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Mechanisms of powdery mildew resistance in the Vitaceae family

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SUMMARY

The cultivated grapevine, *Vitis vinifera*, is a member of the Vitaceae family, which comprises over 700 species in 14 genera. *Vitis vinifera* is highly susceptible to the powdery mildew pathogen *Erysiphe necator*. However, other species within the Vitaceae family have been reported to show resistance to this fungal pathogen, but little is known about the mechanistic basis of this resistance. Therefore, the frequency of successful *E. necator* penetration events, in addition to programmed cell death (PCD) responses, were investigated in a representative genotype from a range of different species within the Vitaceae family. The results revealed that penetration resistance and PCD-associated responses, or combinations of both, are employed by the different Vitaceae genera to limit *E. necator* infection. In order to further characterize the cellular processes involved in the observed penetration resistance, specific inhibitors of the actin cytoskeleton and secretory/endocytic vesicle trafficking function were employed. These inhibitors were demonstrated to successfully break the penetration resistance in *V. vinifera* against the nonadapted powdery mildew *E. cichoracearum*. However, the use of these inhibitors with the adapted powdery mildew *E. necator* unexpectedly revealed that, although secretory and endocytic vesicle trafficking pathways play a crucial role in nonhost penetration resistance, the adapted powdery mildew species may actually require these pathways to successfully penetrate the plant host.

INTRODUCTION

Grapevine powdery mildew, *Erysiphe necator*, is the most economically important disease of viticulture worldwide because of the high susceptibility of the cultivated grapevine species, *Vitis vinifera*, to this pathogen. *Erysiphe necator* is of North American origin, and has subsequently spread through Europe by the introduction of American grapevines (Gadoury and Pearson, 1991). Several members of the Vitaceae family have been reported as being susceptible to *E. necator*, including members of the genera *Ampelopsis*, *Cissus*, *Parthenocissus* and *Vitis* (Boubals, 1961). Conversely, there are several reports of *E. necator* resistance in different Vitaceae members. For example, North American *Vitis* species, such as *V. riparia*, *V. aestivalis* and *V. rupestris*, which have co-evolved with the pathogen, are thought to be less susceptible than *V. vinifera* to *E. necator* (Boubals, 1961; Cadle-Davidson *et al*., 2010; Fung *et al*., 2008).

Plants have evolved several layers of defence to prevent pathogen penetration and colonization. Preformed constitutive physical barriers, such as leaf surface wax or preformed antimicrobial secondary metabolites, prevent the entry of the majority of plant pathogens (Thordal-Christensen, 2003). Powdery mildew is an obligate biotrophic pathogen which needs access to plant host nutrients for growth and reproduction. Therefore, the powdery mildew spore (conidium) forms an infection structure (appressorium) to generate sufficient pressure to rupture the plant cell wall. As the fungus penetrates the plant cell, it forms a feeding structure (haustorium), which is surrounded by the extrahaustorial membrane, a continuum of the plant plasma membrane but with a unique composition (Koh *et al*., 2005). The plant can respond to this invasion with inducible defences. The endocytic and secretory membrane trafficking pathways are now emerging as important components of penetration and basal resistance. For example, the recognition of pathogen ingress is mediated by plasma membrane-localized receptors, which become internalized via endocytosis following the binding of pathogen-associated molecular patterns (PAMPs) (Robatzek *et al*., 2006). Chitin and ergosterol can be considered as fungal PAMPs (Granado *et al*., 1995; Miya *et al*., 2007). The endocytosis of these PAMP receptors activates defence responses, including pathogenesis-related (*PR*) gene expression and callose deposition at the site of pathogen contact (Clay *et al*., 2009; Gomez-Gomez *et al*., 1999). Papillae are formed to reinforce the cell wall under the site of pathogen contact through the polarized secretion of materials, such as callose, phenolics and hydrogen peroxide (McLusky *et al*., 1999; Mellersh *et al*., 2002;

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Thordal-Christensen *et al*., 1997). In addition to secretory vesicles, many other cellular components also become polarized at the site of pathogen interaction, including the actin cytoskeleton and organelles such as peroxisomes, the nucleus, Golgi and endoplasmic reticulum (Freytag *et al*., 1994; Lipka *et al*., 2005; Takemoto *et al*., 2003). It has been proposed that the actin cytoskeleton may facilitate the delivery of secretory vesicles. The depolymerization of the actin cytoskeleton through the use of fungal-derived inhibitors (cytochalasins) or, genetically, by the overexpression of actin depolymerization factors (*ADF*) have both been found to break penetration resistance to powdery mildew (Kobayashi *et al*., 1997; Miklis *et al*., 2007; Yun *et al*., 2003). Furthermore, Kobayashi and Hakuno (2003) have demonstrated that callose deposition at pathogen entry sites is dependent on the actin cytoskeleton, which is also consistent with the model that actin is required for secretory vesicle delivery.

The SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) family includes proteins which mediate vesicle secretion by the facilitation of membrane fusion events (Pratelli *et al*., 2004). A specific plasma membrane-localized member of the syntaxin subgroup of SNARE proteins in *Arabidopsis* (PEN1; SYP121) and in barley (ROR2) has been shown to play an important role in penetration resistance against powdery mildew (Collins *et al*., 2003). PEN3, another protein required for penetration resistance against powdery mildew in *Arabidopsis* (Stein *et al*., 2006), is also implicated in polarized secretion to the papillae. This ATP-binding cassette (ABC) transporter protein is required for callose deposition and functions in a pathway distinct from PEN1 (Bednarek *et al*., 2009; Clay *et al*., 2009; Consonni *et al*., 2006; Lipka *et al*., 2005; Stein *et al*., 2006). Both of these proteins have also been shown to accumulate in papillae at the site of pathogen interaction (Meyer *et al*., 2009).

In the overwhelming majority of cases, plants successfully block the invasion by pathogens through the effective operation of these preformed and inducible penetration resistance pathways. However, some pathogens have evolved mechanisms to suppress PAMP receptor-mediated penetration resistance through the secretion of effector (previously virulence factor) proteins (Bent and Mackey, 2007; Caplan *et al*., 2008; Stergiopoulos and de Wit, 2009). In response to this, some plants have evolved a second layer of defence involving programmed cell death (PCD). PCD is typically triggered when a specific recognition event occurs between a plant resistance (*R*) gene product and a pathogen-secreted effector protein (previously avirulence protein) (Flor, 1971). This rapid, localized cell death effectively restricts the growth of biotrophic pathogens, such as powdery mildew (Peterhänsel *et al*., 1997). The majority of characterized plant *R* genes encode proteins which contain leucine-rich repeat (LRR) domains, a central nucleotide-binding site (NBS) and a variable N-terminus comprising either a Toll/interleukin-1 receptor (TIR) domain or coiled-coil (CC) domain (Jones and Takemoto, 2004).

