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ORIGINAL PAPER

Resistance to *Erysiphe necator* in the grapevine 'Kishmish vatkana' is controlled by a single locus through restriction of hyphal growth

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Abstract *Vitis vinifera* 'Kishmish vatkana', a cultivated grapevine from Central Asia, does not produce visible symptoms in response to natural or artificial inoculation with the fungus *Erysiphe necator* Schwein., the casual agent of powdery mildew. 'Kishmish vatkana' allowed pathogen entry into epidermal cells at a rate comparable to that in the susceptible control *Vitis vinifera* 'Nimrang', but was able to limit subsequent hyphal proliferation. Density of conidiophores was significantly lower in 'Kishmish vatkana' (33.6 ± 8.7 conidiophores mm^{-2}) than in 'Nimrang' (310.5 ± 24.0 conidiophores mm^{-2}) by 120 h after inoculation. A progeny of 310 plants from a 'Nimrang' × 'Kishmish vatkana' cross were scored for the presence or absence of visible conidiophores throughout two successive seasons. Phenotypic segregation revealed the presence of a single dominant allele termed Resistance to *Erysiphe necator* 1 (*REN1*), which was heterozygous in 'Kishmish vatkana'. A bulked segregant analysis was carried out using 195 microsatellite markers uniformly distributed across the entire genome. For each marker, association with the resistance trait was inferred by measuring in the bulks the ratio of peak intensities of the two alleles inherited from 'Kishmish vatkana'. The phenotypic locus was assigned to linkage group 13, a genomic region in which no disease resistance had been reported previously. The *REN1* position was restricted to a 7.4 cM interval by analyzing the 310 offspring for the segregation of markers that surrounded the target region. The closest markers, VMC9H4-2, VMCNG4E10-1 and UDV-020, were located 0.9 cM away from the *REN1* locus.

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Introduction

Erysiphe necator Schwein., is a biotrophic ascomycete which causes powdery mildew (PM) disease in grapes (*Vitis* sp.) and other plants in the Vitaceae family. *Erysiphe necator* is native to the North American continent. During the 1840s, it crossed the Atlantic on grape cuttings imported to Europe, and then spread to other continents where grapevines are cultivated. European grapevine (*Vitis vinifera* L.) is susceptible to the fungus, which colonizes the epidermal cells of photosynthetic tissues, and spreads through subsequent asexual cycles in the absence of host hindrance (Pearson 1988).

Infection begins with conidial germination on the epidermis, formation of primary appressorium, and the adhesion of the resulting sporeling to the cuticle through deposition of an extracellular matrix (Rumbolz et al. 2000). The fungus breaks through the cell wall of the epidermal cell with a penetration peg, which emerges from the appressorium. The pathogen then forms a feeding structure, termed haustorium, which becomes intimately associated with the host plasma membrane. Nutrients are absorbed by the haustorium and utilized for the development of epiphytotic hyphae. As the hyphae elongate, they develop additional appressoria and haustoria, and give rise to conidiophores and conidia. Successful formation of a functional haustorium on the primary appressorium is a requirement for entering the subsequent stages of fungal development.

Natural sources of disease resistance are normally found in geographic regions where populations of pathogens and host plants co-evolved. The germplasm native to temperate zones of North America was investigated by grape breeders for sources of PM resistance. Parallel to breeding, the genetic bases of PM resistance were also studied in several of these *Vitis* species, including *V. cinerea* and *V. rupestris* (Dalbó et al. 2001), and the complex hybrids of *V. aestivalis*, *V. berlandieri*, *V. cinerea*, *V. labrusca*, *V. lincecumii*, *V. riparia* and *V. rupestris* (Fischer et al. 2004). PM resistance has also been explored in grapes of the genus *Muscadinia*. The first studied resistance locus against PM was *Run1* (Donald et al. 2002), termed in accord to the former name of the fungus (Resistance to *Uncinula necator* 1). *Run1* was introgressed from *M. rotundifolia* by conventional breeding and was localised on linkage group (LG) 12 (Barker et al. 2005). Genes in this locus are thought to enable epidermal cells to go through rapid death at the penetration site (Pauquet et al. 2001). A major quantitative trait locus (QTL) for resistance was found on LG 15 in the *Vitis* hybrids 'Regent', 'Villard Blanc' and Gf.Ga-47-42 (Akkurt et al. 2006; Welter et al. 2007), but in these hybrids the physiological mechanism of resistance has yet to be determined. More recently, wild *Vitis* species from the Far East, mainly China, have attracted increasing attention for resistance breeding. Some accessions within the species *V. amurensis*, *V. romanetii*, *V. piazezkii*, *V. davidii*, *V. liubanensis* and *V. bashanica* were reported to be PM resistant (Wan et al. 2007). The type of resistance against a non-native pathogen found in these species, with a substantial variation in resistance and susceptibility among the genotypes of each species, stimulates further investigations.

Vitis vinifera and other *Vitis* species are interfertile. In traditional breeding, cultivated grapevines and various *Vitis* species have been hybridised and then backcrossed to *V. vinifera* in order to introgress resistance traits into fruiting genotypes. A few fertile offspring have also been obtained from crosses between *V. vinifera* and *M. rotundifolia*, despite the fact that hybridisation is normally hampered by the different chromosome number in the *Vitis* and *Muscadinia* genomes (Bouquet 1986). In either approaches to resistance breeding, however, the consequences of linkage drag of undesired traits could not be completely eliminated, even after successive cycles of backcrossing. Furthermore, the high level of heterozygosity of the grape genome makes it impossible to restore many of the original features of the *V. vinifera* parents, even when deleterious alleles are not inherited from the non-domesticated parent.

