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REVIEW ARTICLE

Current understanding of grapevine defense mechanisms against the biotrophic fungus (*Erysiphe necator*), the causal agent of powdery mildew diseaseWenping Qiu¹, Angela Feechan² and Ian Dry³

The most economically important disease of cultivated grapevines worldwide is powdery mildew (PM) caused by the ascomycete fungus *Erysiphe necator*. The majority of grapevine cultivars used for wine, table grape, and dried fruit production are derived from the Eurasian grape species *Vitis vinifera* because of its superior aroma and flavor characteristics. However, this species has little genetic resistance against *E. necator* meaning that grape production is highly dependent on the frequent use of fungicides. The integration of effective genetic resistance into cultivated grapevines would lead to significant financial and environmental benefits and represents a major challenge for viticultural industries and researchers worldwide. This review will outline the strategies being used to increase our understanding of the molecular basis of *V. vinifera* susceptibility to this fungal pathogen. It will summarize our current knowledge of different resistance loci/genes that have evolved in wild grapevine species to restrict PM infection and assess the potential application of these defense genes in the generation of PM-resistant grapevine germplasm. Finally, it addresses future research priorities which will be important in the rapid identification, evaluation, and deployment of new PM resistance genes which are capable of conferring effective and durable resistance in the vineyard.

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INTRODUCTION

Grapevine (*Vitis* spp.) has been cultivated for human consumption for over 7000 years. Few horticultural crops have had more historical, cultural, and social impacts than grapevine. Grapevines are estimated to be cultivated on over 7.6 million of hectares of land worldwide¹. The majority of wine grape cultivars are derived from the species *Vitis vinifera* which originated in Eurasia but are highly susceptible to the pathogens and pests that are thought to have evolved on the wild grapevines native to North America. The ascomycete fungus, *Erysiphe necator* (syn. *Uncinula necator*), the causal agent of grapevine powdery mildew (PM) disease, was inadvertently introduced into Europe from North America in the 1850s and caused significant losses to viticultural production². The fungus has subsequently spread to other grape-growing regions throughout the world and has changed the practice of viticulture by requiring the use of frequent and prophylactic spray programs. Indeed, a report on the use of fungicides in the European Union over the period 2001–2003 indicated that while viticulture only accounted for 3.3% of the agricultural area, a staggering 81,000 tonnes of active substance were applied annually to grapevines in European vineyards, which represented 67% of all fungicides applied to crops in the EU³. Not only does this translate into increased production costs for growers, but there is also the potential impact of these chemicals on the health of beneficial organisms in the vineyard⁴ and vineyard workers⁵, as well as increased carbon emissions generated from their frequent application. Thus, the integration of effective genetic resistance into grape cultivars would reduce the dependence of viticulture on chemical inputs, leading to significant financial, health, and environmental benefits.

Wild North American *Vitis* spp. including *V. rotundifolia* (syn. *Muscadinia rotundifolia*), *V. rupestris*, *V. riparia*, and *V. aestivalis* are more resistant to PM than European *V. vinifera* cultivars⁶. As early as the late 1800s, grape breeders began introgressing genetic resistance from the North American *Vitis* spp. into *V. vinifera*, resulting in the generation of many *Vitis* interspecific 'French–American' hybrids. However, commercial adoption of these new grape cultivars has been limited, due to the reduced quality of wine made from these resistant hybrids. Selected accessions of a number of wild Chinese *Vitis* species^{7,8} have also been reported to show strong resistance to PM, but apart from some specific examples described below, little information is available regarding the genetic basis of PM resistance or the quality of wine produced from *Vitis* interspecific 'French–Chinese' hybrids.

THE BIOTROPHIC FUNGAL PATHOGEN – *ERYSIPHE NECATOR*

There is insufficient space in this short review to provide a detailed description of the biology, ecology, and epidemiology of grapevine PM. Instead, readers are directed to an excellent review published by Gadoury *et al.*⁹ However, a brief description of the infection process is presented here in order to understand the resistance strategies used by the grapevine host to restrict fungal invasion and colonization.

Erysiphe necator is an obligate biotrophic fungus that relies fully on a host cell in photosynthesis-active tissues to complete its life cycle. Once a conidiospore of *E. necator* lands on the epidermis of photosynthesis-active tissues, it germinates to form a lobed appressorium. Based on studies with other PMs, it is likely that germination involves the secretion of fungal lytic enzymes such as lipases, esterases, and cutinases¹⁰ which leads to the release of long-chain

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fatty acid derivatives which enhance fungal germination and development^{10,11}. From the lower surface of the appressorium, a penetration peg emerges which penetrates the cell wall and invades the host epidermal cell to form a specialized intracellular structure called a haustorium. The haustorium is an interface between the fungus and the host cell that facilitates the dynamic exchange of molecules derived from both fungal and host cells. The fungus retrieves hexoses, amino acids, vitamins, and other nutrients from host cells, through the haustorium, while at the same time secreting proteins to suppress host defences. If the establishment of the haustorium and the uptake of nutrients is successful, the fungus continues to spread via hyphae across the surface, producing more appressoria and haustoria at regular intervals. After 5–25 days, sporulation occurs in the form of conidiophores perpendicular to the epidermis on which chains of asexual conidia are produced and spores are released to start a new cycle of infection¹².

