Strategies for *RUN1* Deployment Using *RUN2* and *REN2* to Manage Grapevine Powdery Mildew Informed by Studies of Race Specificity

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ABSTRACT

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The Toll/interleukin-1 receptor nucleotide-binding site leucine-rich repeat gene, “resistance to *Uncinula necator* 1” (*RUN1*), from *Vitis rotundifolia* was recently identified and confirmed to confer resistance to the grapevine powdery mildew fungus *Erysiphe necator* (syn. *U. necator*) in transgenic *V. vinifera* cultivars. However, sporulating powdery mildew colonies and cleistothecia of the heterothallic pathogen have been found on introgression lines containing the *RUN1* locus growing in New York (NY). Two *E. necator* isolates collected from *RUN1* vines were designated NY1-131 and NY1-137 and were used in this study to inform a strategy for durable *RUN1* deployment. In order to achieve this, fitness parameters of NY1-131 and NY1-137 were quantified relative to powdery mildew isolates collected from *V. rotundifolia* and *V. vinifera* on vines containing alleles of the powdery mildew resistance genes *RUN1*, *RUN2*, or *REN2*. The results clearly demonstrate the race

specificity of *RUN1*, *RUN2*, and *REN2* resistance alleles, all of which exhibit programmed cell death (PCD)-mediated resistance. The NY1 isolates investigated were found to have an intermediate virulence on *RUN1* vines, although this may be allele specific, while the Musc4 isolate collected from *V. rotundifolia* was virulent on all *RUN1* vines. Another powdery mildew resistance locus, *RUN2*, was previously mapped in different *V. rotundifolia* genotypes, and two alleles (*RUN2.1* and *RUN2.2*) were identified. The *RUN2.1* allele was found to provide PCD-mediated resistance to both an NY1 isolate and Musc4. Importantly, *REN2* vines were resistant to the NY1 isolates and *RUN1REN2* vines combining both genes displayed additional resistance. Based on these results, *RUN1*-mediated resistance in grapevine may be enhanced by pyramiding with *RUN2.1* or *REN2*; however, naturally occurring isolates in North America display some virulence on vines with these resistance genes. The characterization of additional resistance sources is needed to identify resistance gene combinations that will further enhance durability. For the resistance gene combinations currently available, we recommend using complementary management strategies, including fungicide application, to reduce populations of virulent isolates.

The wild North American grapevine species *Vitis rotundifolia* was recognized as early as 1889 as a source of resistance to powdery mildew (25). In 1919, a partially fertile hybrid was created between *V. rotundifolia* ($2n = 40$) and *V. vinifera* ($2n = 38$) (10). Subsequent backcrosses (BCs) revealed that powdery mildew resistance was inherited as a single dominant locus, “resistance to *Uncinula necator* 1” (*RUN1*), mapping to a region on chromosome (Chr) 12 (1,27). Recently, a Toll/interleukin-1 receptor (TIR) nucleotide-binding site leucine-rich repeat (*NB-LRR*) gene from the *RUN1* locus was confirmed to confer resistance to *Erysiphe necator* (syn. *U. necator*) in transgenic *V. vinifera* wine grape cultivars (15). The *RUN1* gene is typical of most plant resistance (*R*) genes which encode proteins with LRR domains, a central NB, and a variable N

terminus comprising either a TIR domain or coiled-coil domain (8). The resistance mediated by *RUN1* is associated with rapid programmed cell death (PCD), which prevents *E. necator* hyphal growth and sporulation (15).

Eastern North America is the likely center of origin for *E. necator*, because isolate populations collected from this region are much more genetically diverse than those found in Europe and Australia, where only genetic groups A and B have been found (2,17). Although *RUN1* appears to confer resistance to isolates in North America, Europe, and Australia, it can be overcome by an isolate (group M) from the southeastern region of North America, to which *V. rotundifolia* is native (15). Recently, powdery mildew cleistothecia were found on *RUN1* introgression lines growing in a permanent research vineyard in New York State from 2008 to 2010 (6). This suggests that naïve powdery mildew populations in New York may have evolved to overcome *RUN1* or, alternatively, that isolate variants were already present that could overcome *RUN1*. This supports previous observations from other crops that individual *NB-LRR* genes can be overcome in the field by rapidly evolving pathogen populations (3,4,13). Resistance conferred by a single dominant *R* gene is often short lived in the field. For example, resistance conferred by the powdery mildew *R* gene *PI2*, used in apple breeding programs, was found to have broken down in experimental orchards after only 6 years (7).

Therefore, complementary disease management strategies are needed to extend the durability of *NB-LRR* genes. In breeding for

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urable grapevine powdery mildew resistance, the most common strategy has been to combine multiple *R* genes in new cultivars in an attempt to improve resistance levels and durability (14). Pyramiding *R* genes has been demonstrated to be effective in other crop plants. For example, combining *R* genes against late blight in potato led to an additive resistance response (36). Combining four different *R* genes in rice against rice blast, although not additive, was more efficient for maintaining resistance durability in the field (35). Several powdery mildew *R* gene combinations have also been introduced into elite wheat cultivars using molecular markers (23).

The ability to combine multiple powdery mildew *R* genes in grape breeding lines has only recently become possible due to the molecular mapping of several *R* loci. For example, in *V. rotundifolia*, several allelic variants have been indicated by simple-sequence repeat (SSR) fingerprints at both the *RUN1* locus on Chr 12 and *RUN2* locus on Chr 18 (33). In addition, the first mapped powdery mildew *R* gene, *REN2* from *V. cinerea*, maps to Chr 14 and confers partial resistance to grapevine powdery mildew (9,32). However, unlike *RUN1* (15), the mechanisms underlying the resistance mediated by *RUN2* and *REN2* are yet to be determined.