Muscadinia rotundifolia, a grapevine species native to south-eastern USA, is a source of strong resistance to *E. necator* infection. This resistance cosegregates with the *Run1* locus, which has been found to contain a family of TIR-NBS-LRR resistance gene candidates (Dry *et al*., 2009). Similarly, the *REN1* locus from the *V. vinifera* cultivar 'Kishmish vatkana', which is native to central Asia, also displays PCD-associated resistance to *E. necator* (Hoffmann *et al*., 2008). However, these are the few examples in which resistance mechanisms against *E. necator* have been characterized in any detail. We have therefore undertaken a detailed investigation of the resistance responses in a representative genotype from a range of different species within the Vitaceae family to *E. necator* infection. Our results reveal a range of different responses to limit powdery mildew infection. These include penetration resistance and PCD-associated resistance, or combinations of both. Furthermore, using inhibitors of endocytic and secretory vesicle trafficking pathways, we demonstrate the different roles played by these pathways in the penetration resistance of grapevine species to both nonadapted and adapted powdery mildew species.

RESULTS

Characterization of resistance mechanisms to *E. necator* infection within the Vitaceae

In order to investigate possible sources of resistance to *E. necator* within the Vitaceae, representatives of the genera *Vitis*, *Muscadinia*, *Ampelopsis*, *Cissus* and *Parthenocissus* were inoculated with the adapted powdery mildew species *E. necator*. Forty-eight hours after inoculation, the infection stage of germinated conidia (appressorium, haustorium), together with the presence or absence of PCD, was scored using trypan blue which stains both fungal structures and dead host cells. Two types of *E. necator* resistance were observed: penetration resistance and PCD induction. Different frequencies of these resistance mechanisms in the species investigated resulted in four outcomes to *E. necator* inoculation (Fig. 1): susceptibility, partial resistance, PCD-associated resistance and penetration resistance.

Both *V. vinifera* and *A. brevipedunculata* were found to be susceptible to *E. necator*, with the majority (71%–79%) of infection attempts leading to the development of a haustorium within penetrated epidermal cells and subsequent formation of secondary hyphae (Fig. 1A,B). The remaining 19%–24% of germinated conidia formed appressoria, but were unable to penetrate epidermal cells. PCD was only observed in 1%–5% of cells containing haustoria. This high level of successful penetration, combined with a low incidence of PCD in penetrated cells, enables the pathogen to complete its asexual life cycle on these

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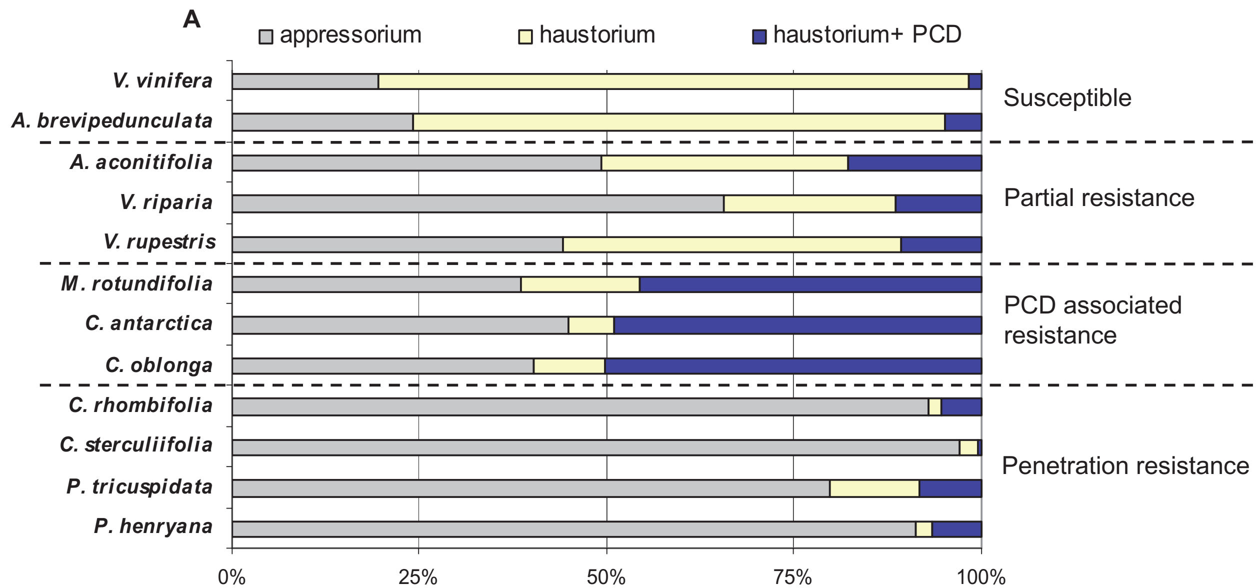

