant reduced incidence of its incorporation into papillae resembles the trafficking of GFP-AtPEN1 to the papilla, which was recently shown to be dependent on the ARF-GEF, GNOM (Nielsen et al. 2012). Given that VvMLO3 trafficking is BFA dependent, it would be interesting to test whether VvMLO3 trafficking is also GNOM dependent. Furthermore, the inhibition of GFP-VvPEN1 and VvMLO4-GFP trafficking to the site of powdery mildew attack by the PI3K inhibitor WM suggests a role for endocytosis in the redistribution of PM material to the site of fungal attack. BFA and WM were recently found to alter the dynamics and distribution of clathrin-coated pits, which are required for selective endocytosis (Ito et al. 2012). Therefore, both the penetration resistance protein VvPEN1 and the susceptibility factors VvMLO3 and VvMLO4 are likely co-trafficked via the same endocytic pathway from the PM to the site of powdery mildew challenge.

MLO proteins are required by adapted powdery mildews to successfully infect the plant cell. This suggests that these pathogens may exploit MLO functions to suppress basal defense responses (Panstruga 2005). The inhibition of VvMLO3 and VvMLO4 trafficking and localization to the site of powdery mildew challenge reduces the penetration success of an adapted powdery mildew pathogen (Fig. 6C and D). Therefore, the accumulation of VvMLO3 and VvMLO4 at the site of powdery mildew attack is correlated with their function as susceptibility factors.

Large vesicular membrane compartments (possibly representing MVB) are found in barley *mlo* mutants around the site of powdery mildew ingress (Collins et al. 2003). The cargo that might be transported by these membrane bodies has not yet been elucidated. Although callose accumulates in the papilla, this poly-carbohydrate is not delivered via AtPEN1 (Meyer et al. 2009). However, analogous to AtPEN1, the secretion and deposition of callose was found to be dependent on the ARF-GEF, GNOM (Nielsen et al. 2012). It is interesting to note that callose deposition at pathogen entry sites has also been shown to be inhibited by CE application (Kobayashi and Hakuno 2003). The ABC transporter protein AtPEN3 is likewise required for callose deposition (Clay et al. 2009) and its own accumulation at attempted *B. graminis* f. sp. *hordei* infection sites is disrupted by CE treatment (Underwood and Somerville

2008). This requirement of callose deposition for both BFA- and CE-sensitive pathways requires further investigation.

MLO has been shown to negatively regulate both actin-dependent and actin-independent penetration resistance pathways (Miklis et al. 2007). However, the actin cytoskeleton inhibitor CE does not appear to affect VvMLO or VvPEN1 trafficking in *Arabidopsis* (Fig. 4B and H) or onion (Fig. 5D to F). This is in agreement with the previous findings of Bhat and associates (2005), who, by overexpressing the actin depolymerizing factor ADF3, showed that MLO and ROR2/PEN1 accumulation at the site of attempted powdery mildew penetration in barley cells was independent of the actin cytoskeleton. Therefore, MLO negatively regulates an actin-dependent defense pathway but is not itself trafficked by an actin-mediated transport process. Because CE application has been reported to disrupt the accumulation of PEN3-GFP at the site of *B. graminis* f. sp. *hordei* attack (Underwood and Somerville 2008), it may be this actin cytoskeleton-dependent pathway that MLO inhibits. Furthermore, because our data demonstrate that CE does not affect the trafficking of VvPEN1 (Fig. 4B and 5D), the PEN1-dependent resistance pathway (Collins et al. 2003; Consonni et al. 2006; Stein et al. 2006) may be the actin-independent pathway inhibited by MLO (Miklis et al. 2007).

Our previous experiments showed that BFA and WM have opposite effects on adapted and nonadapted powdery mildew penetration rates in grapevine (Feechan et al. 2011). The co-trafficking of VvMLO and VvPEN from the PM to the papilla via a BFA- and WM-sensitive pathway may now provide an explanation for this observation (Supplementary Fig. S3). In the presence of a nonadapted powdery mildew species, PEN1 and MLO are endocytosed and redirected to the site of pathogen challenge. Under these circumstances, the AtPEN1 and AtPEN2/AtPEN3 pathways provide penetration resistance. In the presence of an adapted powdery mildew species, again both MLO and PEN1 undergo focal accumulation but both PEN1- and PEN2/PEN3-mediated resistance is dampened by MLO. In the presence of WM or BFA, the endocytosis and focal accumulation of MLO and PEN1 are inhibited and only the focal accumulation of PEN3 is possible due to its independent trafficking via the actin cytoskeleton (Underwood and Somerville 2008). Therefore, following WM or BFA treatment, penetration rates of a nonadapted powdery mildew increase, because PEN1 is not localized to the site of powdery mildew attack. However, penetration rates do not equal that of an adapted powdery mildew species (Fig. 6C and D) due to the continued functioning of the PEN2/PEN3 defense pathway. Conversely, the penetration rates of an adapted powdery mildew decrease in response to WM and BFA treatment (Fig. 6C and D), because MLO is also no longer localized to the site of penetration, possibly curbing the suppression of the PEN2/PEN3-mediated resistance. Consequently, in the presence of these endocytosis inhibitors, the penetration rates of adapted and nonadapted powdery mildew species are similar.