Understanding the mechanisms of resistance and their potential impacts on resistance durability requires knowledge of pathogen biology. Infection of the plant host by *E. necator* begins with conidial germination on the surface of green plant tissue, followed by the formation of a primary appressorium. A penetration hypha from the appressorium breaks through the cuticle and cell wall of the epidermal cell and is subtended by a globose feeding structure (haustorium), which invaginates the epidermal cell membrane (19). Successful formation of a functional haustorium is a requirement for further fungal development. The haustorium is surrounded by a plant cell-derived extrahaustorial membrane (EHM). The fungus secretes effector proteins into the apoplast of the host (39) or across the EHM

into the cytosol (38). The direct or indirect detection of these cytosolic effectors by NB-LRRs can trigger a rapid PCD resistance response. Thus, the strength and mechanism of resistance to powdery mildew, including resistance conferred by the *R* genes described above, can be characterized from the quantification of fungal development and PCD induction at 48 to 72 h postinoculation (hpi) (16,21,29,30).

The objective of the current study was to assess strategies for *RUN1* deployment and *R* gene pyramiding in grapevine by studying the race specificity of the *RUN1*, *RUN2*, and *REN2* alleles. The study of race specificity was enabled by a diverse collection of *E. necator* isolates obtained in a series of population genetic studies (2,17) and following the discovery of naïve isolates naturally infecting *RUN1* vines in Geneva, NY (6).

MATERIALS AND METHODS

Grapevine germplasm development and genotyping. The grapevine germplasm described here was provided as *in vitro* plantlets by CSIRO or as green cuttings by the University of California-Davis or Cornell University. Potted vines were maintained in the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) greenhouse in Geneva, NY to harvest optimum-quality leaves for uniform disease evaluations. *V. vinifera* ‘Portan’ and ‘Tempranillo’ expressing *RUN1* (*MrGA10*) were generated as described previously (15). Of the three Portan *RUN1* and three Tempranillo *RUN1* *in vitro* plantlets transferred from CSIRO to USDA-ARS, all three Portan vines but only one of the Tempranillo vines were successfully established and grown from *in vitro* plantlets. Details of parentage and resistance allele status of the other germplasm described here are provided in Tables 1 and 2. *V. rotundifolia* ‘Thomas’ was a grandparent of NC6-15, a seedling from the original *V. vinifera* × *V. rotundifolia* introgression of *RUN1*

TABLE 1. Allelic profile of simple-sequence repeat (SSR) markers linked to the resistance loci *RUN1* and *RUN2* for cultivars and introgression lines used in this study^a

Genotype	<i>R</i> alleles	Parentage	<i>RUN1</i>		<i>RUN2</i>			
			VMC8g9	VMC4f3.1	UDV108	VMC7f2		
Cabernet Sauvignon	Susceptible	<i>V. vinifera</i>	169	194	169	175	211	239
NC6-15	<i>RUN1</i>	Malaga × <i>V. rotundifolia</i> G52 (Thomas × Hope)	<u>153</u>	172	185	189	203	237
Thomas	<i>RUN1 RUN1.2a</i>	<i>V. rotundifolia</i>	<u>132</u>	<u>153</u>	189	199	205	207
BC5:3294-R23	<i>RUN1</i>	NC6-15 pseudo backcross 5 to Cabernet Sauvignon	<u>153</u>	169	175	185	239	276
Trayshed	<i>RUN1.2a/bRUN2.2</i>	<i>V. rotundifolia</i>	<u>132</u>	<u>133</u>	179	189	219	219
e6-23	<i>RUN1.2b</i>	F2-35 × T6-32 OP (F2-35 × Trayshed)	<u>133</u>	169	169	179	231	241
e1-78	<i>RUN1.2a</i>	T6-31 OP (F2-35 × Trayshed) × Grenache	<u>132</u>	155	179	202	211	231
JB81-107-11	<i>RUN2.1</i>	[(UCD Y14-14 × Grenache) × Magnolia] × Verdelet (S. 9110)	135	172	175	205	<u>203</u>	223

^a SSR alleles that are linked to the resistance are underlined. The following resistance alleles were identified based on the SSR marker VMC8g9: *RUN1* (153 bp), *RUN1.2a* (132 bp), and *RUN1.2b* (133 bp). The following resistance alleles were identified based on the SSR marker UDV108: *RUN2.1* (203 bp) and *RUN2.2* (219 bp).

TABLE 2. Resistance gene status of *RUN1* and *REN2* breeding lines used in this study^a

Genotype	Parentage	<i>RUN1</i>		<i>REN2</i>	
		VMC8g9 159 bp	UDV-058 154 bp	VVIP26 144 bp	UDV-025 124 bp
119 (NY06.0506.02)	Arandell × Eger 99-11 (37-17-102)	+	+	+	+
123 (NY06.0506.01)		+	+	+	+
4 (NY06.0514.04)	NY95.0308.01 × Eger 99-11 (37-17-108)	–	–	+	+
11 (NY06.0514.03)		+	+	–	–
17 (NY06.0514.06)	...	+	+	+	+
59 (NY06.0512.01)	Eger 99-11 (37-16-072) × Cayuga White	+	+	–	–
61 (NY06.0512.02)		+	+	–	–
100 (NY06.0509.02)	Eger 99-11 (37-16-082) × Arandell	+	+	+	+
113 (NY06.0509.01)		+	+	+	+
Chardonnay	...	–	–	–	–

^a Symbols: + and – indicate presence and absence, respectively, of the resistance allele.