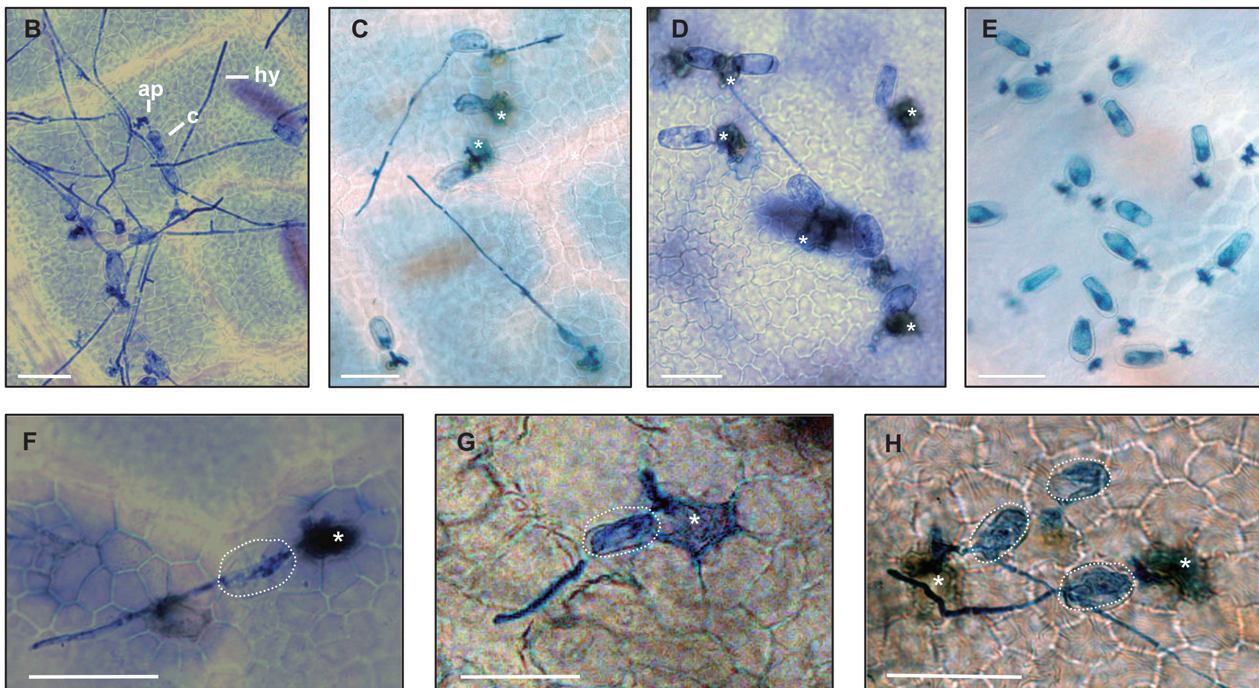

Fig. 1 Susceptibility of different Vitaceae species to *Erysiphe necator* infection. The outcome of *E. necator* infection on leaves 48 h post-inoculation. (A) Frequency of *E. necator* penetration attempts on different Vitaceae members, which concluded in appressorium formation but no penetration, successful penetration and haustorium formation. or a haustorium followed by programmed cell death (PCD) of the penetrated epidermal cell. The frequency of these outcomes in each Vitaceae species leads to susceptibility, partial resistance, PCD-associated resistance or penetration resistance. Each data point is based on three biological replicates (leaves) on which a minimum of 100 germinated conidia were scored. The data shown are representative of the results obtained in at least two independent experiments. (B–H) Trypan blue staining following *E. necator* inoculation. (B) Susceptible *Vitis vinifera*. (C) Partially resistant *V. riparia*. (D) PCD-mediated resistance in *Muscadinia rotundifolia*. (E) Penetration resistance in *Parthenocissus tricuspidata*. (F) PCD response in *M. rotundifolia*. (G) PCD response in *Cissus antarctica*. (H) PCD response in *C. oblonga*. Asterisks indicate cells which have undergone PCD as stained by trypan blue. Broken white circles indicate the position of a conidium. ap, appressorium; c, conidium; hy, hypha. Scale bars, 50 μm.

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hosts with the development of significant amounts of sporulating hyphae (data not shown).

Ampelopsis aconitifolia, *V. riparia* and *V. rupestris* all showed increased resistance to *E. necator* relative to *V. vinifera*. Penetration resistance in these vines was greater than that found in *V. vinifera*, with between 44% and 65% of germinated appressoria failing to penetrate epidermal cells (Fig. 1A,C). Significantly increased levels of PCD were also observed following penetration (10%–17%) compared with those found in the susceptible vines *V. vinfera* and *A. brevipedunculata*. The combined action of increased penetration resistance and increased PCD per penetration considerably restricted the development of *E. necator* on these hosts, in comparison with *V. vinifera*, such that there was little or no sporulation observed 7 days after inoculation (data not shown)

Muscadinia rotundifolia, *C. antarctica* and *C. oblonga* were found to show complete resistance to *E. necator* and did not support any sporulation. Resistance in these three species was characterized by a rapid induction of PCD responses with 45%–50% of germinated conidia (Fig. 1A,D). The PCD responses of *C. antarctica* and *C. oblonga* to *E. necator* were very similar to that found in *M. rotundifolia*, where hyphal growth was rapidly arrested following epidermal PCD (Fig. 1F–H). They also exhibited similar levels of penetration resistance (38%–44%) to those found in the partially resistant group, which includes *A. aconitifolia*, *V. riparia* and *V. rupestris*.

The final subgroup of Vitaceae species includes *C. rhombifolia*, *C. sterculiifolia, P. tricuspidata* and *P. henryana*. These species were also completely resistant to *E. necator* infection. Similar results were also obtained with *C. discolor* (data not shown). This group displayed high levels of penetration resistance to *E. necator*, with between 79% and 95% of germinated spores that formed an appressorium failing to successfully penetrate epidermal cells, as shown by the absence of a haustorium (Fig. 1A,E).

Characterization of nonhost penetration resistance in *V. vinifera*

Figure 1 illustrates that different genotypes within the Vitaceae family show different levels of penetration resistance to *E. necator*. For example, *V. vinifera* shows a low (19%) penetration resistance to *E. necator*, whereas *M. rotundifolia* and *P. tricuspidata* show intermediate (38%) and strong (79%) penetration resistances, respectively. Penetration resistance is normally considered to be the major component of nonhost resistance (NHR) to nonadapted powdery mildew pathogens. This penetration resistance requires the actin cytoskeleton, polarized secretion and papilla formation (Kobayashi *et al*., 1997; Schmelzer, 2002; Staiger, 2000). Therefore, we asked whether the penetration resistance observed in Vitaceae members, to the adapted mildew *E. necator*, requires the same components as NHR to a nonadapted powdery mildew species.

In order to address this question, we first investigated the components involved in the penetration resistance in *V. vinifera* against the nonadapted cucurbit powdery mildew species *Erysiphe cichoracearum. V. vinifera* was found to display strong nonhost penetration resistance to *E. cichoracearum*, with only 21% ± 5% of penetration attempts successful, compared with 77% ± 10% when infected with the adapted powdery mildew *E. necator* (Fig. 2). To examine the mechanistic basis of this NHR in *V. vinifera*, different cell machinery inhibitors were employed to block actin cytoskeleton polymerization and vesicle trafficking pathways. Cytochalasin E (CE) is a fungal-derived metabolite which blocks the polymerization of the actin cytoskeleton and has been reported previously to break NHR in several plant species (Kobayashi *et al*., 1997). Brefeldin A (BFA) is a fungal toxin commonly employed to study vesicle-mediated protein secretion and endocytosis (Robinson *et al*., 2008), whereas wortmannin (WM) inhibits the endocytic trafficking of plasma membrane proteins and disrupts the vacuolar sorting of these proteins (Kleine-Vehn *et al*., 2008; Wang *et al*., 2009).