How, then, does an adapted powdery mildew species exploit the presence of host MLO proteins at the site of attempted penetration to suppress PEN1-mediated resistance? A recent study may shed some light on this; the C-terminal half of AtMLO2 was reported as a target for the *Pseudomonas syringae* effector HopZ2 and AtMLO2 was demonstrated to be required for full susceptibility to *P. syringae* using *Atmlo2* mutants (Lewis et al. 2012). Therefore, AtMLO2 might be a direct target for bacterial and, possibly, fungal pathogen effectors. With the recent identification of a suite of powdery mildew candidate effectors (Godfrey et al. 2009; Pedersen et al. 2012; Spanu et al. 2010), it will be interesting to test whether any of these directly target MLO proteins.

In conclusion, VvPEN1 and VvMLO3/VvMLO4 share the same trafficking pathway and undergo endocytic recycling from the PM to the site of powdery mildew attack. This focal accumulation is correlated with the outcome of powdery mildew penetration.

MATERIALS AND METHODS

Plant material and cultivation.

The *A. thaliana pen1-1* and *Atmlo2-5 mlo6-2 mlo12-1* mutants in the Col-0 genetic background have been described before (Collins et al. 2003; Consonni et al. 2006). Plants were grown in chambers with a cycle of 14 h of light and 10 h of darkness and maintained at 24°C.

Stable transformation and transient gene expression.

Constructs were transformed into *Atpen1* and *Atmlo2 mlo6 mlo12* mutants using the floral dip method (Clough and Bent 1998). For transient gene expression, onion cells were transformed by particle bombardment. Onion epidermal strips on 1% agar were bombarded with vectors coated on gold particles. For three shots, 10 mg of 1 µM gold particles (Bio-Rad, Gladesville, Australia) in 200 µl of ethanol were vortexed for 2 min, spun down for 10 s in a microfuge, drained, washed twice with sterile water, and resuspended in 170 µl of 40% glycerol. While gently vortexing, 10 µl of the plasmid solutions (150 ng/µl), 10 µl of cold 0.1 M spermidine, and 25 µl of 2.5 M CaCl₂ were added drop wise and the resulting mixture was incubated on ice for 10 min. The particles were spun down, washed with 70% ethanol, and resuspended in 24 µl of cold 100% ethanol, and 6-µl aliquots were placed onto sterile filter holders. After bombardment, tissue was stored in the dark for 24 h before fluorescence microscopy.

Phylogenetic analysis.

Protein sequence alignments were performed with ClustalW v2.0 with default settings (Thompson et al. 1994). The aligned sequences were used to generate a phylogenetic tree using the neighbor-joining algorithm (Saitou and Nei 1987) with CLC Genomics Workbench (CLCbio, Aarhus, Denmark). Support for the branching arrangements was evaluated by bootstrap analysis using 100 replicates. Translated grapevine sequences showing homology to the Qa-SNARE subfamily of proteins from *A. thaliana* (Lipka et al. 2007) were identified using a BLASTP search of the V1 gene prediction of genome assembly of *V. vinifera* genotype PN40024 (12× coverage) (Jaillon et al. 2007) available at the University of Padua's CRIBI Biotechnology Center website.

Powdery mildew inoculation studies.

E. necator (isolate APC, provided by E. Scott, University of Adelaide, SA, Australia) was maintained on detached leaves of *V. vinifera* Cabernet Sauvignon using an 8- to 10-day rotation as previously described (Donald et al. 2002). *E. cichoracearum* was maintained on cucumber (*Cucumis sativus*) plants grown in chambers with a cycle of 16 h of light and 8 h of darkness and maintained at 22°C. *Arabidopsis* leaves were infected with *E. cichoracearum* or *E. necator* using a fine paintbrush.

Inhibitor studies.

All chemical inhibitors were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Inhibitor stocks were dissolved and maintained in DMSO at the following concentrations: BFA, 2 mg/ml; WM, 4 mg/ml; and CE, 2 mg/ml. Inhibitor stocks were dissolved in sterile water to achieve indicated working concentrations. Inhibitors were infiltrated into the

abaxial side of *Arabidopsis* leaves using a 1-ml blunt syringe. *Arabidopsis* leaves were left for 2 h before GFP visualization. Onion epidermal strips were floated on inhibitor solutions for 2 h prior to fluorescence microscopy.