All grapes of Mediterranean and Near East origin, belonging to the species *V. vinifera*, were presumed to be susceptible to American native pathogens. This assumption was based on widespread observations of many varieties at many locations. Since the pathogen is inherently able to grow on this species, it has been argued that there is little probability of finding PM resistance in *V. vinifera* due to the relatively short time span that has been available for co-evolution between the host and the newly introduced pathogen, as well as due to the effect of clonal propagation of the crop. However, core genetic resources that contributed to modern *V. vinifera* cultivars are still dispersed through Armenia, Iran, regions around the Black Sea, and the Republics of Central Asia (This et al. 2006). Part of this germplasm, sexually propagating outside of cultivation, may have evolved after the pathogen arrival more than the Western Europe cultivars. Resistance specificities towards non-native pathogens might be developing under natural conditions in *V. vinifera* and in Asian species. Because of historical and geographical isolation, most of the *V. vinifera* germplasm maintained in the Near East had been out of the reach of European and New World grape breeders. These resources were studied and utilised only by local breeders. Filippenko and Stin (1977) identified an accession of *V. vinifera*, named 'Dzhandzhal kara', that is resistant to PM, and used this material in a breeding program in Michurinsk, Russia. PM inoculation studies conducted during the 1960s led to the identification of nine resistant cultivars out of 392 *V. vinifera* accessions originally from Armenia, Moldova, Russia, Georgia and Uzbekistan (reviewed in Vojtovic 1987). One of these accessions, 'Kishmish vatkana', was found to be free of PM infections under heavy disease pressure in the germplasm collection of the Uzbek Research Institute for Horticulture, Viticulture and Enology, Tashkent, Uzbekistan. In 1992, 'Kishmish vatkana' was imported to Pécs, Hungary, and in 2005, to Mountain Grove, Missouri, USA. This cultivar, grown under field and greenhouse conditions in Hungary and under greenhouse conditions in Missouri, was confirmed to be resistant to PM strains that naturally occurred at those locations (P. Kozma and L. Kovács, unpublished). Leaves and berries remain asymptomatic to the disease during the growing season, with only senescent leaves occasionally allowing limited

growth of PM colonies. Resistance was consistently more robust in 'Kishmish vatkana' than in 'Dzhandzhal kara'.

'Kishmish vatkana' has large, loose clusters with anthocyanin-pigmented berries containing soft remains of aborted seeds. It is currently cultivated for fresh fruit consumption in Eastern Uzbekistan. Several morphological features, including hairless shoot tip and wedge-shaped leaf blade, as well as biological traits such as hermaphroditic flowers associated with stenospermocarpy, are consistent with the taxonomical placement of 'Kishmish vatkana' within the species *V. vinifera* subsp. *sativa* proles orientalis subproles antasiatica, according to the classification of Negrul (1968). By virtue of its domesticated traits, fruiting characteristics and innate disease resistance to PM, 'Kishmish vatkana' may become an important genetic resource for resistance breeding.

In this paper, we present (1) the comparative histological characterisation of PM infection in 'Kishmish vatkana' and the susceptible host *V. vinifera* 'Nimrang'; (2) the inheritance of the phenotypic trait in 310 F1 individuals obtained from a cross of 'Nimrang' × 'Kishmish vatkana'; (3) the chromosomal localization of the resistance locus; and (4) the comparison of the genetic control and type of response in 'Kishmish vatkana' with other sources of PM resistance in grape. We termed the PM resistance locus in 'Kishmish vatkana' Resistance to *Erysiphe necator* 1 (*REN1*), based on the original scientific name of the fungus (Saccardo 1882).

Materials and methods

Plant material

Vitis vinifera 'Kishmish vatkana', the PM resistant cultivar studied in this paper, was obtained in the form of dormant cuttings from the Uzbek Research Institute for Horticulture, Viticulture and Enology, Tashkent, Uzbekistan. *Vitis vinifera* 'Nimrang' is a table grape susceptible to PM, which is cultivated in the same geographic region as 'Kishmish vatkana'. 'Nimrang' was used as a negative (susceptible) control plant for comparative inoculation studies. The seedling '04-10/325' is a PM resistant offspring of 'Nimrang' and 'Kishmish vatkana'. The seedling '01-1/867' is a PM resistant genotype selected from a cross between the hybrid 'VRH3082-1-42' (*M. rotundifolia* × *V. vinifera* backcrossed to *V. vinifera* cultivars for four generations, Pauquet et al. 2001) and the hybrid 'Petra' (*Vitis amurensis* × *V. vinifera* backcrossed to *V. vinifera* cultivars for two generations). Since 'Petra' is as susceptible to PM as most *V. vinifera* cultivars, '01-1/867' was expected to have inherited PM resistance from the ancestor *M. rotundifolia* 'G52'. Because of its resistance to PM, '01-1/867' was chosen as a positive (resistant) control plant for inoculation studies. Biological replicates of 'Kishmish vatkana', 'Nimrang' and the seedlings '04-10/325' and '01-1/867', were obtained by vegetative propagation as own-rooted cuttings for inoculation and histological tests. A cross between 'Nimrang' as seed parent (bearing functionally female flowers) and 'Kishmish vatkana' as pollen parent was performed in 2004. Of the resulting F_1 progeny, 310 seedlings, designated 04-10, were grown for inoculation tests in potting soil in a greenhouse during the seasons of 2005 and 2006. Subsequently, own-rooted vines of the progeny were planted and maintained at the Experimental Station of the Research Institute of Viticulture and Enology, Pécs, Hungary. Genomic DNA was extracted from young leaves as described by Doyle and Doyle (1990).

Artificial inoculation and microscopic observations

An *E. necator* isolate was collected from a vineyard in southern Missouri, and maintained on *V. vinifera* 'Cabernet Sauvignon' leaves (source leaves) cultured on agar plates. The pathogen was sub-cultured onto fresh leaves every ten days. The test plants of 'Kishmish vatkana', 'Nimrang' and the resistant offspring '04-10/325' and '01-1/867', were grown under greenhouse conditions during spring and summer, and were inoculated by touching the adaxial epidermis of the third and fourth fully expanded leaves beneath the apex with sporulating colonies on the surface of source leaves. Plants were then incubated in the greenhouse, with temperatures ranging from 22 to 27°C, with relative humidity ranging from 72 to 96% and without supplemental lighting. Inoculations were repeated three times. Inoculated leaves were collected at 24, 48, 72 and 120 h post-inoculation (hpi), and fixed in 3:1 ethanol:glacial acetic acid until chlorophyll was removed. Leaf segments were incubated in water for 1–2 h and transferred to a mix of lactic acid:glycerol:water (1:1:1, v/v/v) for at least 12 h. Subsequently, leaf segments were incubated for 5 min with 0.1% (w/v) aniline blue in lacto-glycerol to stain fungal structures. Samples were then examined using an Olympus BX41 microscope using visible light. The frequency of host cell entry was quantified by counting the number of primary appressoria that developed a hypha out of 100 primary appressoria observed on each of the inoculated leaves. The level by which a plant supported completion of fungal life cycle was quantified by counting the number of conidiophores per mm^2 of leaf blade. Plant reaction was quantitatively expressed as the number of epidermal cells associated with a primary appressorium that underwent browning, out of the total number of epidermal cells associated with a primary appressorium. Histological assays were repeated three times for each of the three observations. Data were subjected to analysis of variance; mean separation was done

using Fisher's least significant difference multiple comparison test ($P \leq 0.05$).