PLANT DEFENSES AGAINST BIOTROPHIC FUNGAL PATHOGENS

There are two main strategies that plants use to restrict the invasion and growth of biotrophic fungal pathogens: penetration resistance and programmed cell death (PCD)-mediated resistance (Figure 1). Penetration resistance blocks the breach of the cell wall and membrane by the germinated spore and thus prevents the formation of the haustorium. The PCD-mediated resistance is exerted inside the penetrated epidermal cell and induces the death of invaded cell, thereby terminating the supply of nutrients required by the biotrophic fungus for further growth and development.

The innate immune responses in a plant cell happen, consecutively and are interconnected, in two basic forms: pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI)¹³. PTI is activated by the interaction of extracellular pattern-recognition receptors in the plasma membrane of the host cell and pathogen-specific molecules that are released from a pathogen¹⁴. The fungal PAMP chitin, which is a major constituent of fungal cell walls, is released during infection by PM and is detected by a LysM (*lysin motif*)-receptor-like kinase¹⁵. This, in turn, activates the mitogen-activated protein kinase cascade which triggers multiple defense responses, including the generation of reactive oxygen species, defense gene activation, biosynthesis/signaling of plant stress/defense hormones, phytoalexin biosynthesis, and cell wall strengthening¹⁶. PTI is the first line of defense and provides protection against the majority of microbes that plants face. However, through evolution, certain isolates have become 'adapted' to a specific host through the development of effector proteins that are secreted into the plant cell to suppress PTI and enable the pathogen to become virulent on the host¹³. Over time, selected plant species in which PTI had been compromised, acquired additional receptors (resistance (R) proteins) that specifically recognize these effectors, leading to ETI. R proteins interact with the effector directly, or indirectly through partner proteins, leading to the induction of defense responses that share overlapping pathways with PTI¹⁷. ETI is most commonly associated with PCD (observed as a hypersensitive response) which prevents biotrophic pathogens, including PM, from obtaining nutrients and completing its life cycle.

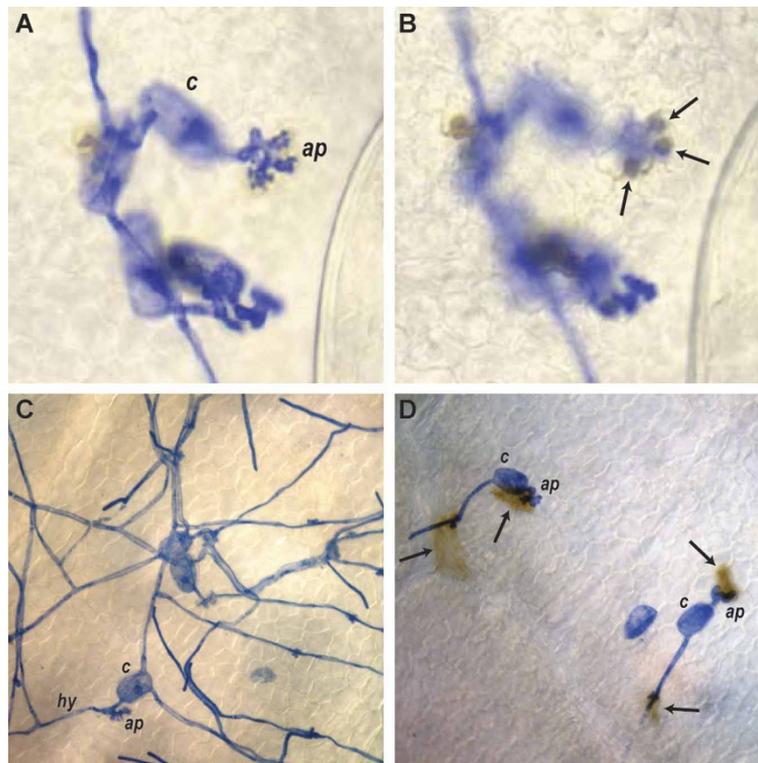


Figure 1. Mechanisms of grapevine defense against the biotrophic fungal pathogen powdery mildew (*E. necator*). Grapevine powdery mildew spores were inoculated onto detached leaves of *M. rotundifolia* (A & B), *V. vinifera* cv. Cabernet Sauvignon (C) and a *V. vinifera* backcross progeny plant containing the powdery mildew resistance gene *MrRUN1* (D). Leaf samples were collected after 2 days and fixed and stained with Coomassie brilliant blue to visualize fungal structures. Panels A and B represent the same field of view but are focused at different levels to show the germinated conidium (c) and appressoria (ap) on the surface of the leaf (panel A) and the globular papillae (arrows) beneath the appressoria (panel B) which are blocking penetration and haustoria formation. Panel C shows normal growth of *E. necator* hyphae (hy) across the leaf surface of a susceptible grapevine cultivar. Panel D shows the induction of *MrRUN1*-mediated programmed cell death in penetrated epidermal cells (arrows) which effectively halts further growth of this biotrophic pathogen.