carried out by Detjen in 1919 (10). NC6-15 was backcrossed to *V. vinifera* 'Cabernet Sauvignon', and BC5-3294-R23 is derived from five generations of this pseudo-backcrossing to different *V. vinifera* cultivars to avoid inbreeding depression, with the final cross also to Cabernet Sauvignon. 'Trayshed' is another powdery-mildew-resistant *V. rotundifolia* cultivar and is homozygous for the *RUN1.2* locus on Chr 12 in addition to carrying another resistance locus, *RUN2.2*, on Chr 18. Powdery-mildew-resistant breeding lines e1-78 and e6-23 lack *RUN2.2* and represent both haplotypes (*RUN1.2a* and *RUN1.2b*) of Trayshed for Chr 12. The breeding line JB81-107-11 that was previously used to map the *RUN2.1* locus on Chr 18 has a complex background (30), including *V. rotundifolia* 'Magnolia' as a grandparent. The *REN2* locus from *V. cinerea* was inherited from the 'Arandell' (31) or NY95.0308.01 parent (Table 2). For studies involving *REN2*, the *RUN1* locus was inherited from one of four Eger 99-11 full-sibling progeny (37-16-072, 37-16-082, 37-17-102, and 37-17-108) resulting from pollinating a *RUN1* donor, 3082-1-42, with SK 90-2/19, an interspecific hybrid breeding line with *V. amurensis*, 'Traminer', 'Bianca', and 'Riesling' background.

To confirm the presence of the *R* loci, DNA was extracted from young leaf material using the DNeasy 96 Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's directions or by modified cetyltrimethylammonium bromide procedure (33). Concentration and purity of DNA was determined by NanoDrop spectrophotometer (Wilmington, DE), and samples were normalized to 10 ng/μl. The predictive allele sizes of the SSRs used in this study—VMC8g9 (1); UDV-025 and UDV-058 (11); VMC4f3.1, UDV108, VMC7f2 (33), and VVIP26 (GenBank BV140645.1)—are described in Tables 1 and 2. Fluorescently labeled forward primers were used to incorporate a fluorescence signal during the following thermocycling conditions: 30 s at 95°C; followed by 35 cycles of 45 s at 95°C, 45 s at 58°C, and 45 s at 72°C; followed by 10 min at 72°C. Detection by capillary electrophoresis for Table 2 was performed at the Core Laboratories Centre at Cornell University using an ABI 3730 DNA Analyzer (Life Technologies, Carlsbad, CA) with GeneScan-500 Liz Size Standard (Life Technologies). The SSR data were analyzed using Genographer 2.1.4 software. For Table 1, capillary electrophoresis detection was performed at the University of California-Davis using an ABI 3500

DNA Analyzer (Life Technologies) with GeneScan-500 Liz Size Standard (Life Technologies). The SSR data were analyzed using GeneMapper 4.1 software.

Collection and maintenance of *E. necator* isolates. All *E. necator* isolates were maintained on surface-sterilized detached leaves of susceptible *V. vinifera* 'Chardonnay' on 1% water agar in petri dishes, as previously described (6). Isolate transfer was completed on a 10-day propagation cycle by dry tap transfer (i.e., gently tapping conidia from an infected leaf held above a new propagation leaf in an open petri dish). All inoculated leaves were incubated at 25°C under a cycle of 16 h of light and 8 h of darkness.

Isolates used in this study included two New York (NY) isolates collected from *V. vinifera*, LNYM ('Merlot'; Lockport, NY) and NY19 (Chardonnay; Burdett, NY) (2); two NY1 isolates collected from *RUN1* full-sibling vines (with the parentage Eger 99-11 [37-17-104] × Arandell) (6,31), NY1-137 (progeny 44-7-137) and NY1-131 (progeny 44-7-131); and one isolate collected from *V. rotundifolia* in Georgia, Musc4 (2,15). Each isolate belongs to a different haplotype based on multilocus sequencing and SSR analysis (2,17).

Powdery mildew disease assays. The fourth fully expanded leaf (glossy and translucent) was collected and used for all experiments described here. At the conclusion of the incubation period described for each experiment, leaves were cleared and stained with either trypan blue or Coomassie blue. For experiments visualized by trypan blue staining, whole leaves were inoculated with *E. necator* conidia by dry tap transfer of cultures at 10 days postinfection (dpi), as described above. At 48 hpi, infected leaves were stained in 10 ml of trypan blue (lactic acid/phenol/dH₂O/glycerol = 1:1:1:1 by volume, and 2 mg of trypan blue) in a 15-ml Falcon tube at 90°C for 1 h, as previously described (22). Leaves were cleared in chloral hydrate (2.5 g/ml) overnight and rinsed several times in water before mounting on a microscope slide in 50% glycerol. Trypan blue stains fungal structures and is a vital stain for cell death. At minimum, 100 germinated spores were scored on a leaf of each genotype using a compound light microscope. Infection structures were categorized as follows: (i) germinated spore with a haustorium and appressorium (Fig. 1C and D), (ii) germinated spore with a secondary hypha at least twice as long as the spore (Fig. 1A, B, and E), and (iii) the frequency of

Fig. 1. Representative resistance responses of grapevines containing *RUN1* to inoculation with different *Erysiphe necator* isolates. Leaves were sampled 48 h postinoculation. **A**, Tempranillo *RUN1* transgenic vines inoculated with LNYM show high levels of programmed cell death (PCD) induction in penetrated cells with some formation of long secondary hyphae (hy). **B**, Control Tempranillo leaves inoculated with LNYM where long secondary hyphae are formed without PCD. **C**, Rapid PCD induction with little secondary hyphae formation on BC5:3294-R23 introgression lines inoculated with LNYM. **D**, PCD induction on BC5:3294-R23 vines inoculated with NY1-137 with some secondary hyphae formation. **E**, Musc4 forms long secondary hyphae without PCD on BC5:3294-R23 vines. Trypan blue is a vital stain for dead cells. PCD is indicated by black arrows. Scale bar = 50 μm.