Progeny phenotyping

Progeny plants were cultivated under greenhouse conditions during the growing seasons of 2005 and 2006. PM inoculum was provided from a mixture of sensitive vines exposed to natural strains of *E. necator* from southern Hungary (i.e. *V. vinifera* 'Cardinal', 'Sugraone', 'Queen of Vineyards', 'Olympia' and 'Narancsízu'), which were trellised to create a uniform canopy over the progeny plants. The growth of PM on the source vines initiated in the spring of 2005 from spores overwintered in the greenhouse. Once infections spread uniformly across the source vines, test plants were transferred into the greenhouse. Progeny plants began showing PM symptoms on the adaxial leaf epidermis when shoots reached the five to seven-leaf stage. From this stage, test plants were scored three times at three-week intervals in both 2005 and 2006. Phenotypic scores were assigned according to the OIV 455 scale established by the Organisation Internationale de la Vigne et du Vin, (IPGRI, UPOV, OIV 1997). Genotypes with no visible conidiophores and no PM symptoms (OIV class 9) were assigned to the resistant category, whereas genotypes with masses of conidiophores detectable with the unaided eye (other OIV classes) were collapsed in the susceptible category. This phenotypic scoring is consistent with that used for the characterisation of *M. rotundifolia*-derived PM resistance by Pauquet et al. (2001).

Bulked segregant analysis

Two DNA bulks were prepared by grouping DNA extracted from resistant and susceptible genotypes of the 04-10 cross population, respectively. Each DNA bulk was normalised as follows. The amount of DNA from each seedling was determined using a spectrophotometer and by quantitative PCR amplification of the single-copy gene *UFGT* (Goto-Yamamoto et al. 2002). Quantitative PCR was carried out as reported in Castellarin et al. (2006), except that genomic DNA was used as a template. From each of the two phenotypic classes, fifteen DNA samples were selected for bulking based on their overlapping threshold cycles and comparable fluorescence intensity after 25–27 PCR cycles.

A set of 291 microsatellite markers scattered over the entire genome was selected from the consensus map of Doligez et al. (2006) and integrated with additional markers present in the consensus map of Di Gaspero et al. (2007). Each additional marker was projected onto the map of Doligez et al. (2006) in the interval between the surrounding two markers shared by the two maps, in a position at the center of the interval. Details of markers and map positions are given in Supplementary Material S1.

PCR amplification of all SSR markers in the two parents and in the two DNA bulks, as well as capillary electrophoresis and allele calling, were carried out as described by Di Gaspero et al. (2007). The contribution of each allele inherited from 'Kishmish vatkana' to the resistant and the susceptible DNA bulks was estimated using the area below the allele peak. If *A* and *a* are the alleles of a given heterozygous SSR marker in 'Kishmish vatkana', the ratio $Area_A/Area_a$ in the resistant DNA bulk was compared to the ratio $Area_A/Area_a$ in the susceptible DNA bulk. For multilocus markers with *n* peaks segregating from 'Kishmish vatkana', the ratio $Area_A/Area_a$ was calculated between all $n(n - 1)/2$ combinations of peaks.

Local linkage map around the *REN1* locus

Bulked segregant analysis (BSA) indicated a number of SSR loci on LG 13 with alleles that were in linkage disequilibrium with the resistance trait. SSR loci of that LG were analysed together with the phenotypic scores in the whole population of 310 F_1 individuals.

PCR amplification and capillary electrophoresis were carried out as described above. A local linkage map of 'Kishmish vatkana' LG 13 was constructed using CarthaGene 0.999R (de Givry et al. 2005). Linkage was determined at a LOD of 9.0 and a maximum distance threshold of 30 cM. Marker order was determined using the 'build 5' command, optimised by using algorithm 'taboo' (command 'greedy 3 1 1 15') and locally refined by testing all possible marker orders within a sliding window of five markers (command 'flips 5 2 1'). The position of the *REN1* locus was confirmed by constructing a framework map with marker order supported by LOD values increasing from 3.0 to 8.0. Map distances were calculated using the Kosambi function. Recombinant plants between the most tightly linked markers and the *REN1* locus were phenotypically confirmed in the 2007 season using the same procedure as described above.

Results

Host-fungus interaction in 'Kishmish vatkana'

Histological observations revealed that conidial germination, germ tube formation, and development of the appressorium occurred in a similar manner on leaves of 'Kishmish vatkana' and the susceptible grapevine 'Nimrang' (Fig. 1). The rate of successful cell entry by *E. necator* on the leaf epidermis was comparable in the two genotypes. The formation of a primary hypha was an indication of successful cell penetration and the establishment of a compatible