PAMP-TRIGGERED IMMUNITY AGAINST POWDERY MILDEW IN GRAPEVINE

The cultivated grapevine, *V. vinifera* is resistant to species of PM that are not adapted to grapevine. For example, the 'non-adapted' PM species, *Erysiphe cichoracearum* that causes PM disease of cucurbits, shows much lower rates of penetration of grapevine epidermal cells than *E. necator*¹⁸ and, as such, is unable to establish a successful infection. Penetration resistance represents the major component of PTI against non-adapted PMs in most plant species and has been shown by forward genetic screens, in Arabidopsis and barley, to involve the combined action of at least three PENETRATION (PEN) genes: *PEN1*, *PEN2*, and *PEN3*^{19–21}. *PEN1* is a member of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family which includes proteins which mediate membrane fusion events²² and is proposed to have a role in the trafficking of secretory vesicles to the plasma membrane that contain cargo required for penetration resistance against PM. *PEN2* and *PEN3* function in the same penetration resistance pathway in Arabidopsis which is independent of *PEN1*²⁰. *PEN2* is a myrosinase involved in the biosynthesis of antimicrobial molecules that are delivered to the site of PM penetration via *PEN3* which is an ATP-binding cassette transporter^{23,24}.

As grapevine is a woody perennial and much more difficult to transform, it has not been possible to use similar forward genetic screens to identify basic components of PTI against *E. necator*. However, accumulating evidence from studies using other experimental approaches indicates that the *PEN1*- and *PEN2/PEN3*-like pathways are also important components of PTI in grapevine. The first piece of evidence comes from inhibitor studies. Penetration resistance of grapevine against the non-adapted PM species *E. cichoracearum* was shown to be compromised by the actin cytoskeleton inhibitor cytochalasin¹⁸ which was subsequently shown, in Arabidopsis, to inhibit the focal accumulation of *PEN3* under the site of PM penetration²³. This suggests a role for a *PEN3*-like protein in PTI against non-adapted PM in grapevine, but as yet, no orthologues of the Arabidopsis *PEN3* protein have been identified in any

other plant. Recently a *PEN1* orthologue from grapevine (*VvPEN1*) was cloned and its functional complementation of the Arabidopsis *pen1* mutant demonstrated²⁵. A *VvPEN1*-GFP fusion protein was also shown to accumulate under the site of PM penetration as has been demonstrated for *PEN1* in Arabidopsis and barley²⁶. The accumulation of *PEN1* and *VvPEN1* under the site of attempted PM penetration is inhibited by the endomembrane trafficking inhibitors brefeldin A and wortmannin^{25,27}. Feechan *et al.*¹⁸ also demonstrated that penetration resistance against the non-adapted PM species *E. cichoracearum*, in grapevine, was compromised by brefeldin A and wortmannin, suggesting the existence of a *PEN1*-mediated secretory pathway that is also an important component of PTI against PM in grapevine.

EFFECTOR-TRIGGERED IMMUNITY AGAINST POWDERY MILDEW IN GRAPEVINE

The most important class of *R-genes* in plants are those encoding proteins with nucleotide binding (NB) site – leucine-rich repeat (LRR) domains²⁸. These NB-LRR proteins specifically recognize the microbial effector molecules secreted during infection and initiate ETI which is highly effective against biotrophic pathogens such as PM. The genomes of perennial woody plants appear to possess a larger number of NB-LRR resistance genes than annual herbaceous plants which most probably reflects the more diverse range of pathogens that perennial plants have to deal with over their lifespan²⁹.

The grapevine genome encodes a large family of NB-LRR genes that are clustered in tandem repeats in genomic regions^{30,31}. Tandem repetitive and paralogous *R-genes* constitute a reserved genetic army that can be activated whenever there is a need to battle against invading pathogens. It is therefore reasonable to expect that a significant amount of genomic diversity exists within the large number of accessions of wild *Vitis* species. To date, nine loci have been identified from a range of different grape species native to North America, China, and Central Asia, which are thought to contain *R-genes* that confer strong resistance to *E. necator* (Table 1). The chromosomal location of these loci have been genetically mapped using molecular markers, although the exact

Table 1. Major resistance loci in grapevine species that confer resistance to the powdery mildew fungus *Erysiphe necator*

<i>R</i> -locus	Source of resistance	Origin of <i>R</i> -loci	Resistance mechanism	Chromosome	<i>R</i> -gene type	References
<i>RUN1</i>	<i>M. rotundifolia</i> 'Thomas'	North America	PCD of penetrated cell (rapid)	12	<i>TIR-NB-LRR</i> gene (<i>MrRUN1</i>) functionally confirmed in transgenic susceptible cultivars	32
<i>RUN2</i>	<i>M. rotundifolia</i> 'Magnolia'/ 'Trayshed'	North America	PCD of penetrated cell (lower frequency compared to <i>RUN1</i>)	18	n.d.	35,84
<i>REN1</i>	<i>V. vinifera</i> 'Kishmish vatkana'	Central Asia	PCD of penetrated cell (significantly slower and lower frequency than <i>RUN1</i>) – hyphal growth and sporulation restricted	13	Maps to a <i>CC-NB-LRR</i> gene cluster	34,39,40
<i>REN2</i>	<i>V. cinerea</i> 'Illinois 547-1'	North America	PCD of penetrated cell (slower than <i>RUN1</i>)	14	n.d.	35,85
<i>REN3</i>	Interspecific hybrid 'Regent'	North America	n.d.	15	n.d.	86
<i>REN4</i>	<i>V. romanetii</i>	China	PCD of penetrated cell (rapid)/callose encasement of haustorium ^a	18	n.d.	37
<i>REN5</i>	<i>M. rotundifolia</i> 'Regale'	North America	Post-penetration but mechanism not reported	14	n.d.	36
<i>REN6</i>	<i>V. piasezkii</i>	China	PCD of penetrated cell (very rapid) ^b	9	n.d.	Riaz S, 2015, unpublished data
<i>REN7</i>	<i>V. piasezkii</i>	China	n.d.	19	n.d.	Riaz S, 2015, unpublished data

n.d. – not determined.