Vitis vinifera leaves were treated with increasing concentrations of CE, BFA and WM prior to *E. cichoracearum* inoculation, and the penetration rate of germinated spores was scored. In each case, inhibitor pretreatment was found to partially compromise nonhost penetration resistance to *E. cichoracearum* in *V. vinifera* leaves, with the most statistically significant increase in fungal penetration, relative to the control treatment [dimethylsulphoxide (DMSO) only], observed at 10 μM CE, 25 μM BFA and 40 μM WM (Fig. 3). The mean increases in penetration using these optimum concentrations were found to be 26% ± 6%, 17% ± 2% and 19% ± 1% following CE, BFA and WM pretreatment, respectively, over three independent experiments (data not shown). Higher concentrations of CE, BFA and WM

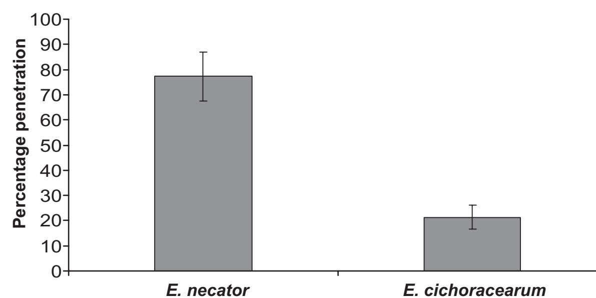

Fig. 2 Penetration efficiency of the adapted powdery mildew *Erysiphe necator* and the nonadapted powdery mildew *E. cichoracearum* on *Vitis vinifera*. Successful penetration was scored by the presence/absence of a haustorium. Each data point represents the mean ± standard deviation of three independent experiments within which a minimum of 100 germinated conidia were scored on three leaves.

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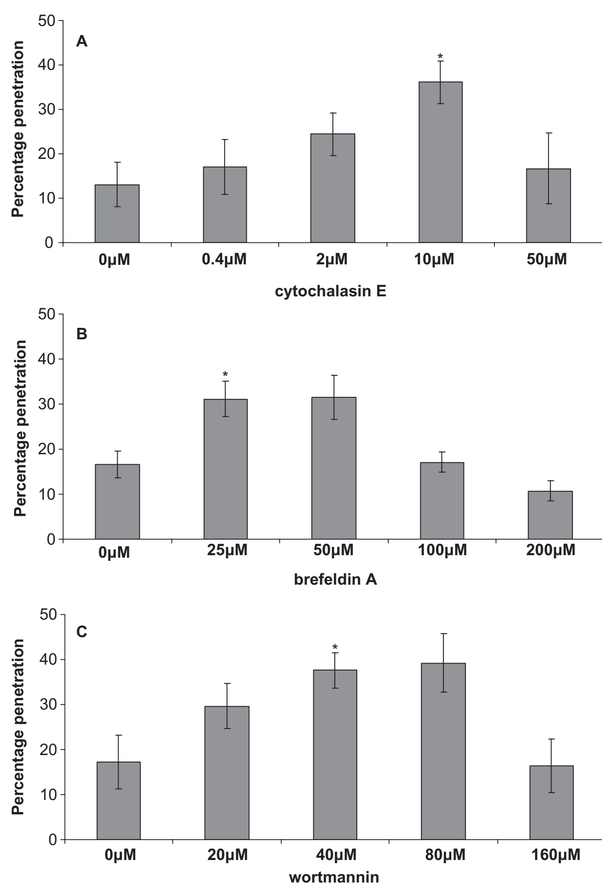

Fig. 3 Penetration frequency of the nonadapted powdery mildew *Erysiphe cichoracearum* on *Vitis vinifera* following treatment with increasing concentrations of cytochalasin E (A), brefeldin A (B) and wortmannin (C). Successful penetration was scored by the presence/absence of a haustorium. Each data point ± standard deviation is based on three biological replicates (leaves) on which a minimum of 100 germinated conidia were scored. Asterisks indicate a significant difference from the negative dimethylsulphoxide (DMSO) control ($P < 0.01$; Student's *t*-test).

decreased the penetration frequency of germinated spores, suggesting that these concentrations were toxic to powdery mildew growth (Fig. 3).

These results confirm that actin cytoskeleton polymerization and vesicle trafficking are important processes for the establishment of nonhost penetration resistance in *V. vinifera*.

Characterization of host penetration resistance in different Vitaceae species

To investigate whether there was mechanistic overlap between the penetration resistance displayed by different Vitaceae family members against the adapted powdery mildew species *E. necator* (Fig. 1A) and the nonhost penetration resistance displayed by *V. vinifera* against the nonadapted species *E. cichoracearum* (Figs 2 and 3), the effect of the inhibitors CE, BFA and WM on the penetration resistance displayed by *V. vinifera*, *M. rotundifolia* and *C. rhombifolia* was examined.

The pretreatment of *V. vinifera* leaves with 10 μM CE increased significantly the penetration efficiency of *E. necator* from 67% ± 6% in the control treatment (0.2% DMSO) to 80% ± 4% (Fig. 4A). This suggests that a component of the resistance displayed by *V. vinifera* to *E. necator* penetration involves the same cellular machinery as that employed against the nonadapted powdery mildew species *E. cichoracearum* (Fig. 3A), as both require the actin cytoskeleton. However, CE was not found to break the penetration resistance to *E. necator* in *M. rotundifolia* or *C. rhombifolia* (Fig. 4A), which suggests that the penetration resistance displayed in these two species against *E. necator* is not dependent on actin cytoskeleton function.

In contrast with the results obtained with the nonadapted powdery mildew *E. cichoracearum* (Fig. 3A), pretreatment with BFA was found to decrease the penetration efficiency of *E. necator* on *V. vinifera* from 81% ± 5% in the control treatment (0.4% DMSO) to 47% ± 4% (Fig. 4B). However, as with CE treatment (Fig. 4A), BFA was found to have no effect on the penetration efficiency of *E. necator* on *M. rotundifolia* or *C. rhombifolia* (Fig. 4C). As with BFA, WM treatment was also found to decrease the penetration efficiency of *E. necator* on *V. vinifera* from 82% ± 5% in the control treatment (0.4% DMSO) to 47% ± 1% (Fig. 4C). Again this is contrary to the observed effect of WM on the nonhost penetration resistance of *V. vinifera* against *E. cichoracearum* (Fig. 3C). Interestingly, WM was also effective at reducing the level of *E. necator* penetration on *M. rotundifolia* from 67% ± 6% in the control treatment (0.4% DMSO) to 42% ± 1% (Fig. 4C). However, as with CE and BFA, WM had no significant effect on the penetration efficiency of *E. necator* on *C. rhombifolia* (Fig. 4C).

It was surprising that pretreatment with 25 μM BFA and 40 μM WM led to a decrease in penetration efficiency in the compatible interaction between *E. necator* and *V. vinifera* (Fig. 4B,C), as the same concentration of inhibitors led to an increase in penetration by the nonadapted powdery mildew species *E. cichoracearum* (Fig. 3B,C). To rule out the possibility of any direct inhibitory effects of BFA and WM pretreatment on *E. necator*, at these concentrations, a dose–response curve was constructed using the nonhost species *Arabidopsis thaliana* treated with increasing concentrations of BFA and WM prior to *E. necator* inoculation (Fig. S1, see Supporting Information). It was necessary to carry out the dose–response curve on a nonhost plant species for *E. necator* as, if BFA and WM do decrease the penetration efficiency in compatible interactions, any toxic or inhibitory effects on penetration would be indistinguishable. The penetration efficiency of *E. necator* on the nonhost *Arabidopsis* increased significantly following pretreatment with 25 μM BFA and 40 μM WM, demonstrating that these concentrations are not toxic to *E. necator* (Fig. S1).