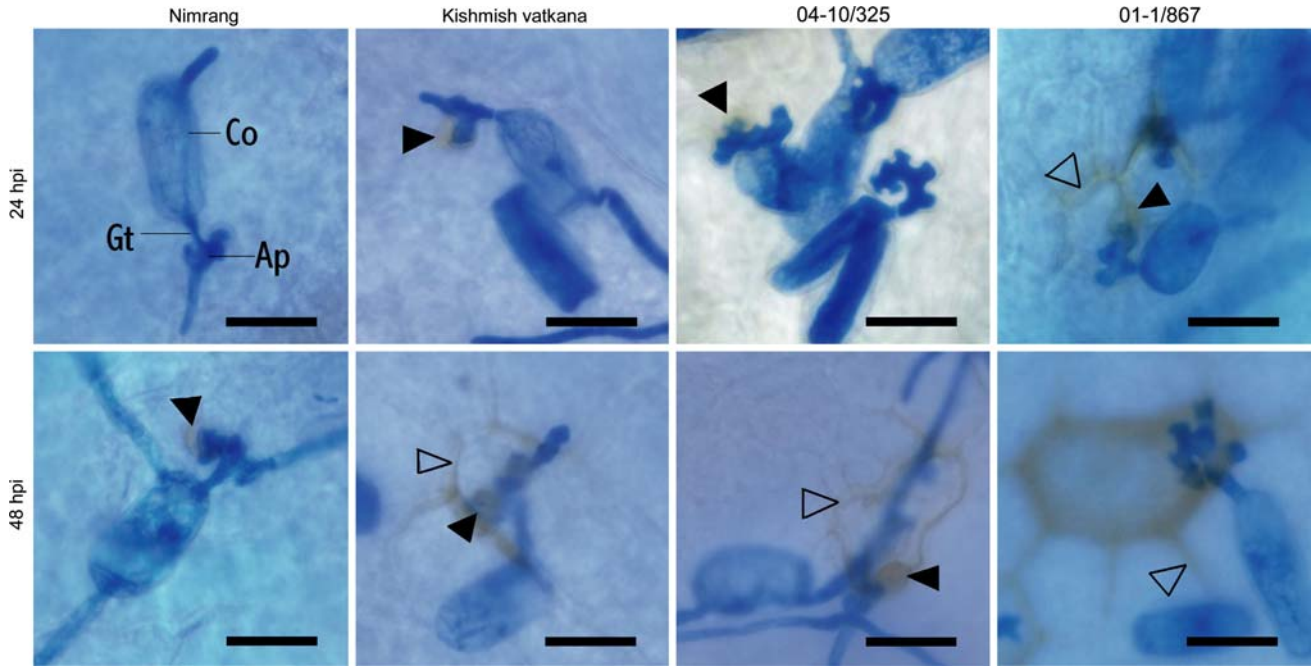

Fig. 1 Interaction between *E. necator* and epidermal cells on the adaxial side of leaves in 'Kishmish vatkana', a susceptible plant (Nimrang), a 'Kishmish vatkana' offspring (4-10/325), and a *M. rotundifolia*-derived plant (01-1/867) within the first 48 hpi. *Filled triangles* mark epidermal cell browning beneath the appressorium, *open triangles* mark epidermal cell browning along cell walls. The length of the scale bars is 20 µm. *Co* conidium; *Gt* germ tube; *Ap* appressorium

interaction. The percentage of appressoria which gave rise to primary hyphae was not significantly different ($P \leq 0.05$) in 'Kishmish vatkana' and 'Nimrang' at 24, 48 and 72 hpi (Fig. 2). The offspring '04-10/325', which showed a macroscopic phenotype similar to that in the resistant parent 'Kishmish vatkana', also supported the development of primary hyphae at a comparable rate (Figs. 1, 2). On leaves of 'Kishmish vatkana', 'Nimrang' and '04-10/325', the

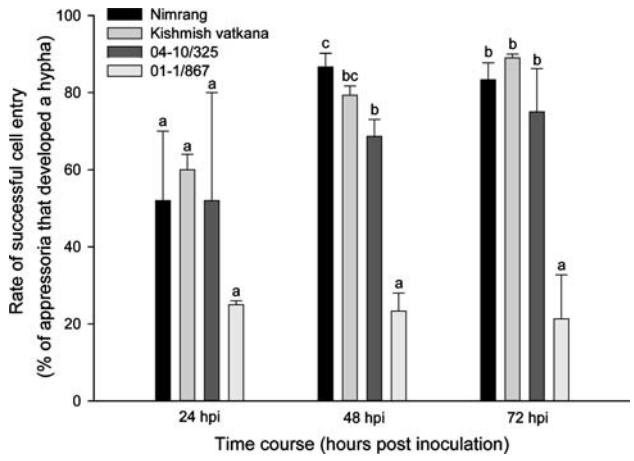

Fig. 2 Rate of successful entry of host epidermal cells by *E. necator* in 'Kishmish vatkana', a susceptible plant (Nimrang), a 'Kishmish vatkana' offspring (04-10/325), and a *M. rotundifolia*-derived plant (01-1/867). The rate of successful entry was estimated as the percentage of primary appressoria that developed a primary hypha. Bars not marked by the same letter indicate values that differ significantly ($P \leq 0.05$)

percentage of primary appressoria that supported hyphal growth ranged from 52 to 60% at 24 hpi, from 69 to 87% at 48 hpi and from 75 to 89% at 72 hpi. By contrast, in the genotype '01-1/867', which was expected to have inherited PM resistance from *M. rotundifolia*, the rate of successful formation of primary hyphae was 25, 23 and 21% of the total number appressoria at 24, 48 and 72 hpi, respectively. Thus, host cell entry was significantly lower ($P \leq 0.05$) in '01-1/867' than in any of the other genotypes, including 'Kishmish vatkana', at 48 and 72 hpi (Fig. 2).

Hyphal branching was not prevented in 'Kishmish vatkana', but hyphal growth was restricted relative to 'Nimrang'. As a result, only sparse colonies were observed in 'Kishmish vatkana' at 120 hpi compared to the profuse fungal growth present in 'Nimrang' (Fig. 3a). The number of conidiophores per mm^2 of leaf surface was determined at 120 hpi and served as an estimation of the fungal invasion (Fig. 3b). *Erysiphe necator* produced a significantly lower number of conidiophores in 'Kishmish vatkana' (on average 33.6 ± 8.7 mm^{-2}) than in 'Nimrang' (on average 310.5 ± 24.0 mm^{-2}). Conidiophore density was not significantly different ($P \leq 0.05$) among the resistant genotypes, that is 'Kishmish vatkana', its resistant offspring '04-10/325', and the *M. rotundifolia*-derived genotype '01-1/867', even though PM formed the fewest conidiophores in absolute terms on '01-1/867' (on average 1.7 ± 0.6 conidiophores mm^{-2}).