PCD induction (see arrows in Fig. 1A, C, and D). The frequency of (ii) secondary hyphae formation or (iii) PCD was quantified relative to the proportion of spores that had successfully penetrated. One to two leaves were scored in two independent experiments for data presented in Figure 2. Two leaves were scored in three independent experiments for data presented in Figure 3. For comparisons between grapevine genotypes within isolate treatment and between isolate treatments within grapevine genotype, *P* values were calculated using a Student's *t* test and are presented in Supplementary Table S1.

Fig. 2. Responses of grapevines containing *RUN1* to inoculation with different *Erysiphe necator* isolates. Percentage of **A**, programmed cell death (PCD) or **B**, secondary hyphae formation on the introgression line BC5:3294-R23 (carrying the *RUN1* locus) and *Vitis vinifera* Portan and Tempranillo lines with or without the *RUN1* transgene following inoculation with the isolates LNYM, NY1-137, or Musc4. At least 100 germinated spores were scored 48 h post-inoculation after trypan blue staining for cell death. Asterisks indicate a significant difference from the relevant control cultivars Portan or Tempranillo, hashes indicate a significant difference versus the same line challenged with LNYM, and double asterisks indicates a significant difference versus the same line challenged with NY1-137 ($\alpha < 0.05$; Student's *t* test). Each data point is the mean $\pm$ standard deviation of two independent experiments where ($n = 2$).

For experiments visualized by Coomassie blue staining, inoculum spore suspensions were made by resuspending conidia (10 dpi) in 150 μ l of distilled water with 0.1% Tween-20 (Sigma-Aldrich, St. Louis), and adjusted to approximately 1×10^5 *E. necator* conidia/ml based on hemacytometer counts. Leaf discs were drop-inoculated with a 10- μ l spore suspension. After 48 h, leaf discs were cleared in 3:1 (vol/vol) ethanol/acetic acid, changing the solution three to four times until the tissue was completely bleached, and then transferred to 50% ethanol for long-term storage. The solution was briefly replaced for 10 to 30 s with Coomassie stain (Coomassie Brilliant Blue R-250 [Sigma-Aldrich] at 0.12 g/liter in 50% [vol/vol] methanol and 10% [vol/vol] glacial acetic acid) to stain the mycelium, then rinsed with several changes of water and mounted for viewing on a microscope slide in 50% glycerol. Infection structures were categorized as

Fig. 3. Responses of grapevines containing *RUN1* alleles or *RUN2* to inoculation with different *Erysiphe necator* isolates. Percentage of **A**, programmed cell death (PCD) and **B**, secondary hyphae formation on *V. rotundifolia* cultivars or breeding lines, 48 h postinoculation with the *E. necator* isolates LNYM, NY1-137, or Musc4. Hashes indicate a significant difference versus the same line challenged with LNYM and the double asterisks a significant difference versus the same line challenged with NY1-137 ($\alpha < 0.05$; Student's *t* test). At least 100 germinated spores were scored. Each data point is the mean $\pm$ standard deviation of three independent experiments ($n = 2$).

follows: 100 spores per leaf disc were observed with a compound light microscope and categorized as (i) germinated spore with an appressorium (Fig. 4A), (ii) germinated spore with a secondary hypha at least twice as long as the spore (Fig. 4B), or (iii) germinated spore with multiple or branching secondary hypha (Figs. 4D and 5A and B). Colony success rate was quantified as the proportion of spores in category (iii) out of those that successfully penetrated; that is, (ii) and (iii). The colony success rate equation is an improvement over that of Ramming et al. (30), which calculated the proportion of (iii) out of all spores, resulting in an artifact of combining both penetration resistance and colony resistance. For data presented in Figures 4, 5, and 6 and Tables 3 and 4, leaves were collected from two replicate shoots per vine and from two replicate vines per genotype. The experiment was conducted twice.

For measuring colonization and sporulation, whole leaves were drop-inoculated with 1×10^5 conidia/ml in four 10- μ l interveinal locations per leaf. Mycelium growth and sporulation were scored under $\times 15$ magnification at 10 dpi and rated according to Miclot et al. (2012) (24) for mycelia, where 9 = absent, 7 = scattered, low density, 5 = widespread, low density, 3 = widespread, locally dense, and 1 = widespread, dense; and for sporulation, where 9 = absent, 7 = scattered, low density of conidia, 5 = scattered, high density of conidia, 3 = widespread, moderate density of conidia, and 1 = widespread, high density of conidia. Images were taken using QImaging camera with QCapture 3.1.2 software. Four replicate leaves were used per isolate–vine combination, and experiments were repeated three times.

RESULTS

***RUN1* resistance specificity to diverse isolates.** Given that the NY1-137 isolate was observed growing on introgression lines containing the *RUN1* locus in a vineyard in New York (6), we decided to further assess the race specificity of the *RUN1* transgenic vines. Following inoculation with the *E. necator* isolates LNYM, Musc4, and NY1-137, PCD and the growth of secondary hyphae

was scored as a measure of resistance in *RUN1* transgenics, BC5:3294-R23 vines (a *RUN1* introgression line) (1), and the untransformed *V. vinifera* Portan and Tempranillo.