^a Feechan A, 2015, unpublished data.

^b Dry I, 2015, unpublished data.

position on the chromosome in question is only currently known for the Resistance to *Uncinula necator* (*RUN1*) locus which is co-located with a locus for Resistance to *Plasmopora viticola* (downy mildew) (*RPV1*) on chromosome 12³².

RUN1 is one of the three PM resistance loci that have been identified from different accessions of the wild North American grapevine species *M. rotundifolia* (Table 1) and is, to date, the only pathogen resistance locus that has been cloned from any grapevine species³². Sequencing of the *RUN1*/*RPV1* locus revealed that it contains a family of seven putative Toll/interleukin-1 receptor (TIR)-NB-LRR-type *R-genes*. However, only one of the candidate *TIR-NB-LRR* genes at the locus (*MrRGA10*) was found to confer strong resistance to PM when transformed into a range of susceptible *V. vinifera* cultivars including Shiraz, Tempranillo, and Portan. This gene, designated *MrRUN1*, confers complete resistance against isolates from Australia, North America, and France by rapidly inducing PCD in penetrated epidermal cells³². However, a PM isolate (Musc4) collected from the southeastern region of North America³³, to which *M. rotundifolia* is native, was found to be capable of breaking *MrRUN1* resistance³² indicating that the effector recognized by the *MrRUN1* protein has either been mutated or completely lost from the Musc4 isolate.

The other two PM resistance loci identified in *M. rotundifolia* are located on different chromosomes to *RUN1* (Table 1). Allelic variants of the *RUN2* locus, *RUN2.1* and *RUN2.2* on chromosome 18, have been identified in the *M. rotundifolia* cultivars 'Magnolia' and 'Trayshed', respectively³⁴ whereas *RUN1* is thought to have originated from the cultivar 'Thomas'³⁵. Interestingly, while *RUN2.1*-mediated PM resistance does not appear to be as strong as *RUN1*, it is not broken by the Musc4 isolate making it a potential candidate for pyramiding with *RUN1*³⁵. *REN5* was derived from the *M. rotundifolia* cultivar 'Regale' and maps to the upper portion of chromosome 14³⁶. The mechanism underlying the resistance mediated by *REN5* is yet to be determined but appears to be initiated at the post-penetration phase³⁶.

Recent research has demonstrated that wild Chinese *Vitis* species also represent an important source of major dominant *R-genes* for PM resistance. *REN4* has been successfully introgressed into *V. vinifera* from the Chinese species *V. rotundifolia* and shown to segregate as a single dominant *R-locus*³⁷. *REN4* resistance was initially reported to be associated with high levels of penetration resistance and did not appear to be dependent on the induction of PCD³⁷. However, more recent studies indicate that *REN4*-mediated resistance occurs post-penetration and involves two different mechanisms – penetrated epidermal cells either undergo PCD or the haustoria become encased in callose thereby effectively blocking nutrient uptake (Feechan A, 2015, unpubl. data). Interestingly, this dual resistance phenotype is reminiscent of the type of response

mediated by the broad spectrum PM resistance gene *RPW8.2* from Arabidopsis which has a unique structure in terms of other known *R* proteins³⁸. Another important observation is that *REN4* resistance is not broken by the Musc4 isolate³⁵ suggesting that *REN4* targets a different *E. necator* effector to that recognized by the *RUN1* protein. This is most probably the result of *REN4* co-evolving with different *E. necator* isolates in China to those in North America.

The wild Chinese grapevine species *V. piasezkii* also appears to contain at least two PM resistance loci, designated *REN6* and *REN7*, on chromosomes 9 and 19 respectively (Riaz S, 2015, unpublished data). A comparison of the resistance responses of the *REN6*, *REN4*, and *RUN1* loci, against the same Australian *E. necator* isolate, in the same genetic background, indicated that PCD initiation is most rapid in penetrated cells containing *REN6*, with less than 5% of appressoria producing a secondary hypha, compared to ~15% and 30% in grapevines containing *REN4* or *RUN1* respectively (Dry I, 2015, unpublished data).

Finally, it is now clear that certain accessions of *V. vinifera* from Central Asia also contain a major *R-gene* that, while less effective than *RUN1*-mediated resistance, still significantly restricts PM growth and sporulation. Two *V. vinifera* cultivars, 'Kishmish vatkana' and 'Dzhandzhakara', originating from Uzbekistan, were shown to induce PCD in penetrated epidermal cells at a higher frequency than susceptible vines, but the speed of the PCD induction was much slower than that observed in a genotype containing *RUN1*³⁹. As a result, more PM hyphal growth and sporulation is observed on *REN1* plants than on *RUN1* plants, but this is still much less than observed on susceptible *V. vinifera* cultivars. The *REN1* locus has been mapped to a 1.4 Mbp region on chromosome 13⁴⁰. The syntenous region in the PN40024 *V. vinifera* reference genome contains a cluster of *CC-NB-LRR* genes⁴⁰, but no data have yet been published to indicate what candidate *R-genes* are present in this region in 'Kishmish vatkana' or 'Dzhandzhakara'. Riaz and co-workers³⁴ subsequently identified an additional six *V. vinifera* and two *V. vinifera* subsp. *sylvestris* accessions from Central Asia that also contained a *REN1*-like locus. Based on genetic marker analysis, they concluded that the *REN1*-like resistance in *V. vinifera* subsp. *sylvestris* was most likely the progenitor of the resistance in the Central Asian *V. vinifera* accessions.