Epidermal cells contacted by the appressoria displayed various levels of browning when examined with a light

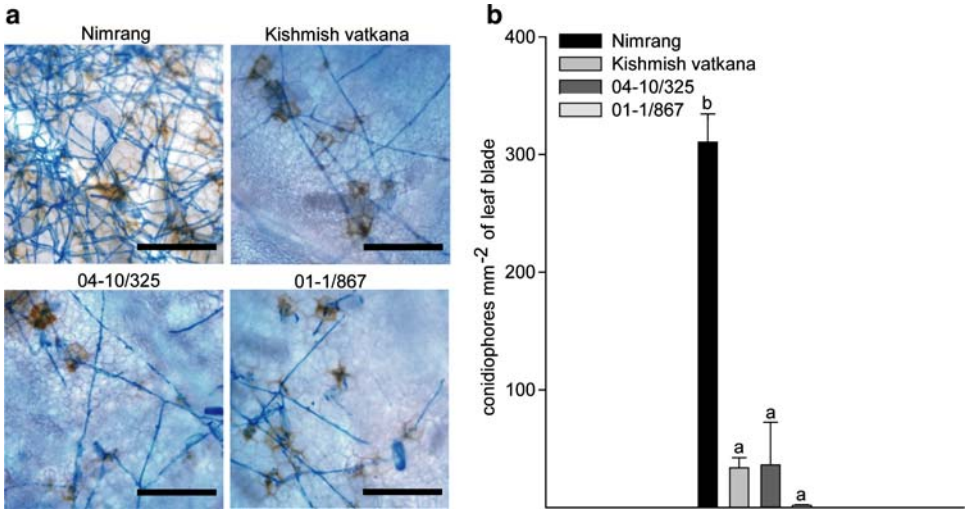

Fig. 3 Hyphal development (**a**) and conidiophore density (**b**) of *E. necator* at 120 hpi in 'Kishmish vatkana', a susceptible plant (Nimrang), a 'Kishmish vatkana' offspring (04-10/325), and a *M. rotundifolia*-derived plant (01-1/867). The length of the scale bars is 100 µm. *Bars* not marked by the same letter indicate values that differ significantly ($P \leq 0.05$)

microscope (Fig. 1). Based on the histological location, two types of browning could be differentiated. The first type was restricted to a well-defined circular area beneath the appressorium (Fig. 1; solid triangles). This type of browning occurred invariably, and without any discernible difference in terms of intensity, frequency, or timing of appearance among resistant and susceptible grapevines. The second type of browning initially developed along the cell walls (Fig. 1; open triangles), gradually spread to the entire epidermal cell, and in some instances, to neighbouring cells as well. The frequency of this second type of browning was assayed at 24, 48 and 72 hpi (Fig. 4). Diffused browning along the walls of attacked cells was not different between 'Kishmish vatkana' and 'Nimrang' at any of the three time points, but it occurred more frequently in the *M. rotundifolia*-derived genotype '01-1/867' than in the other genotypes at 24 and 48 hpi ($P \leq 0.05$). In '01-1/867', most attacked epidermal cells developed a brown discoloration along the wall as early as 24 hpi (Figs. 1, 4), and the affected cell turned entirely brown by 48 hpi. Furthermore, browning often spread to the cell wall of neighbouring cells (Fig. 1). In 'Kishmish vatkana', '04-10/325' and 'Nimrang', browning was in most cases less pronounced and intense within the first 48 hpi than in '01-1/867' (Fig. 1).

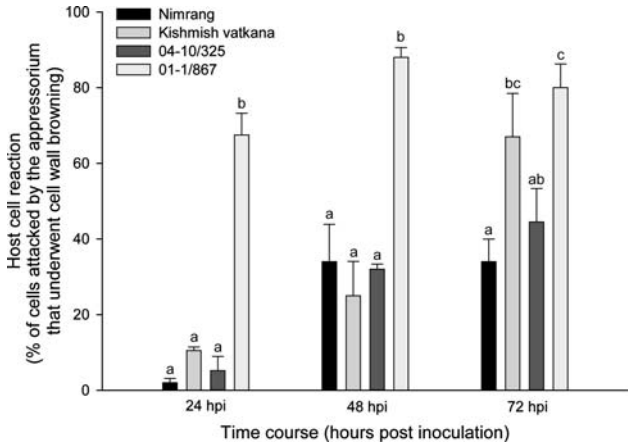

Fig. 4 Plant cell reaction to penetration attempt by PM at the pathogen entry site in 'Kishmish vatkana', a susceptible plant (Nimrang), a 'Kishmish vatkana' offspring (04-10/325), and a *M. rotundifolia*-derived plant (01-1/867). Among the epidermal cells attacked by an appressorium, the percentage of cells that developed cell wall browning was scored. *Bars* not marked by the same letter indicate values that differ significantly ($P \leq 0.05$)

Genetic determinants of PM resistance in 'Kishmish vatkana'

The inheritance of PM resistance was studied in 310 offspring of the F1 population 'Nimrang' × 'Kishmish vatkana' by observing the presence or absence of visible hyphae and conidiophores. Of these, 150 F1 individuals were symptomatic and showed masses of conidiophores detectable to the naked eye; and 160 F1 individuals were asymptomatic. This classification was based on observations in two consecutive years, repeated three times each year. This segregation ratio (1:1.07, $\chi^2 = 0.32$) fits a monogenic control of the trait, with 'Kishmish vatkana' heterozygous at that locus and carrying a dominant allele for PM resistance. Under greenhouse conditions, formation of sparse PM colonies occurred on senescing basal leaves of 58% individuals previously scored as resistant. The type of plant reaction displayed on the adaxial side of the leaf blade by resistant and susceptible parents, and the two categories of offspring are summarised in Table 1. While some additional features visible to the unaided eye (e.g. senescence-like chlorosis and small necrotic lesions) showed a tendency to occur only in the progeny that did not support visible PM growth, they were not consistent among different leaves within the same plant and could not be used for segregation analysis.