A high frequency of PCD was observed in epidermal cells associated with the penetration of LNYM isolates on both BC5:3294-R23 vines (94%) (Figs. 1C and 2A) and transgenic *RUN1* vines (Figs. 1A and 2A) (64 to 81%), compared with the untransformed Portan (1%) and Tempranillo (10%) (Figs. 1B and 2A). The high frequency of PCD in penetrated epidermal cells slightly reduced secondary hyphae formation at 48 hpi in the *RUN1* transgenics (77 to 89%) compared with the untransformed cultivars (95 to 99%) (Figs. 1A and B and 2B). The lowest rates of secondary hyphal growth were on BC5:3294-R23 vines (Figs. 1C and 2B).

Following challenge with the NY1-137 isolate, BC5:3294-R23 (86%) and *RUN1* transgenic lines of Tempranillo (71%) and Portan (40 to 46%) were also found to have significantly higher frequencies of PCD in penetrated epidermal cells than the untransformed controls (Figs. 1D and 2A). Although Portan *RUN1* vines still had a significantly increased frequency of PCD compared with the untransformed vines, rates of PCD were significantly lower (24 to 30%) than the same vines infected with the LNYM isolate (Fig. 2A). This suggests that *RUN1* resistance in the Portan transgenic vines is partially compromised by the NY1-137 isolate. Interestingly, the frequency of PCD induction in Portan *RUN1* vines in response to NY1-137 penetration was significantly lower than that observed in Tempranillo *RUN1*. PCD induction was also significantly reduced, albeit to a very low level, in BC5:3294-R23 inoculated with NY1-137 compared with LNYM (Fig. 2A). The formation of secondary hyphae was similar in all of the lines inoculated with NY1-137 with the exception of BC5:3294-R23 progeny which had much lower rates of secondary hyphae formation (Figs. 1D and 2B). Consistent with our previous findings (15), no significant increase in PCD was found on BC5:3294-R23 vines nor on any of the *RUN1* vines following infection with the Musc4 isolate, and all lines had high rates of secondary hyphae formation (Figs. 1E and 2B) indicating the ineffectiveness of *RUN1*-mediated resistance against this particular isolate.

Fig. 4. Representative resistance responses of *RUN1REN2* introgression lines to inoculation with different *Erysiphe necator* isolates at 3 days postinoculation. Coomassie blue staining of cleared leaves shows **A**, LNYM conidia with necrotic host epidermal cells beneath the appressoria, indicative of programmed cell death (PCD) (black arrows); **B**, NY1-131 and **C**, NY1-137 with PCD and some secondary hyphae (hy); and **D**, Musc4, which formed colonies with no visible PCD response. Scale bars = 50 μ m.

In order to test whether the differences in PCD observed between isolates would affect the fitness of the isolates, including colony formation and subsequent conidiation, we expanded the study to include more isolates and a longer incubation to allow conidiation. Colony density per field of view (the number of times a hypha crosses a vertical or horizontal transect) and conidiation were scored at 9 dpi. According to our data, NY1-131 and NY1-137 isolates are intermediate in virulence between LNYM and Musc4 on *RUN1* vines, and fungal growth is sufficient for conidiation (Table 3). Surprisingly, an NY isolate from *V. vinifera* (NY19) had a similar capacity to colonize and conidiate on *RUN1* vines, despite being collected from *V. vinifera*, suggesting that virulence on *RUN1* vines is not a rare trait in NY populations of *E. necator* (Table 3).

Race specificity of *RUN1* and *RUN2* alleles. In light of the existence of field isolates capable of breaking *RUN1*-mediated resistance and with a view to pyramiding grapevine powdery mildew *R* genes, the race specificity of several different *RUN1* and *RUN2* alleles from *V. rotundifolia*, as defined in Materials and Methods, was investigated in cultivars and breeding lines. Grapevine genotypes carrying various combinations of the *RUN1* and *RUN2* alleles (Table 1) were inoculated with powdery mildew isolates LNYM, NY1-137, and Musc4 and scored for percent PCD in penetrated epidermal cells. In response to LNYM infection, the *RUN1.2a/b*-containing genotypes, which include e1-78, e6-23, and Trayshed, as well as the *RUN1*-only genotype BC5:3294-R23 and the *RUN1RUN1.2a* genotype Thomas, all displayed similar levels of PCD (Fig. 3A) and secondary hyphae formation (Fig. 3B). However, the BC5:3294-R23 vine was found to have a small but significant reduction in PCD (~10%) in response to NY1-137 compared with LNYM (Fig. 3A), which was also observed in the *RUN1* source Thomas (~3%) despite carrying both *RUN1* and *RUN1.2a* alleles. No significant differences were found in secondary hyphae formation for any of the *RUN1*- or *RUN1.2a/b*-containing vines when challenged with LNYM or NY1-137. Induction of PCD (8 to 13%) was markedly reduced and secondary hyphae formation was high (97 to 99%) for all *RUN1* and *RUN1.2a/b* genotypes (Fig. 3) inoculated with Musc4.

In contrast, the *RUN2.1* breeding line JB81-107-11 (33) was found to have an intermediate PCD response (60 to 65%) to all three *E. necator* isolates investigated (Fig. 3A). Thus, although the *RUN2.1*-mediated resistance response in terms of PCD induction (Fig. 3A) appears to be lower than that induced by the *RUN1*, *RUN1.2a*, or *RUN1.2b* loci, it does not appear to be broken by the Musc4 isolate. However, the frequency of secondary hyphal formation was found to be significantly lower on JB81-107-11 when challenged with LNYM (86%) and NY1-137 (81%) than Musc4 (98%) (Fig. 3B). It should be noted that Trayshed, which combines *RUN1.2a/b* and *RUN2.2* alleles, displayed PCD and secondary hyphal formation rates similar to other genotypes containing *RUN1.2a* or *RUN1.2b* only (Fig. 3). This suggests that, unlike *RUN2.1*, *RUN2.2* is ineffective against the Musc4 isolate (Fig. 3).