The existence of major *R-gene* resistance against *E. necator* in *Vitis* species native to China and Central Asia brings into question the current dogma that this pathogen is native to North America and was spread to all grape-growing regions from this one source. It seems more likely that *E. necator* isolates have been in existence in Central Asia and China for a much longer period than previously thought to explain the evolution of these *R-genes* in the wild grape species from these regions³⁴.

Table 2. Genes from wild grapevine species postulated to be involved in resistance to the powdery mildew fungus *Erysiphe necator*

Gene	Description	<i>Vitis</i> species	Function and phenotype	References
<i>VaEDS1</i>	Enhanced Disease Susceptibility ortholog	<i>V. aestivalis</i> 'Norton'	Defense pathway regulator – complements Arabidopsis <i>eds1</i> mutant. Constitutively high expression in <i>V. aestivalis</i> resistant genotype and regulated by SA and PM	41,49
<i>VpPR10.1</i>	Pathogenesis-related protein 10	<i>V. pseudoreticulata</i> 'Baihe-35-1'	Antifungal activity. Increases resistance to PM in agroinfiltrated grapevine leaves	71
<i>VpALDH2B4</i>	Aldehyde dehydrogenase	<i>V. pseudoreticulata</i> 'Baihe-35-1'	Activation of SA signaling? Enhanced resistance to PM when overexpressed in Arabidopsis	43
<i>VpWRKY1</i>	WRKY domain Transcription factor	<i>V. pseudoreticulata</i> 'Baihe-35-1'	Transcriptional activator of defense-related genes. Enhanced resistance to PM when overexpressed in Arabidopsis	42
<i>VpRFP1</i>	C4C4-type RING finger protein	<i>V. pseudoreticulata</i> 'Baihe-35-1'	Transcriptional activator of defense-related genes? Enhanced resistance to PM when overexpressed in Arabidopsis	45
<i>VpEIRP1</i>	E3 ubiquitin ligase <i>Erysiphe necator</i> -induced C3HC4 RING finger protein	<i>V. pseudoreticulata</i> 'Baihe-35-1'	Ubiquitination and degradation of a negative transcriptional regulator of defense? Enhanced resistance to PM when overexpressed in Arabidopsis	44

DOWNSTREAM GENES IMPLICATED IN RESISTANCE TO POWDERY MILDEW

In addition to the isolate-specific *R*-genes that confer ETI in penetrated epidermal cells (Table 1), a number of other genes have also been implicated in PM resistance in certain wild *Vitis* species (Table 2). These genes have been identified because (i) they show increased transcription during PM infection and/or show differential expression levels between PM-resistant wild *Vitis* species and susceptible *V. vinifera* cultivars and (ii) they confer increased levels of resistance to PM when overexpressed transiently in grapevine leaves or stably transformed into wild-type or mutant lines of the model species *A. thaliana*^{41–45}.

One example of this is the cultivar Norton which is derived from the North American grapevine species *V. aestivalis* and which is highly resistant to *E. necator* in comparison to *V. vinifera* cv. Cabernet Sauvignon⁴⁶. Investigation of the PM-responsive transcriptome of the two grapevine species revealed that an ortholog of the Arabidopsis *Enhanced Disease Susceptibility 1 (EDS1)* was differentially expressed in Norton and Cabernet Sauvignon. *EDS1* transcription was induced in response to PM in Cabernet Sauvignon whereas its transcription levels were constitutively high in Norton and always exceeded the levels induced in Cabernet Sauvignon⁴¹. The level of salicylic acid was also found to be significantly higher in Norton than in Cabernet Sauvignon under non-PM-infected conditions⁴⁶. *EDS1* has been previously shown to regulate resistance to host-adapted biotrophic pathogens in Arabidopsis in a SA-dependent manner^{47,48}. Constitutively expressed *VaEDS1* complemented the function of the mutant *eds1* gene in Arabidopsis⁴¹ and rendered the *pen2/eds1* mutant resistant to PM. The *VaEDS1* promoter was also shown to be inducible by SA and PM⁴⁹. These results strongly suggest that the constitutively high levels of SA and SA/PM-responsive *EDS1* in Norton may account for the elevated resistance of this genotype to PM.