Table 1 Symptoms of powdery mildew. Macroscopic phenotypes of the adaxial side of leaves were scored in the parents and 310 offspring of the cross 'Nimrang' × 'Kishmish vatkana' under greenhouse conditions during two consecutive growing seasons

	Host reactions	Visible conidiophores
Kishmish vatkana	Nothing visible, senescence-like chlorosis	NO[a]
Nimrang	Nothing visible	YES
Resistant progeny	Nothing visible, necrotic fine dotted lines, senescence-like chlorosis	NO[a]
Susceptible progeny	Nothing visible, brown spots	YES

[a] Limited sporulation occurred only on senescent basal leaves

Bulked segregant analysis

Out of 291 SSR markers tested, 195 were heterozygous in the resistant parent and hence informative for the BSA. The distribution of the informative markers across the genome is shown in Fig. 5. An average of 10.3 informative markers per chromosome were analysed with an average distance of 8.2 cM between adjacent markers. A region spanning 49 cM on one chromosome arm of LG 7 could not be covered by any heterozygous marker. A gap of 20–30 cM remained in each of LG 6, 8, 10, 11, 13, 15 and 18; a gap of 35 cM in LG 18; and two gaps of 27 and 30 cM, respectively, in LG 16.

Only one group of markers, all belonging to LG 13, displayed an unbalanced dosage in the resistant and the susceptible bulks for the alleles inherited from the resistant parent. If *A* and *a* are the alleles inherited from 'Kishmish vatkana' and Area is the fluorescence intensity of the allelic peak measured as the area below the peak, a significant difference between the ratio $Area_A/Area_a$ in the resistant bulk and the ratio $Area_A/Area_a$ in the susceptible bulk was found for seven markers VMC2C7, UDV-020, VMC9H4-2, VMCNG4E10-1, VMC3B12, VMC3D12, and UDV-038 in decreasing order of magnitude (Fig. 6). All of these markers were linked to each other and identified a single chromosomal region of 47.6 cM, based on marker distances reported by Doligez et al. (2006), which is in accord with the monogenic control of PM resistance. The difference of $Area_A/Area_a$ ratio between the resistant bulk and the susceptible bulk peaked up to 32 in this interval and was lower than 5 (mean 0.85 ± 1.02 standard deviation) for markers of all linkage groups other than LG 13 (Fig. 6).

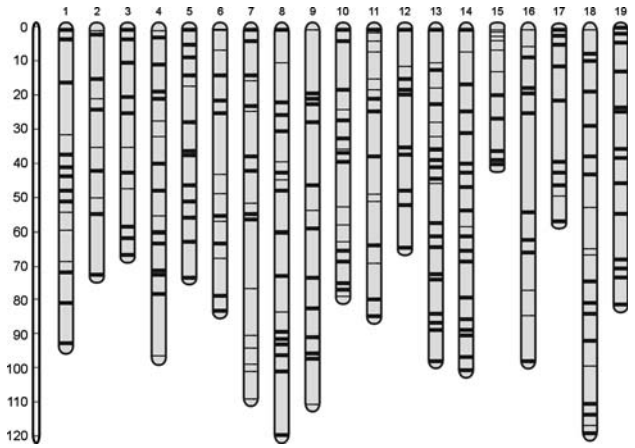

Fig. 5 Distribution of 291 SSR markers used for BSA across the 19 grapevine chromosomes. Markers heterozygous in the resistant parent 'Kishmish vatkana', and hence informative for BSA, are in *thick* lines, homozygous markers are in *thin* lines. Map distances are scaled to the white reference bar on the left and expressed in cM Kosambi. Marker orders and distances were drawn according to the consensus maps of Doligez et al. (2006) and Di Gaspero et al. (2007) as described in the text

Genetic map around the *REN1* locus

A local map of the LG 13 has been constructed, with twelve SSR loci that span the 46.6-cM interval where the PM resistance locus was identified by BSA (Fig. 7). The *REN1* locus was robustly placed in this LG at a LOD of 9.0. Four co-segregating loci, two amplified by the markers VMC9H4-2 and VMCNG4E10-1, respectively, and

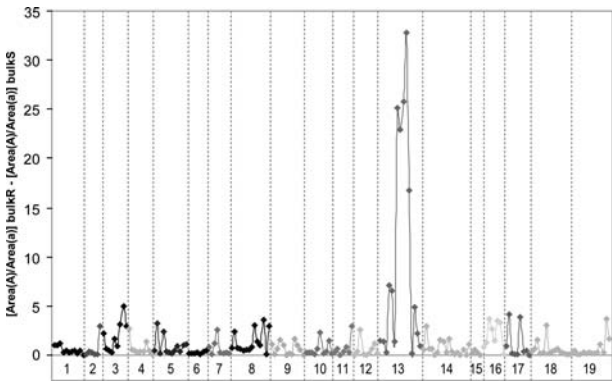

Fig. 6 Bulked segregant analysis using 195 SSR markers dispersed on the 19 grapevine chromosomes. Linkage groups are reported on the *x*-axis, and markers within each linkage group are represented by dots and ordered from left to right according to the consensus maps of Doligez et al. (2006) and Di Gaspero et al. (2007). For each marker, the differential contribution to the resistant DNA bulk and the susceptible DNA bulk of each allele inherited from 'Kishmish vatkana' were analysed using the area below the allele peak. If *A* and *a* are the alleles of a given SSR marker in 'Kishmish vatkana', the ratio $Area_A/Area_a$ in the resistant DNA bulk was compared to the ratio $Area_A/Area_a$ in the susceptible DNA bulk, and expressed as the absolute value of the difference $[Area_A/Area_a]_{bulkR} - [Area_A/Area_a]_{bulkS}$ in the *y*-axis. For multilocus markers with *n* peaks segregating from 'Kishmish vatkana', the difference $[Area_A/Area_a]_{bulkR} - [Area_A/Area_a]_{bulkS}$ was calculated between all $n(n-1)/2$ combinations of peaks and the highest score was reported in this graph