Microscopy.

Detached leaf material was sampled at 48 h post powdery mildew inoculation and stained in trypan blue with heat for 2 to 5 min according to Koch and Slusarenko (1990). Fungal structures were visualized using a Zeiss Axioscope 2 light microscope. Fluorescent proteins were visualized using a Zeiss Axioscope FS CFP/YFP/GFP/Rhodamine filter set. The presence of germinated powdery mildew spores was identified using propidium iodide (Sigma-Aldrich) staining with the rhodamine (red) filter (filter setting 15; excitation wavelength, 546/12 nm; emission wavelength, 590 nm). After locating a germinated spore, the filter was switched to the GFP position (filter setting 10; excitation wavelength, 450 to 490 nm; emission wavelength, 515 to 565 nm) to score for the presence or absence of GFP accumulation beneath the appressorium.

Cloning and constructs.

VvPEN1 (VIT08s0032g01150) was amplified from *V. vinifera* Cabernet Sauvignon root cDNA using 5'-ATAGAATTCA TGAACGATCTCTTCTCCG-3' (*EcoRI* site bold) and 5'-ATC AAGCTTCTACGCCTGAGTAGGTGGAGG-3' (*HindIII* site bold), subcloned into pBLUNT (Invitrogen, Carlsbad, CA, U.S.A.) and verified by DNA sequencing before ligation into pART7 (Gleave 1992) and pC'GFP (a modified version of vector pART7 with an added mGFP5 coding region) then pART27 (Gleave 1992).

Cloning of VvMLO3, VvMLO6, and VvMLO17 cDNA was described previously (Feechan et al. 2008). VvMLO4 was amplified from reverse-transcribed RNA isolated from *V. vinifera* Cabernet Sauvignon leaves using 5'-TTGAGTTGTAAAGCC AGGAAATG-3' and 5'-CATCAGCCTCGTACCTGTGG-3'. VvMLO4 was cloned into pBLUNT and verified via DNA sequencing. VvMLO cDNAs were cloned into pDONR221 or pDONR223, then introduced into pAM-PAT gateway expression vectors (GenBank AY436765) and pGWB5 (sGFP fusion vector) (Nakagawa et al. 2007). pGWB6 was used as an empty vector control for free (cytoplasmic) sGFP localization (Nakagawa et al. 2007). pCLB1301 (modified binary vector pCambia1300 with a 35S::mGFP5-ER expression cassette from pBIN35S::mGFP-ER) (Haseloff et al. 1997) was used to visualize mGFP5 targeting to the endoplasmic reticulum. VvMLO3 cDNA was amplified using 5'-GACGGTACCGATA TGGCTAAGGGATCAAAG-3' (*KpnI* site bold) and 5'-CGA AAGCTTGTAGTTCTTGCTCTTTTATCGAATG-3' (*HindIII* site bold), then cloned into pBLUNT (Invitrogen). VvMLO3 was subcloned into ER082 (Tucker et al. 2012) to form a C-terminal fusion with the coding sequence of Venus YFP. The VvMLO3-vYFP polylinker cassette was inserted into pART7. VvMLO4 cDNA was amplified using 5'-GCACTCGA GTTGAGTTGTAAAGCCAGGAAATGGC-3' (*XhoI* site bold) and 5'-GCGGGATCCGTCCTCTCTCTTCGATCAAATGA-3' (*BamHI* site bold), then cloned into pBLUNT. VvMLO4 was subcloned into ER082. The VvMLO4-vYFP cassette was inserted into pART7. VvPEN1 cDNA was amplified using 5'-ATCGGTAACATGAACGATCTCTTCTCCGGTT-3' (*KpnI* site bold) and 5'-ATCCCCGGGCGCCTGAGTAGGTGGAG G-3' (*SmaI* site bold), then subcloned into pC'CFP to make an N-terminal CFP fusion.

Gene expression analysis.

Total RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and DNase-treated according to the

manufacturer's instructions. cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Castle Hill, NSW, Australia) with the Oligo (dT)20 primer according to the manufacturer's instructions. Semiquantitative RT-PCR was performed with equal amounts of cDNA to confirm the expression of *VvPEN1* (using 5'-CCCGGCTAATCATGA ACGATCT-3' and 5'-ACGGGCTACGCCTGAGTAG-3'), *VvMLO3*, *VvMLO4*, *VvMLO6*, and *VvMLO17* using specific primers described previously (Feechan et al. 2008). The *Arabidopsis* housekeeping gene *ELONGATION FACTOR1 ALPHA* (*AtEF1A*; At5g60390) was amplified using the primers 5'-TGAGCACGCTCTTCTTGTCTTCA-3' and reverse primer 5'-GGTGGTGGCATCCATCTTGTACA-3' to serve as an internal standard.

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