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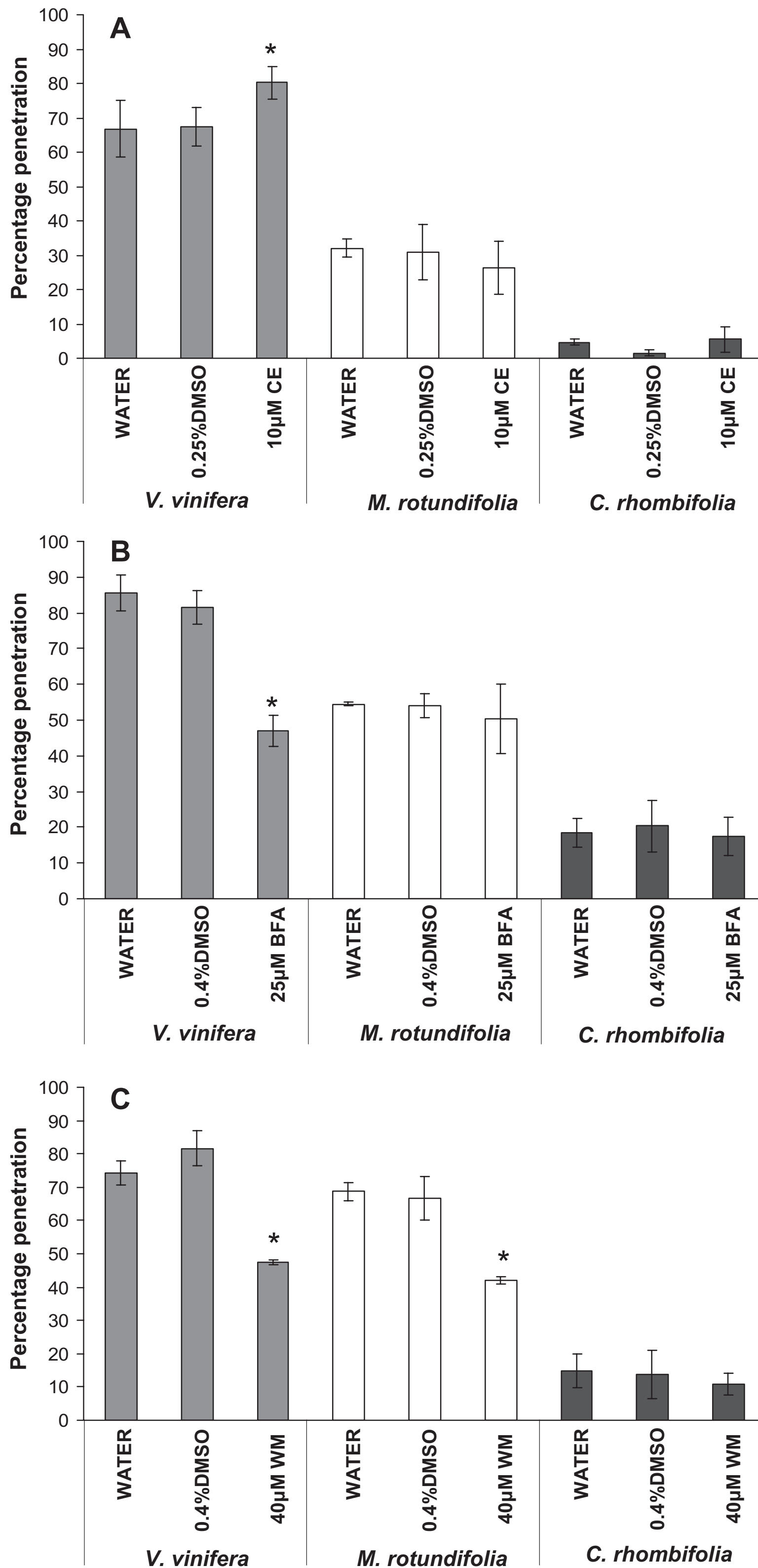

Fig. 4 Penetration frequency of the adapted powdery mildew *Erysiphe necator* on the Vitaceae species *Vitis vinifera*, *Muscadinia rotundifolia* and *Cissus rhombifolia* following treatment with the following inhibitors: (A) cytochalasin E (CE), 0.25% dimethylsulphoxide (DMSO) or water; (B) brefeldin A (BFA), 0.4% DMSO or water; (C) wortmannin (WM), 0.4% DMSO or water. Successful penetration was scored by the presence/absence of a haustorium. Each data point ± standard deviation is based on three biological replicates (leaves) on which a minimum of 100 germinated conidia were scored. The data shown are representative of the results obtained with at least two independent experiments. Asterisks indicate a significant difference from DMSO controls ($P < 0.05$; Student's *t*-test).

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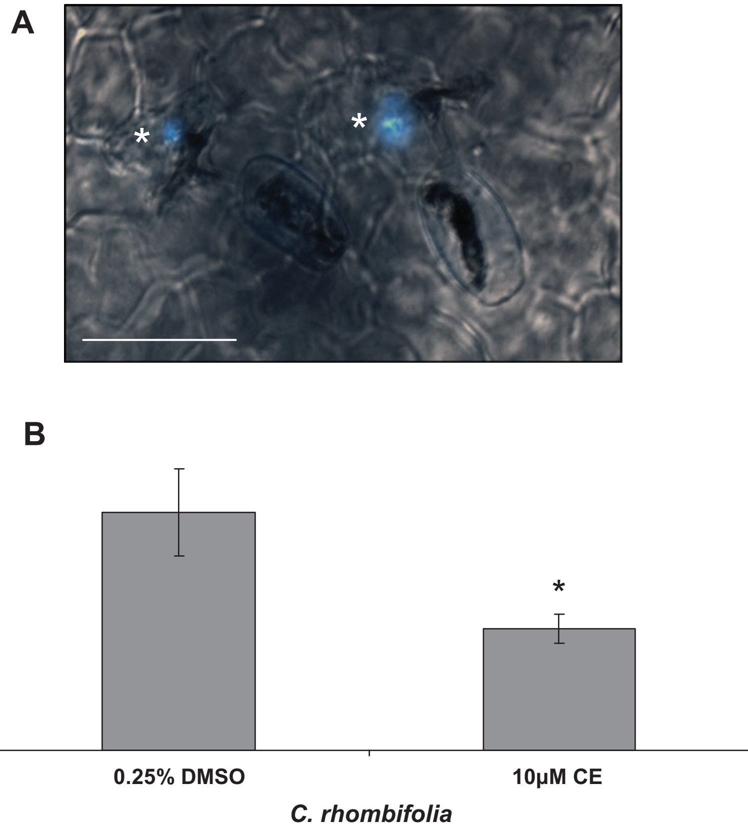