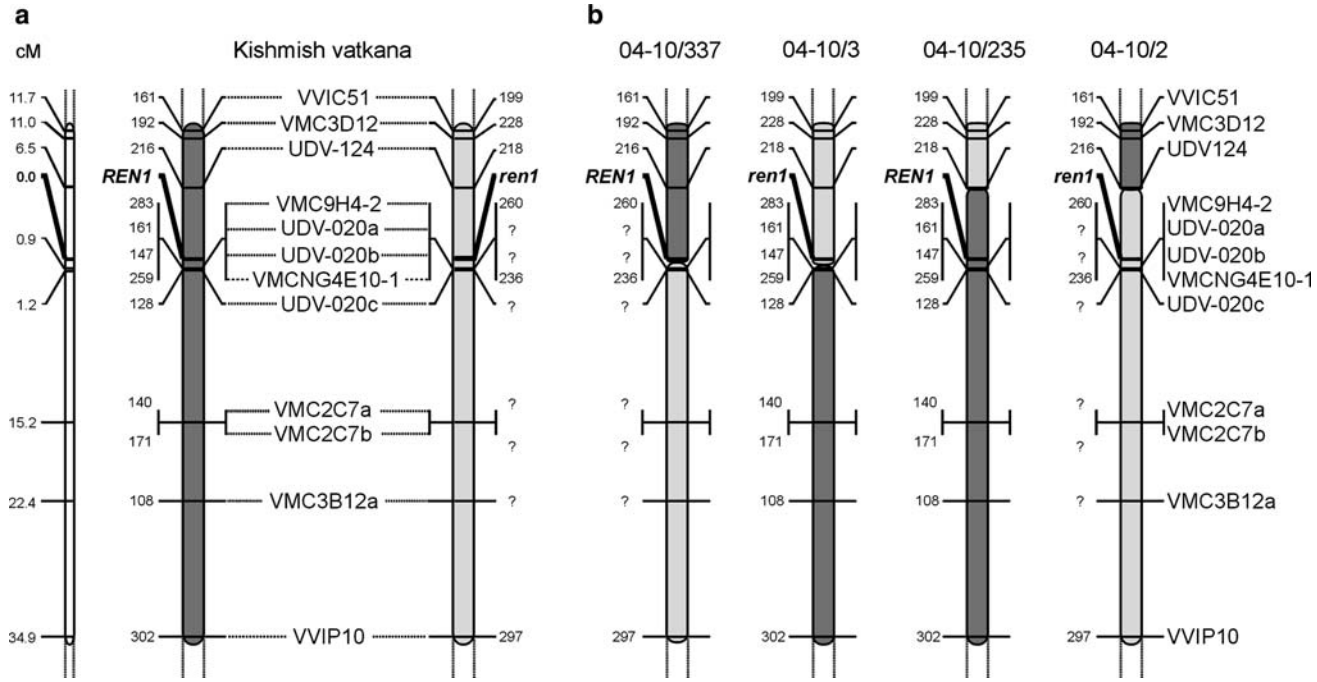

Fig. 7 Local map around the *REN1* locus in a 46.6-cM region on LG 13 in 'Kishmish vatkana' (**a**) and in four F1 individuals (**b**). **a** The homolog carrying the resistance allele *REN1* is shown in *dark grey*; the homolog carrying the susceptibility allele *ren1* is shown in *light grey*; SSR markers are reported in the middle of the two homologs; allele length of each marker on each homolog is reported external to the corresponding homolog, and is expressed in base pairs; multi-locus markers (UDV-020 and VMC2C7) were suffixed by letter *a*, *b*, or *c*. A question mark was used when the allelic peak in the susceptible homolog could not be identified ('null' or not-amplified alleles). Marker distances are reported on the reference bar on the left as cumulative distance from the *REN1* locus on each side of the locus, and are expressed in cM Kosambi. **b** Crossover points in chromosome 13 inherited from 'Kishmish vatkana' of two resistant F1 individuals ('04-10/337' and '04-10/235') and two susceptible F1 individuals ('04-10/3' and '04-10/2') that showed recombination between the *REN1* locus and the most tightly linked markers

two co-amplified by the multilocus marker UDV-020, were placed 0.9 cM away from the *REN1* locus. The most likely position of *REN1* was identified in the 7.4-cM interval shown in Fig. 7. We also built a framework map with marker order supported by increasing LOD values starting from 3.0. The position of *REN1* remained confined in an 8.6-cM interval between UDV-124 and UDV-020c (allele 128 bp) with a LOD of 7.0. Three point analysis was performed with MapMaker (Lander et al. 1987) and showed that log-likelihood differences among all possible orders between *REN1* and markers VMC9H4-2, VMCNG4E10-1, UDV-020a, UDV-020b, and UDV-020c were comprised between 0.0 and -0.05. The haplotypes, in coupling with the *REN1* or *ren1* alleles on the two homologous chromosomal regions in 'Kishmish vatkana', are reported in Fig. 7. Out of the 310 progeny plants, three individuals were found in which a recombination event took place between the *REN1* locus and the three most tightly linked markers VMC9H4-2, VMCNG4E10-1, and UDV-020. Each recombinant inherited the chromosome 13 from 'Kishmish vatkana' with a one-point crossover between the two homologs in the 46.6-cM region surrounding the *REN1* locus.

The whole chromosomal region of 'Kishmish vatkana', flanked by the markers VVIC51 and VVIP10, showed a recombination rate comparable to other grapevines such as *V. vinifera* 'Cabernet Sauvignon', 'Chardonnay', 'Bianca' (Di Gaspero et al. 2007), and 'Grenache' (Adam-Blondon et al. 2004), in that the genetic interval between the same markers ranged from 41.5 to 46.6 cM. By contrast, recombination was apparently suppressed in the regions surrounding the *REN1* locus in 'Kishmish vatkana'. The interval flanked by marker UDV-124 on one side of the *REN1* locus, and by the co-segregating markers VMC9H4-2 and VMCNG4E10-1 on the other side, was 7.4 cM in 'Kishmish vatkana' based on 310 available meioses. The equivalent interval was larger in 'Bianca' and 'Chardonnay', and corresponded to 15.4 and 20.2 cM, respectively, even when estimated on the basis of a lower number of meioses (Di Gaspero et al. 2007). In particular, markers VMC9H4-2 and VMCNG4E10-1 which did not recombine in 310 meioses from 'Kishmish vatkana', and were placed 0.9 cM away from the *REN1* locus, did recombine in other grape genotypes, as witnessed by their genetic distance of 3.2 cM (Di Gaspero et al. 2007) and 3.4 cM (Doligez et al. 2006) in integrated maps of four and nine grapevines, respectively.