***REN2*- and *RUN1*-mediated resistances are additive.** The *REN2* locus, which maps to Chr 14, provides partial resistance to grapevine powdery mildew and is derived from *V. cinerea* (9,32). Because *REN2* originates from a completely different genetic background, it might be a useful candidate for pyramiding with *RUN1*. To test the ability of *REN2* to complement *RUN1* resistance, the powdery mildew isolates LNYM, NY19, NY1-131, NY1-137, and Musc4 were tested for posthaustorial colony success rate on breeding lines with *RUN1* or *REN2*. The breeding lines were genotyped using four SSR markers to detect the presence of *RUN1* and *REN2* *R* genes (Table 2).

All isolates established colonies on Chardonnay with similarly high efficiency (88 to 92%) (Figs. 5A and 6). In contrast, vines containing *REN2* or *RUN1* displayed marked resistance to *E. necator* colony formation (Figs. 5B and C and 6), and vines combining the two *R* genes showed a trend of additive increased resistance to powdery mildew colony formation (Figs. 5A to D and 6). For example, LNYM established colonies at success rates of 30% on *REN2* vines, 6% on *RUN1* vines, and 2.6% on *RUN1REN2* vines (Fig. 6). Rates of colony formation were also maintained at significantly lower rates on *RUN1REN2* vines inoculated with isolates NY-19, NY1-131, and NY1-137 than vines with either *REN2* or *RUN1* *R* genes alone. Musc4

Fig. 5. Representative resistance responses of grapevines containing either the *RUN1* or *REN2* to inoculation with the *Erysiphe necator* NY19 isolate at 48 h postinoculation. Coomassie blue staining of cleared leaves shows **A**, frequent colony development with long secondary hyphae (hy) on *Vitis vinifera* Chardonnay, with no visible host response; **B**, colony development on *REN2* with some conidia stopped at the appressorial (a) stage and some necrotic host epidermal cells indicative of programmed cell death (PCD) (black arrows); **C**, some PCD and rare, long secondary hyphae on a *RUN1* introgression indicating the ability of some conidia to form a colony; and **D**, high frequency of PCD on a *RUN1REN2* introgression line. Scale bar = 50 μ m.

established colonies on all vines (Figs. 4A and 6) with high efficiency, although colony formation was slightly lower than the Chardonnay controls (90%) in *REN2* (72%), *RUN1* (61%), and *RUNIREN2* (52%) vines (Fig. 6).

To examine whether the minor but statistically significant reductions in colony success rate observed at 3 dpi between NY19, NY1-131, and NY1-137 would affect later fitness parameters, we quantified colony density and sporulation at 10 dpi compared with Musc4. All isolates grew well and sporulated extensively on detached leaves from Chardonnay. Independent of whether they contained *RUN1* or both *RUN1* and *REN2*, leaves inoculated with NY19 formed scattered, low-density colonies (rating 7) but did not sporulate. However, colony development at 10 dpi was visibly reduced on leaves inoculated with either NY1-131 or NY1-137, when both *RUN1* and *REN2* (rating 7) were present, compared with leaves from vines that contained *RUN1* alone (rating 5) (Table 4). Despite the widespread colony development of NY1-131, NY1-137, and Musc4 on *RUN1* vines, only Musc4 was

able to sporulate within 10 dpi. Similarly, only Musc4 sporulated on *RUNIREN2* leaves within this 10-dpi experiment (Table 4).

DISCUSSION

During infection, pathogens secrete small effector molecules to suppress basal immune responses in plant cells and thereby facilitate penetration and colonization. Through evolution, certain plant species have evolved a second line of defense, referred to as effector-triggered immunity, whereby these pathogen effector molecules are recognized either directly or indirectly by R proteins that respond by triggering PCD (i.e., a hypersensitive response) (8,13). However, R-gene-mediated defense is typically short lived due to the strong selection pressure that such resistance can exert upon pathogen populations, resulting in the rapid selection and subsequent increase of virulent isolates.

RUN1 was previously introduced into *V. vinifera* Portan and Tempranillo by genetic transformation and demonstrated to provide resistance to Australian and French powdery mildew isolates (15). Grapevines expressing *RUN1* limit fungal development through PCD of the challenged epidermal cells and, therefore, fungal growth is arrested shortly after penetration; however, short secondary hyphal growth may occur between penetration and the onset of PCD (Fig. 1A). The ability of the fungus to form secondary hyphae may be indicative of a slower recognition or PCD response. If a pathogen subsequently evolves effectors that evade this recognition, a new pathogen race emerges, and resistance is overcome. Transgenic vines containing *RUN1* displayed a strong PCD response to powdery mildew isolates collected from New York (LNYM and NY19) but not to isolate Musc4 collected from *V. rotundifolia* in Georgia (2,15). However, the discovery of isolates NY1-131 and NY1-137, growing on *RUN1* introgression lines in an experimental vineyard in New York (6), indicated that these *RUN1*-virulent *E. necator* isolates may have arisen quickly (from planting of *RUN1* vines in 2008 to collection of isolates from *RUN1* vines in 2010). The current study confirms this original observation and demonstrates that these isolates are indeed more virulent on *RUN1* transgenics and introgression lines compared with the LNYM isolate (Figs. 2, 3, and 6). However, it is also clear that the virulence of these isolates on *RUN1* genotypes is intermediate between the avirulent LNYM isolate and that of the virulent Musc4 isolate, which completely evades *RUN1* recognition. Musc4 originates from the same region of southeastern North America as *V. rotundifolia*, the source of *RUN1*. Whereas the coevolution of Musc4, along with *V. rotundifolia*, has led to the loss of Musc4 recognition by *RUN1*, resulting in pathogenic specialization (18), it would appear that the NY1-131 and NY1-137 isolates are intermediate in their evolution to avoid *RUN1* detection. Furthermore, NY19 and LNYM were collected from commercial vineyards and,