Fig. 5 *Erysiphe necator*-induced callose deposition in the papillae of *Cissus rhombifolia*. (A) Merged bright field and fluorescence image of callose under appressoria marked by an asterisk. (B) Frequency of papillary callose following cytochalasin E or 0.25% dimethylsulphoxide (DMSO) treatment, scored by the presence of callose under spores with an appressorium. Leaves were stained with 0.1% aniline blue. Each data point ± standard deviation is based on three biological replicates (leaves) on which a minimum of 100 germinated conidia were scored. The data shown are representative of the results obtained in two independent experiments. Scale bar, 50 μm. Asterisk indicates a significant difference from DMSO control ($P < 0.05$; Student's *t*-test).

These results suggest that the vesicle trafficking pathways are not required for host penetration resistance to the adapted powdery mildew, *E. necator*, in the Vitaceae species examined. On the contrary, BFA- and WM-inhibited pathways appear to be partially required for successful *E. necator* penetration on *V. vinifera*, whereas only the WM-inhibited endocytic pathway is required for successful *E. necator* penetration on *M. rotundifolia*.

Penetration resistance in *C. rhombifolia* is not dependent on papillae formation

The results in Fig. 4 show that the penetration resistance of *C. rhombifolia* to *E. necator* was not influenced significantly by treatment with the inhibitors CE, BFA or WM, despite the fact that at least one of these cell machinery inhibitors was found to affect the penetration resistance in *V. vinifera* and *M. rotundifolia*. This suggests that the strong penetration resistance observed in *C. rhombifolia* to *E. necator* may not be caused by an active cellular response, but may be a result of a preformed physical or chemical barrier to penetration.

In order to investigate this further, we looked for the presence of callose-containing papillae in *C. rhombifolia* epidermal cells in response to *E. necator* inoculation. *Cissus rhombifolia* leaves were inoculated with *E. necator* and analysed for callose deposition by aniline blue staining. Over three inoculations, callose-containing papillae (Fig. 5A) were detected beneath 65% ± 7% *E. necator* appressoria on *C. rhombifolia*. As callose deposition, at the site of pathogen entry, has been shown to be dependent on actin (Kobayashi and Hakuno, 2003), we also investigated the effect of CE treatment on papillary callose formation in *C. rhombifolia*. CE was found to reduce the incidence of callose-containing papillae from 58% ± 10% to 30% ± 3% (Fig. 5B). Thus, although the inhibition of actin cytoskeleton function by CE does not lead to a reduction in penetration resistance to *E. necator* in *C. rhombifolia* (Fig. 4A), it does reduce the incidence of papillary callose deposition (Fig. 5B), suggesting that the resistance of *C. rhombifolia* to *E. necator* penetration is not dependent on papillary callose deposits.

DISCUSSION

Previous studies have investigated the susceptibility of *Vitis* species, including *V. vinifera* cultivars and wild *Vitis* species from North America and China, to grapevine powdery mildew, *E. necator* (Doster and Schnathorst, 1985; Eibach, 1994; Staudt, 1997; Wan *et al*., 2007). Some information is also available regarding the susceptibility of species from the Vitaceae genera

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Ampelopsis, *Cissus* and *Parthenocissus* (Boubals, 1961). More recently, Cadle-Davidson *et al*. (2010) have reported the results of a comprehensive study involving 1025 *Vitis* accessions analysed under the same conditions, with the same *E. necator* isolate, to look at the variation in foliar resistance to powdery mildew. However, none of these studies has investigated the biological basis of any observed resistance to *E. necator* infection.

This study has characterized, for the first time, the relative importance, in different members of the Vitaceae family, of the two major factors regulating susceptibility to *E. necator*: penetration efficiency and PCD induction in penetrated cells. However, it is important to note that the quantification of these two resistance mechanisms in the different Vitaceae species examined only relates to the specific *E. necator* isolate used in this study. The relative levels of penetration and PCD-associated resistance observed may vary significantly when challenged with different isolates which have undergone pathogenic specialization (Gadoury and Pearson, 1991). Furthermore, intraspecific variation in powdery mildew susceptibility has been observed (Cadle Davidson *et al*., 2010).

Different members of the Vitaceae family display a diverse range of responses to attempted invasion by *E. necator* (Fig. 1A). The cultivated European species *V. vinifera* and the ornamental Asian species *A. brevipedunculata* are highly susceptible to *E. necator*, because of the low levels of penetration resistance and low levels of PCD induction, following penetration, which are insufficient to prevent *E. necator* from completing its life cycle. Conversely, members of the *Cissus* and *Parthenocissus* genera were found to be completely resistant to *E. necator* infection because of the very high levels of penetration resistance.

The remaining Vitaceae species examined appear to utilize a combination of both penetration resistance and PCD-associated resistance to restrict *E. necator* growth, with varying levels of success. All show significantly higher levels of resistance than *V. vinifera* to *E. necator* penetration. However, complete resistance (i.e. absence of any sporulation) was only observed in those species in which this reduced penetration was combined with a highly efficient PCD response in penetrated epidermal cells, i.e. *M. rotundifolia*, *C. antarctica* and *C. oblonga* (Fig. 1A,F–H). Thus, plants within the Vitaceae family appear to have evolved a number of different biological responses in an attempt to restrict *E. necator* invasion.

Characterization of penetration resistance to powdery mildew in the Vitaceae

Penetration resistance is normally considered to be the major component of NHR to nonadapted powdery mildews, and has been shown to involve the actin cytoskeleton and polarized secretion (Underwood and Somerville, 2008). However, little attention has been paid to the mechanistic basis of penetration resistance to an adapted powdery mildew species, and whether this is a distinct resistance pathway or involves similar components to NHR. In order to investigate this, specific cell machinery inhibitors were employed on members of the Vitaceae family showing a wide variation in penetration resistance against *E. necator*. CE blocks the polymerization and elongation of the actin cytoskeleton (Kobayashi *et al*., 1997). BFA is a fungal toxin that interferes with guanine-nucleotide exchange factors (GEFs) that catalyse the activation of the small GTPase ADP ribosylation factor (ARF) (Geldner *et al*., 2003; Jackson and Casanova, 2000), required for vesicle formation. Wortmaninn inhibits phosphatidylinositol 3-kinase (PI3PK), an enzyme involved in the production of phosphatidylinositol 3-phosphate (PI3P), a lipid predominantly of endosomal membranes (Wang *et al*., 2009). CE has been reported previously to break NHR in several plant species (Kobayashi *et al*., 1997); however, to our knowledge, the vesicle trafficking inhibitors BFA and WM have not been used previously to study penetration resistance against powdery mildew.