A large number of studies have also been carried out to determine the genetic basis of PM resistance in certain accessions of the wild Chinese grapevine *V. pseudoreticulata*. At least five different genes have been identified that may contribute to PM resistance in this wild species (Table 2). The transcription factor, *VpWRKY1*, was rapidly induced in *V. pseudoreticulata* within 12 h of inoculation with *E. necator* and the level of expression was found to be correlated with the level of resistance⁴². Furthermore, ectopic expression of *VpWRKY1* in Arabidopsis enhanced resistance to *E. cichoracearum*. Two other genes that appear to be upregulated in the *V. pseudoreticulata* accession Baihe-35-1, in response to PM inoculation, and which both confer resistance to PM when ectopically expressed in Arabidopsis, belong to the Really Interesting New Gene (RING) finger protein gene family^{44,45}. The RING finger domain has been shown to have E3 ligase activity, which is important in ubiquitin-dependent protein degradation⁵⁰. Although plant cells contain hundreds of distinct E3 ligases involved in ubiquitination reactions, in a range of different biological processes, to date there are only limited reports of any RING-type E3 ligases involved in plant defense^{51,52}.

EVIDENCE FOR POWDERY MILDEW SUSCEPTIBILITY GENES IN GRAPEVINE

As described above, adapted PM species are able to successfully penetrate their cognate host by secreting effector proteins that suppress host PTI. However, successful penetration by the adapted PM species has been shown to be dependent on the presence of a functional allele of the Mildew resistance Locus O (*MLO*) in a range of host species including barley⁵³, Arabidopsis⁵⁴, tomato⁵⁵, and pea⁵⁶.

MLO proteins belong to large gene families which are unique to plants and encode seven-transmembrane domain proteins of unknown biochemical activity localized in the plasma membrane⁵⁷. Significantly, only specific *MLO* genes within the family

are capable of acting as PM susceptibility genes and these encode proteins with conserved motifs within the cytoplasmic C-terminal domain of the MLO protein⁵⁸. The mechanism by which MLO proteins act as PM susceptibility factors is unknown. One possibility is that adapted PM species are able to utilize these specific MLO proteins to suppress host PTI, perhaps through the secretion of an effector that targets MLO either directly or indirectly through another protein. Support for this hypothesis comes from the recent observation that the Arabidopsis PM susceptibility protein AtMLO2, also acts as a susceptibility factor for infection by the bacterial pathogen *Pseudomonas syringae* and that AtMLO2 is targeted by the *P. syringae* effector HopZ2⁵⁹.

Based on sequence homology, the presence of the C-terminal conserved motifs and expression kinetics following PM infection, Feechan et al.⁶⁰ identified three *VvMLOs* (*VvMLO3*, *VvMLO4*, and *VvMLO17*) that may act as PM susceptibility genes in *V. vinifera*. *VvMLO3* and *VvMLO4*, but not *VvMLO17*, were subsequently shown to partially rescue an Arabidopsis *mlo2 mlo6 mlo12* triple mutant²⁵. Furthermore, GFP fusions of both *VvMLO3* and *VvMLO4* were demonstrated to localize to the site of PM appressoria formation, in agreement with previous localization studies in barley with HvMLO²⁶. These data strongly support a role for *VvMLO3* and *VvMLO4* as PM susceptibility factors in grapevine. However, despite the generation of numerous single and double *VvMLO3/4* knockout mutants in *V. vinifera* using RNAi techniques, it has not yet been to recover transgenic grapevines with high rates of reduced PM penetration (Feechan A, 2015, unpubl. data). It is interesting to note that PM-resistant *mlo* mutants of barley, tomato, and pea have been identified in naturally occurring segregating populations in which *mlo* is in the homozygous state^{53,55,56}. Therefore, an alternative strategy in grapevine, may be to employ techniques such as EcoTILLING⁶¹ to search for point mutations and/or small insertions/deletions in *VvMLO3* and *VvMLO4* in *V. vinifera* germplasm collections where the mutation, and thus the PM resistance phenotype, is masked by presence of the wild-type MLO allele.

Evidence for the presence of PM susceptibility gene(s) in grapevine also comes from a recent study which used genotype-by-sequencing to identify a QTL for PM susceptibility from Chardonnay named *Sen1* (Susceptibility to *Erysiphe necator* 1)⁶². Isolation and analysis of genes located at the *Sen1* locus will assist in helping us understand the ways that *E. necator* establishes and maintains a compatible biotrophic relationship with the grapevine host cell.

DEVELOPMENTAL CHANGES IN GRAPEVINE RESISTANCE TO POWDERY MILDEW

Age-related or ontogenic resistance has been observed in a number of plant species to viral, bacterial, oomycete, and fungal pathogens⁶³. Both grapevine berries and leaves display ontogenic resistance to PM. This is particularly apparent in developing berries of *V. vinifera* cultivars which are highly susceptible to infection in the first 1–2 weeks after fruit set⁶⁴ but then become increasingly resistant to PM penetration^{65,66}. In contrast, berries of most North American *Vitis* species exhibit strong resistance at all stages of berry development⁶⁷, a constitutive resistance that may have developed during the coevolution of wild *Vitis* species and *E. necator* populations.