Fig. 6. Development of *Erysiphe necator* isolates on grapevine leaves containing *RUN1* or *REN2* relative to *Vitis vinifera* Chardonnay susceptible control. Colony success rate as indicated by the presence of multiple or branching secondary hyphae at 48 h postinoculation. At least 100 germinated spores were scored. Asterisks indicates a significant difference from Chardonnay controls, double asterisks indicates a significant difference from the vines containing *RUN1* only and *REN2* only (additive resistance), hashes indicate a significant difference from the same genotype with LNYM, and daggers indicate a significant difference from the same genotype with NY isolates ($\alpha < 0.05$; Student's *t* test). Each data point is the mean $\pm$ standard deviation of three independent experiments (*n* = 4).

TABLE 3. Mean colony density per field of view (the number of times a hypha crosses a vertical or horizontal transect) and conidiation was scored 9 days postinfection^a

Isolate	<i>Vitis vinifera</i> Chardonnay	Portan <i>RUN1</i>	BC5:3294-R23
LNYM	73.9 (± 10.0)	1.0 (± 0.8)	1.6 (± 1.3)
NY19	82.3 (± 3.4)	19.0 (± 7.8)	4.3 (± 1.5)
NY1-131	81.1 (± 0.8)	19.9 (± 15.4)	1.4 (± 2.0)
NY1-137	61.6 (± 16.4)	11.9 (± 4.1)	4.1 (± 1.5)
Musc4	71.2 (± 1.4)	33.8 (± 9.0)	36.4 (± 17.1)

^a Chardonnay was included as a susceptible control and *Erysiphe necator* Musc4 was included as a positive control for pathogen compatibility. Bold indicates conidiation in two experiments (*n* = 3) and italics indicates conidiation in one experiment. Numbers in parentheses indicate $\pm$ standard deviation.

TABLE 4. *Erysiphe necator* isolate development 10 days postinfection on different grapevine genotypes with or without one, or two resistance genes

Isolate	Genotype <i>R</i> alleles	Mycelia ^a	Conidia ^b
NY19	Chardonnay	1	1
	<i>F1-11/59/61 RUN1</i>	7	9
	<i>F1-100/113/119/123 RUNIREN2</i>	7	9
NY1-131	Chardonnay	1	1
	<i>F1-11/59/61 RUN1</i>	5	9
	<i>F1-100/113/119/123 RUNIREN2</i>	7	9
NY1-137	Chardonnay	1	1
	<i>F1-11/59/61 RUN1</i>	5	9
	<i>F1-100/113/119/123 RUNIREN2</i>	7	9
Musc4	Chardonnay	1	1
	<i>F1-11/59/61 RUN1</i>	5	5
	<i>F1-100/113/119/123 RUNIREN2</i>	5	5

^a Mycelia or colony rating scale: 9 = absent, 7 = scattered, low density, 5 = widespread, low density, 3 = widespread, locally dense, and 1 = widespread, dense (Miclot et al. 2012).

^b Conidia rating scale: 9 = absent, 7 = scattered, low density of conidia, 5 = scattered, high density of conidia, 3 = widespread, moderate density of conidia, and 1 = widespread, high density of conidia (Miclot et al. 2012).

therefore, represent a component of the natural variation of *E. necator* in commercial vineyards. The similarity of NY19 virulence to that of the NY1-131 and NY1-137 isolates suggests that the ability to colonize (Fig. 6; Table 3) and form cleistothecia (6) on *RUN1* vines may be common among naïve New York isolates that have not previously encountered *RUN1*, albeit to a much lower degree than isolates collected from *V. rotundifolia*. The ability to overcome *RUN1* may be indicative of different effector profiles present in the LNYM, NY1, and Musc4 isolates.

The current study also highlights the importance of the genetic background into which the *R* genes are being introgressed or otherwise introduced. Previously, we identified two other putative *R* genes at the *RUN1* locus, *RGA4* and *RGA8*, which may also make minor additive contributions to powdery mildew resistance (15). BC5:3294-R23 contains the whole *RUN1* locus introgressed; therefore, it is possible that the higher levels of resistance observed in this line, in comparison with the transgenic lines containing *RUN1* only (Fig. 2), are due to the additive action of *RGA4*, *RGA8*, and *RUN1* at this locus. However, it is also interesting that *RUN1* conferred higher frequencies of PCD against NY1-137 on Tempranillo than on Portan (Fig. 2), suggesting that genetic background may have a significant effect on the expression of resistance. This has been demonstrated in other pathosystems; for example, the bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) *R* gene *Xa3/Xa26* is differentially regulated by the genetic background of rice and functions better in the *japonica* background than in *indica* (40). Therefore, unknown alleles at other unknown loci may be contributing to powdery mildew resistance among the cultivars and breeding lines used in this study.

The SSR marker VMC8g9 is tightly linked to *RUN1*. BC5:3294-R23 is derived from five generations of pseudo-backcrossing of NC6-15 to different *V. vinifera* cultivars to avoid in-breeding depression. NC6-15 has a VMC8g9 allele combination of 153/172. Thomas, which is a grandparent of NC6-15, shares the 153-bp allele with NC6-15 and BC5:3294-R23. The breeding lines e1-78 and e6-23 are both derived from Trayshed and each has one of Trayshed's *RUN1.2a/b* alleles (132/133) at this locus. Interestingly, Thomas also shares the *RUN1.2a* allele but this was not inherited by NC6-15. This suggests that there may be three different *RUN1* alleles, designated as *RUN1*, *RUN1.2a*, and *RUN1.2b*. They appear to mediate similar PCD responses to the isolates tested, although the *RUN1*-containing vines Thomas and BC5:3294-R23 show a small but significant reduction in PCD level when challenged with NY1-137 compared with LNYM, which was not observed in vines with only the *RUN1.2a/b* allele combinations (Fig. 3). This may suggest either the beginning of evolution to avoid detection by this specific *RUN1* allele (153 bp) or it may reflect the genetic background of BC5:3294-R23, which is predominantly *V. vinifera*.