The increase in penetration of the nonadapted powdery mildew, *E. cichoracearum* (Fig. 3A), and adapted powdery mildew species, *E. necator* (Fig. 4A), on *V. vinifera* leaves, treated with CE, demonstrates that the actin cytoskeleton is partially required for both nonhost and host penetration resistance. This is in agreement with previous inhibitor and genetic studies, which have demonstrated the importance of the actin cytoskeleton in several plant species for NHR (Kobayashi *et al*., 1997; Yun *et al*., 2003) and host penetration resistance in barley (Miklis *et al*., 2007). The actin cytoskeleton is required for callose deposition at pathogen entry sites (Kobayashi and Hakuno, 2003). The *Arabidopsis* mutants of *pen3* show impaired callose secretion responses (Clay *et al*., 2009; Stein *et al*., 2006). Interestingly, the accumulation of PEN3 at attempted barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) infection sites is also disrupted by CE (Underwood and Somerville, 2008).

As with CE, the penetration of *V. vinifera* epidermal cells by the nonadapted powdery mildew species *E. cichoracearum* was also increased significantly in response to treatment with the vesicle trafficking inhibitors BFA and WM. BFA has been reported to inhibit both endosomal vesicle trafficking and Golgi-derived vesicle secretion (Driouich *et al*., 1993; Geldner *et al*., 2003; Sciaky *et al*., 1997; Steinmann *et al*., 1999). WM also inhibits the endocytic trafficking of plasma membrane proteins (Kleine-Vehn *et al*., 2008; Wang *et al*., 2009). For example, WM inhibits the endocytosis of the plasma membrane-localized PAMP receptor FLAGELLIN SENSITIVE 2 (FLS2) in response to the bacterial PAMP flagellin (Robatzek *et al*., 2006). Thus, the increase in penetration observed with the nonadapted powdery mildew

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E. cichoracearum following BFA and WM treatment, suggests that endocytosis and/or vesicle secretion are important for nonhost penetration resistance, possibly through PAMP receptor endocytosis or the exocytosis of cell wall materials at the site of pathogen interaction.

Surprisingly, however, BFA and WM were both found to produce the opposite response against the adapted powdery mildew species *E. necator*. BFA decreased significantly *E. necator* penetration on *V. vinifera*, and WM produced a similar result in both *V. vinifera* and *M. rotundifolia* (Fig. 4B,C). These results suggest that endocytic and secretory vesicle trafficking may, in fact, be required for successful infection by an adapted powdery mildew species. One possible explanation for this result may be that the extramembrane material required to form the host extrahaustorial membrane is provided by these vesicle trafficking pathways. The extrahaustorial membrane is of a unique composition and therefore is probably formed *de novo* by vesicle trafficking (Frei dit Frey and Robatzek, 2009; Koh *et al*., 2005). An alternative explanation may be that the proteins required for pathogen entry are internalized and trafficked by endocytosis. For example, the plasma membrane-localized host protein MLO is required for adapted powdery mildew pathogenicity (Büschges *et al*., 1997; Consonni *et al*., 2006), and has been shown to accumulate at the site of pathogen infection (Bhat *et al*., 2005). A family of VvMLOs has been identified recently in *V. vinifera* (Feechan *et al*., 2008; Winterhagen *et al*., 2008). It is conceivable that the inhibition of vesicle trafficking with BFA and WM could interfere with the turnover and accumulation of VvMLO at the site of *E. necator* infection, leading to reduced penetration efficiency. There is also emerging evidence that fungal pathogen effectors (virulence factors) can enter the plant cell in the absence of the pathogen (Catanzariti *et al*., 2006; Manning and Ciuffetti, 2005; Rafiqi *et al*., 2010), suggesting that the plant host cell machinery may be exploited for effector entry inside the plant cell. Therefore, it is possible that WM and BFA could interfere with the delivery of effectors into the plant cell that are required for the suppression of PAMP-triggered immunity. Support for this theory comes from the recent demonstration that WM inhibits the entry of fungal effectors into plant cells by blocking lipid raft-mediated endocytosis (Kale *et al*., 2010). In summary, our data clearly indicate that the role of endocytosis in adapted powdery mildew infection requires further investigation.

Representative members of the four Vitaceae species examined, *C. rhombifolia*, *C. sterculiifolia*, *P. tricuspidata* and *P. henryana*, were found to be completely resistant to *E. necator* infection, and this was mediated by very high levels of penetration resistance (Fig. 1). However, attempts to modify penetration resistance in *C. rhombifolia* (Fig. 3A–C) or *P. tricuspidata* (data not shown) using CE, BFA or WM were unsuccessful, despite the fact that CE was shown to reduce papillary callose deposition (Fig. 5). These results suggest that the high level of penetration resistance observed in these plants is not dependent on inducible cellular processes involving the actin cytoskeleton and vesicle trafficking. Another explanation may be that the strong penetration resistance found in these plants is the result of a preformed barrier, such as leaf surface wax or preformed antimicrobial secondary metabolites (Thordal-Christensen, 2003). It has been reported that barley powdery mildew *Blumeria graminis* secretes a lipase (LIP1) which has lipolytic activity on epicuticular wax components of the host leaf. The released wax derivatives are important for fungal attachment, growth and pathogenicity (Feng *et al*., 2009). If the composition of the epicuticular wax of a particular plant species is not readily degraded by the powdery mildew-secreted lipase, this may hinder infection.

Sources of PCD-associated resistance to powdery mildew in the Vitaceae

R proteins typically trigger PCD following interaction directly or indirectly with specific pathogen effectors (Axtell and Staskawicz, 2003; Dodds *et al*., 2006; Ellis *et al*., 2007; Mackey *et al*., 2002, 2003). These effectors (avirulence factors) are deployed by the pathogen to gain access to host nutrients and/or to suppress basal plant immunity (Bent and Mackey, 2007; Caplan *et al*., 2008). Therefore, effectors and *R* genes tightly co-evolve, as the effector is under selection pressure to evade detection, whereas the *R* gene is under pressure to retain the ability to detect the effector. As *E. necator* is endemic to the USA, it might be expected that Vitaceae species from this region have evolved *R* gene-mediated resistance to *E. necator* infection pressure.