While the genetic and mechanistic basis of ontogenic resistance to PM in grapevine is still unknown, it is important to note two things. First, that ontogenic resistance to PM is not unique to grapevine. For example, strawberry leaves and fruit also display ontogenic resistance to PM (*Podosphaera aphanis*)⁶⁸. Second, the ontogenic resistance phenotype appears to be the result of an increase in PTI and does not appear to be associated with changes in the availability of nutrients during leaf or berry development. In experiments reported by Ficke et al.,⁶⁵ conidial germination and appressorium formation were unaffected by berry development but the rate of penetration, formation of haustoria, and develop-

ment of secondary hyphae was almost completely halted on older berries. Furthermore, the increased PM resistance could not be ascribed to any changes in cuticle or cell wall thickness in the developing berries⁶⁶. Based on the evidence presented in the previous section, which shows that the successful penetration of epidermal cells, by an adapted PM species, is dependent on MLO-mediated suppression of host PTI, it is tempting to speculate that changes in host *MLO* expression or activity may be involved in this process.

PERSPECTIVES AND CHALLENGES

With the ongoing development of cheaper and faster sequencing technologies it will be possible to undertake complete genome and transcriptome sequencing of an increasing number of wild *Vitis* species that display resistance to *E. necator*. This will facilitate comparative genomics studies leading to the identification of key components in PTI and ETI against *E. necator* in grapevines. Identification and characterization of more grapevine *R-genes* will unveil deeper insights into and shed more light onto the complex genetic mechanisms of grapevine disease resistance and also provide more molecular markers and genes for breeding resistant grapevine cultivars.

With the identification of an increasing number of *R-gene* candidates, it will be essential that techniques are available to functionally characterize these genes. Ideally, this would involve stable transformation of a susceptible *V. vinifera* cultivar with the *R-gene* candidates to challenge them with a range of *E. necator* isolates³². However, *R-loci* typically contain multiple *R-gene* candidates and stable grapevine transformation, despite technical improvements⁶⁹, remains a long and technically challenging process. Alternative strategies are needed to facilitate rapid evaluation of these *R-gene* candidates. One possibility is the use of transient expression systems such as agroinoculation⁷⁰. This approach has been used to demonstrate the anti-fungal activity of VpPR10.1 against *E. necator*⁷¹ (Table 2). It might also be feasible to test grapevine *R-gene* function by transforming into *Arabidopsis* mutants in which PTI has been compromised allowing *E. necator*

to penetrate and form haustoria⁷². Figure 2 shows the results of an experiment in which the PM resistance gene *MrRUN1*, was transformed into the *Arabidopsis pen1-1* mutant. Inoculation of these *pen1-1* mutants with *E. necator* resulted in a significant induction of PCD in transgenic lines containing *MrRUN1* but not in the *pen1-1* control lines³². Furthermore, *MrRUN1*-mediated PCD in *Arabidopsis* was only induced in response to penetration by *E. necator* and not observed with *E. cichoracearum* demonstrating the response is specific to grapevine PM. Thus, this approach could be used to rapidly evaluate multiple *R-gene* candidates before selected genes are introduced into susceptible *V. vinifera* cultivars for final validation.

Due to its mixed reproductive system (sexual and asexual) and large population size, PM is considered to be a pathogen with a high evolutionary potential and therefore a high risk to overcome genetic resistance⁷³. This was demonstrated by the fact that PM resistance conferred by the apple *R-gene* *Pl2* was found to have broken down in experimental orchards after only 6 years⁷⁴. Jones et al.⁷⁵ recently reported on the genome sequencing of five *E. necator* isolates collected from Californian vineyards that had regularly been treated with synthetic fungicides. Their results showed there to be a significant amount of structural variation in the genomes of the different *E. necator* isolates and, in particular, a variation in copy number of the *EnCYP51* gene which is the target of the commonly used sterol demethylase fungicide⁷⁵. This demonstrates that *E. necator* is able to readily respond to strong selection pressures in the field. This has important implications for the potential deployment of major dominant *R-genes* outlined in Table 1 and highlights the importance of using pyramiding strategies involving multiple *R-genes* to maintain durable PM resistance in the vineyard.

While it is generally accepted that pyramiding *R-genes* is an effective approach for increasing the durability of field resistance⁷⁶, it is essential that the *R-genes* to be combined do not rely on recognition of the same *E. necator* effector protein to initiate PCD (defined as the avirulence (Avr) effector), because resistance conferred by both genes would be lost simultaneously should a new isolate evolve in which this effector has been lost or mutated. At