JB81-107-11 is a breeding line with a complex background, including the *V. rotundifolia* genotypes Trayshed and Magnolia. Field studies in California (where only the *E. necator* group B has been found) show that JB81-107-11 is resistant to powdery mildew. This resistance, designated *RUN2.1*, segregates with the SSR marker UDV108 on Ch18, with a resistance allele (202 bp) shared with Magnolia at this locus (33). This previous study also identified resistance at this locus derived from Trayshed but with a different allelic profile, which was designated *RUN2.2* (33). Our results show that, whereas Trayshed (*RUN1.2a/b* and *RUN2.2*) was completely susceptible to the Musc4 isolate, the intermediate resistance response of JB81-107-11 (*RUN2.1*) was maintained at a consistent level across all powdery mildew isolates tested, including Musc4 (Fig. 3). This suggests that *RUN2.1* is able to recognize and mount a resistance response against the Musc4 isolate while *RUN2.2* is not. Such differences in race specificity between *RUN2.1* and *RUN2.2* would not have been detected in the previous studies by Riaz et al. (33) because of their use of group B isolates. Based on our assessment of the race specificity of the different powdery mildew *R* genes sourced from

V. rotundifolia, it would appear that *RUN2.1* (or another *R* gene from JB81-107-11) is a good candidate to pyramid with *RUN1*. However, other *E. necator* isolates are known to overcome JB81-107-11 resistance and produce conidia (30). Studies with root knot nematode (RKN) *R* genes showed that pyramiding two different *R* genes in one genotype suppressed the emergence of virulent RKN isolates. The authors concluded that pyramiding two distinct *R* genes into a single cultivar could be a secure and durable strategy (12). This suggests that the combination of the two *R* genes *RUN1* and *RUN2.1* may provide a strategy for improved durability of resistance to powdery mildew infection in the vineyard.

Another potential option for pyramiding with *RUN1* is the *REN2* powdery mildew resistance locus from *V. cinerea* (9,32). *REN2* significantly reduced infection by all powdery mildew isolates tested, including Musc4 (Fig. 6). Furthermore, our results clearly demonstrate that grapevine genotypes in which both *RUN1* and *REN2* are combined show greater resistance against powdery mildew isolates NY19, NY1-131, and NY1-137, in terms of both colony formation at 48 hpi (Figs. 4A to C and 6) and mycelial growth at 10 dpi (Table 4), than is achieved by *RUN1* alone. Although this additive resistance was also observed at 48 hpi against Musc4, by 10 dpi there was no discernable difference between *RUN1* and *RUN1REN2* vines on the level of Musc4 growth or sporulation (Table 4). There is good evidence to indicate that the durability of major qualitative *R* genes may be influenced by genetic background (26). For example, the *R* gene *pvr2* (potato virus resistance) was defeated at high frequency when introgressed into a susceptible genetic background but it was not overcome when combined with a quantitative trait locus for partial resistance (26). Furthermore, combining *Rlm6* (resistance to *Leptosphaeria maculans*) with quantitative resistance was found to increase the durability of this *R* gene in *Brassica napus* (5). Therefore, it is likely that combining *RUN1* qualitative resistance with *REN2* quantitative resistance may also improve the durability of resistance to *E. necator* isolates.

For every isolate tested except Musc4, frequent host PCD was observed on *RUN1*, *REN2*, and *RUN1REN2* vines at 2 and 3 dpi (Figs. 4 and 5). In spite of this PCD, the fungus was often able to form microscopically visible colonies by 48 hpi and macroscopically visible colonies by 10 dpi (Fig. 6; Table 4). This phenotype of PCD that appears to trail the leading edge of hyphal growth was first described for isolates that overcame *RUN2.1* resistance from JB81-107-11 and was referred to as trailing necrosis (30). As mentioned above, the effector profile of the different *E. necator* isolates may determine the speed of recognition and PCD induction by the host *R* genes. That having been said, the trailing PCD observed here appears to be sufficient to reduce hyphal density and prolong the latent period, resulting in economically significant resistance.

The previous collection, genotyping, and analysis of grapevine powdery mildew isolates (2,17), together with the characterization of these isolates by this and previous studies, can now begin to inform strategies for the deployment of powdery mildew *R* genes in grapevine. However, such studies are still severely limited by well-justified restrictions on the distribution of different powdery mildew isolates to different countries or regions in which new resistant grapevine germplasm may be located. Recently, barley powdery mildew effector candidates were identified (20,34). A subset of these have now been functionally characterized (28,39) and confirmed as having virulence functions. We are now undertaking research to identify the powdery mildew effector candidates secreted by *E. necator* and to determine which of these are recognized by *R* genes such as *RUN1* and *RUN2.1*. The identification of these avirulence effectors will enable us to investigate the effector specificity of different powdery mildew *R* genes, without requiring access to the powdery mildew isolates. This approach has been used successfully to inform potato breeders about the race specificity of genes conferring resistance to late blight *Phytophthora infestans* and assist in devising scientifically sound strategies for pyramiding *R* genes to increase durability in the field (37).

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