PCD-mediated resistance to *E. necator* has been reported previously in back-cross progeny derived from a cross between the North American species *M. rotundifolia* and *V. vinifera*, and this resistance cosegregates with a cluster of TIR-NBS-LRR resistance gene candidates at the *Run1* locus (Barker *et al*., 2005; Dry *et al*., 2009). Our data show that the powdery mildew *R* gene(s) in *M. rotundifolia* mount a very effective PCD response to *E. necator* infection, leading to PCD in approximately 75% of penetrated cells (Fig. 1A,B,F). Other North American species, *V. rupestris* and *V. riparia*, also show increased PCD induction in penetrated cells (approximately 20% and 30%, respectively) compared with *V. vinifera*, as does the Chinese species *A. aconitifolia* (36%; Fig. 1A). Further research is required to determine whether the elevated level of *E. necator*-induced PCD in these species represents recognition by a weak powdery mildew *R* gene or an alternative resistance mechanism.

Surprisingly, very high levels of PCD induction were observed in *E. necator*-penetrated cells of *C. antarctica* and *C. oblonga*, similar to the levels found in *M. rotundifolia* (Fig. 1F–H). This

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suggests the existence of *R* gene products in these two Australian *Cissus* species, which are capable of recognizing effectors secreted from *E. necator*. Such a hypothesis would appear to be at odds with the model of plant resistance gene evolution, which proposes that *R* gene-mediated resistance is the result of a significant period of co-evolution between the host and adapted pathogen. To our knowledge, *E. necator* has only been in Australia since the 1860s (B. Emmett, Dept. of Primary Industries, Victoria, Australia, personal communication). However, this is not the first report of *E. necator*-induced PCD in a Vitaceae species originating from outside of North America. Hoffmann *et al*. (2008) reported the existence of a cultivated grapevine *Vitis vinifera* 'Kishmish vatkana' from Central Asia that showed elevated levels of PCD to *E. necator*. Furthermore, this resistance has been mapped to a single dominant locus (designated *REN1*) that contains a family of NBS-LRR genes. Hoffmann *et al*. (2008) proposed that wild *V. vinifera* populations in Central Asia, unlike the clonally propagated cultivated grapevines in Europe, are undergoing sexual propagation, and may have evolved resistance since the arrival of *E. necator* from North America. An alternative explanation may be that closely related powdery mildew species exist that are endemic to Asia and Australia which share effectors with *E. necator*.

In conclusion, several novel sources of resistance to grapevine powdery mildew have been identified within the Vitaceae family which include both PCD-associated and penetration-based resistance. The characterization of penetration resistance in *V. vinifera* indicates commonalities with other plant species in terms of the role of the actin cytoskeleton and vesicle trafficking pathways against a nonadapted powdery mildew species. However, these results also suggest that the adapted powdery mildew species may actually require the plant host endocytic and secretory pathways for pathogenicity. Further studies are now underway to elucidate which proteins are trafficked by the endocytic and vesicle secretion pathways that are required by adapted powdery mildew species for successful infection.

EXPERIMENTAL PROCEDURES

Plant cultivation

Cuttings of *A. brevipedunculata*, *A. aconitifolia*, *C. antarctica*, *C. oblonga*, *C. rhombilfolia*, *C. sterculiifolia*, *P. tricuspidata* and *P. henryana* were obtained from Adelaide Botanic Garden (Adelaide, SA, Australia). *Vitis riparia*, *V. rupestris* and *M. rotundifolia* were obtained from the variety collection in the Coombe Vineyard of the University of Adelaide (Urrbrae, SA, Australia). At least two individual vines were maintained for each Vitaceae member, with the exception of *P. henryana* and *C. oblonga*. Leaves were sampled from potted vines grown in a temperature-controlled glasshouse at Waite Campus, University of Adelaide (Adelaide, SA, Australia) maintained between 23 and 25 °C. *Arabidopsis thaliana* (Col-0) plants were grown at 24 °C with a 14-h light/10-h dark cycle.

Powdery mildew inoculation studies

Erysiphe necator (isolate APC, kindly provided by Eileen Scott, University of Adelaide, SA, Australia) was maintained on detached leaves of *V. vinifera* cv. Cabernet Sauvignon using an 8–10-day rotation as described previously (Donald *et al*., 2002). *Erysiphe cichoracearum* was maintained on cucumber plants (*Cucumis sativus*) grown at 22 °C with a 16-h light/8-h dark cycle.

Young glossy detached vine leaves of a similar developmental stage (approximately 6 cm in diameter) were inoculated with *E. necator* conidia or *E. cichoracearum* by gently tapping conidia from infected leaves (8 days post-inoculation) above open dishes. The Petri dishes were sealed with Parafilm® and the leaves were incubated at 25 °C under a 16-h light/8-h dark cycle for 48 h. Leaves from *Arabidopsis thaliana* Col-0 plants were infected with *E. cichoracearum*, using a fine paintbrush, and plants were incubated at 25 °C for 48 h.

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; CE, 2 mg/mL. Inhibitor stocks were dissolved in sterile water to achieve the indicated working concentrations. Leaves from *V. vinifera* cv. Cabernet Sauvignon, *M. rotundifolia* and *C. rhombifolia* were immersed in inhibitors, or an appropriate control DMSO solution, or sterile water for 1 h. Vine leaves were rinsed in sterile water and air dried before powdery mildew inoculation. Inhibitors were infiltrated into the abaxial side of *Arabidopsis thaliana* Col-0 leaves using a blunt syringe before powdery mildew inoculation.

Microscopy

Leaf material was harvested 48 h after powdery mildew inoculation and stained in trypan blue with heat for 1 h, according to Koch and Slusarenko (1990). Fungal structures were visualized using a Zeiss (Göttingen, Germany) Axioscop 2 light microscope. Successful penetration was determined by the presence of a fungal haustorium, and PCD by the presence of a blue fungal-penetrated epidermal cell.

To stain for callose, detached leaf material was incubated in 5% $NaHCO_3$ for 2 min before staining with 0.1% aniline blue solution in 50 mM potassium phosphate buffer (pH 7.5) for 1 h. Leaves were rinsed in 10 mM potassium phosphate buffer. Papillary callose was visualized using a Zeiss Axioscope FS CFP (blue light) filter set.

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SUPPORTING INFORMATION

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

Fig. S1. Penetration frequency of the nonadapted powdery mildew *Erysiphe necator* on *Arabidopsis thaliana* following treatment with increasing concentrations of brefeldin A (A) and wortmannin (B). Successful penetration was scored by the presence/absence of a haustorium. Each data point represents the mean ± standard deviation of two independent experiments within which a minimum of 100 germinated conidia were scored on three leaves. Asterisks indicate a significant difference from the negative dimethylsulphoxide (DMSO) control ($P < 0.01$; Student's *t*-test).

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