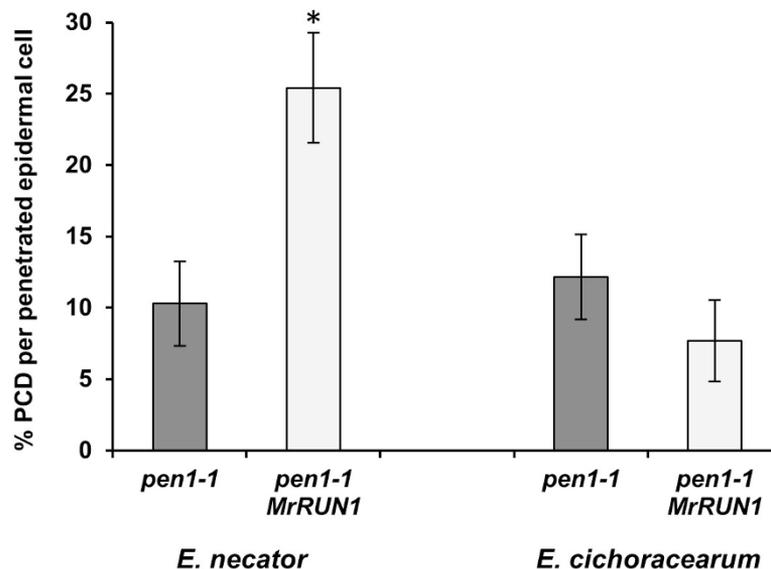


Figure 2. Use of the *Arabidopsis pen1* mutant for rapid screening of candidate grapevine powdery mildew resistance genes. The *pen1-1* mutant line that allows increased penetration of non-adapted powdery mildew species was transformed with the grapevine powdery mildew resistance gene *MrRUN1* and inoculated with either grapevine powdery mildew (*E. necator*) or *Arabidopsis* powdery mildew (*E. cichoracearum*). Programmed cell death (PCD) was estimated by trypan blue staining of inoculated leaves at 2 dpi. 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. Asterisk indicates a statistically significant difference from *pen1-1* ($P < 0.05$; Student's *t*-test).

present, we have only limited information about the range of PM isolates detected by some of the *R*-loci listed in Table 1³⁵ and breeding strategies are currently being employed that are based essentially on the availability of *R*-genes from different *Vitis* species, rather than on any empirical knowledge regarding the isolate specificity of these different *R*-genes. However, the assumption that *R*-genes from different *Vitis* species have different recognition specificities may not always be true. This was clearly demonstrated by a study on resistance to the oomycete pathogen *Phytophthora infestans*, which causes late blight in potato, showing that *R*-genes from three different *Solanum* species were functionally equivalent and recognized the same *P. infestans* effector⁷⁷.

The simplest approach to determining Avr effector recognition specificity is to challenge grape genotypes containing different *R*-loci to PM, with a range of PM isolates with different Avr specificities³⁵. However, we are currently limited by the paucity of *E. necator* isolate collections and by the fact that it is not always feasible to bring the grape genotypes and *E. necator* isolates together in the same country, region, or research facility because of quarantine issues and concerns associated with accidental release of the pathogen. Therefore alternative approaches will be required to enable Avr recognition specificity of PM *R*-genes from different grape species. One such approach is 'Effectormics', a high-throughput functional genomics approach that uses the transient expression of Avr effectors to probe plant germplasm to detect and characterize *R*-genes⁷⁸. However, this is only possible once the Avr effectors have been identified. This will be challenging given that sequencing of the *E. necator* genome has revealed it to contain at least 150 candidate-secreted effector proteins (CSEPs)⁷⁵. Furthermore, unlike functionally characterized Avr effectors from oomycete pathogens, which have a conserved host cell targeting motif (RXLR) in the N-terminal region of their mature protein⁷⁹, there are insufficient Avr effectors characterized from PM species, or for that matter, fungal biotrophic pathogens in general, to use a bioinformatics approach based on conserved domains to enable prediction of the potential Avr candidates from the *E. necator* effectorome. Thus, more targeted approaches may be required involving the use of a comparative genomics approach in which the effectoromes of isolates possessing different pathogenicity specificities are compared⁸⁰ or a map-based cloning approach using PM populations segregating for the Avr effector gene⁸¹.

The identification of Avr effectors will not only provide us with tools to enable testing of the recognition specificity of new *R*-genes without the need to have access to the PM isolate, but will also provide us with a diagnostic test to be able to follow the appearance of resistance-breaking strains in the vineyard. Characterization of the *E. necator* effectorome will also facilitate the identification of the host targets of the effectors that suppress the grapevine PTI pathway as has been done with the *Blumeria graminis* f. sp. *hordei* – barley interaction^{82,83}. This knowledge could possibly be used to modify the host targets to avoid PTI suppression and, in so doing, re-establish PTI against *E. necator* in grapevine.

Further work is also needed to properly characterize the genes and pathways underpinning the quantitative PM resistance displayed by certain wild *Vitis* species. Much of the evidence implicating a role for the genes listed in Table 2, in PM resistance, is based on correlations between an elevated level of gene expression (either constitutive or PM-induced) in the resistant genotype vs. the susceptible genotype and functional assays showing reduced pathogen infection when overexpressed in Arabidopsis. However, in order to prove conclusively that these genes do contribute to PM resistance in these wild grapevine species, it will be necessary to demonstrate that the segregation of resistance is genetically linked to the inheritance of these candidate genes. This will also test the degree to which these genes are able to function in different genetic backgrounds i.e. *V. vinifera* which is essential to know if they are being considered as part of any gene pyramiding strategy.

In summary, we see the following research priorities as critical in the ongoing development of durable and effective PM-resistant grapevine germplasm:

1. Genome and transcriptome sequencing of more PM-resistant wild grape species to identify new *R*-genes.
2. Genome and transcriptome sequencing of multiple virulent and avirulent grapevine powdery mildew isolates to facilitate construction of the *E. necator* effectorome and identify Avr effectors.
3. Functional characterization of *R*-genes from different *Vitis* species in terms of effector recognition specificity.
4. Whole genome association analysis of disease resistance using high-density SNPs to identify genes conferring partial resistance to PM for pyramiding with major *R*-genes.
5. Continued exploration of grapevine genes involved in PM susceptibility, including host targets of PTI suppression by *E. necator* effectors.
6. Further genetic analysis of ontogenic resistance in developing leaves and berries to identify the genes and pathways underlying this process.

COMPETING INTERESTS

The authors declare no conflict of interest.

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