Discussion

We identified partial resistance to PM in the Central Asian grape 'Kishmish vatkana'. This resistance manifests itself in the absence of visible PM colonies in plants, which are inoculated artificially using conidia, or naturally by exposure to sporulating PM colonies. Based on microscopic observations, *E. necator* was able to enter the epidermal cells of 'Kishmish vatkana' and draw nutrients from the host to sustain its initial growth. Hence, *E. necator* is an adapted pathogen on this grape genotype. However, hyphal proliferation and conidiophore density were significantly lower than in a susceptible control, which was symptomatic to PM to the unaided eye.

This phenotypic trait segregated in the progeny of a cross of 'Kishmish vatkana' and a symptomatic grapevine. Presence or absence of visible PM colonies were inherited as a simple Mendelian trait and fit the model of a monogenic segregation. This phenotypic trait was accounted for by a single dominant locus, named *REN1*, which was assigned to LG 13 by BSA. BSA was originally developed for dominant markers, based upon scores of presence/absence of bands in DNA bulks of contrasting phenotypes. BSA also applies to co-dominant markers if a measurement of the dosage of each allele in the bulks is quantitatively reliable for detecting linkage disequilibrium (Fernandez et al. 2006). To maximise the sensitivity of BSA, we have normalized the individual DNA templates using quantitative Real-Time PCR before bulking the pools. The relative contribution of parental alleles to the bulks was then estimated by means of capillary electrophoresis, and quantification of the fluorescence intensity (represented by the area below the fluorescence peak) associated with each allele. The calculation of the parameter $[\mathrm{Area}_A/\mathrm{Area}_a]_{\mathrm{bulkR}} - [\mathrm{Area}_A/\mathrm{Area}_a]_{\mathrm{bulkS}}$, where *A* and *a* are the alleles of a heterozygous marker in the resistant parent and where bulkR and bulkS are the two pooled DNAs of phenotypically contrasting seedlings, enabled us to detect linkage for markers located within a window of at least 47.6 cM surrounding the target locus. This extent of linkage is in agreement with the limit of detection of approximately 25 cM on either side of the target locus, as estimated for AFLP and RAPD dominant markers (Michelmore et al. 1991).

The *REN1* locus was identified in a 7.4-cM interval on LG 13 by the combined analysis of phenotypes and genotypes of an F_1 progeny of 310 seedlings. The *REN1* locus is located on a chromosome of 'Kishmish vatkana' (LG 13) that is different from the chromosome that carries *Run1* in *M. rotundifolia* (LG 12), and different from the chromosome of some *Vitis* interspecific hybrids (LG 15) in which a major QTL for PM resistance was found. In spite of the difference in the genomic location of these loci, it was interesting to observe that the regions around all of these loci share the common feature of being home to several nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes. NBS-LRR members were mapped in the 10-cM surroundings of the *REN1*-linked markers VMCNG4E10-1, VMC9H4-2, and UDV-020 (Di Gaspero et al. 2007). This observation is particularly intriguing in light of the facts that candidates for the *M. rotundifolia RUN*1 gene on LG 12 were four NBS-LRR genes (Barker et al. 2005), and another group of NBS-LRR genes on LG 15 are located in the QTL interval for PM resistance in *Vitis* hybrids (Akkurt et al. 2006; Di Gaspero et al. 2007; Welter et al. 2007).

To gain insight into the physiological basis of PM resistance in 'Kishmish vatkana', histological observations were made during the early stages of the host-pathogen interaction. The establishment of a compatible interaction between *E. necator* and 'Kishmish vatkana' was not prevented. The onset of the pathogenic process was similar to that in PM symptomatic vines within the first 72 h after conidial germination. *Erysiphe necator* was able to traverse the cuticle and the epidermal cell wall to gain access to the host cell membrane of 'Kishmish vatkana'. Conidia of *E. necator* contain sufficient resources for the formation of the germ tube and the appressorium, but not for the subsequent hyphal growth. The formation and elongation of a primary hypha is a sign that the fungus was able to successfully penetrate the host cell and draw resources from it. Based on the observed frequency of primary appressoria that developed an elongating hypha, 'Kishmish vatkana' supported cell entry at the same rate as the susceptible vine 'Nimrang'. Resistance to the pathogen in 'Kishmish vatkana' has resulted into the restriction of hyphal development and the decrease of conidiophore production which are statistically significant compared to symptomatic controls at around 72–120 h after fungal entry.

Epidermal cells of 'Kishmish vatkana' contacted by appressoria developed a brown circular area beneath the appressorium at around 24 hpi. A second type of browning was also observed which initially appeared along the wall of epidermal cells attacked by appressoria, and then proceeded to engulf the entire cell. In some instances, the browning spread from a penetrated cell to neighbouring cells along the walls. Both types of cell discoloration also occurred to some extent in susceptible genotype 'Nimrang', although this phenomenon was detectable especially at the late time point of 120 hpi.

Cell wall browning occurred rapidly in 'Kishmish vatkana' and in the resistant *M. rotundifolia*-derived genotype '01-1/867', but it was detected more frequently ($P \leq 0.05$) in the latter at the early time points of 24 and 48 hpi. In 'Kishmish vatkana', cell wall browning was also less pronounced than that in '01-1/867' at the same time points. These observations are in favour of the hypothesis that defence in 'Kishmish vatkana' may recruit mechanisms

similar to those operating in '01-1/867', although the response is faster and more intensely activated in '01-1/867' than in 'Kishmish vatkana'.

It is tempting to speculate that this rapid and intense cell browning is the result of a hypersensitive response, likely acting through a programmed cell death (PCD). There are several observations, however, that call this into question. First, we repeatedly failed to stain PM-affected epidermal cells in 'Kishmish vatkana' and '01-1/867' with trypan blue, a dye which is absorbed only by dead cells (data not shown). However, we cannot rule out the possibility that morphological features specific to grapevine prevented the entry of trypan blue into dead cells. Second, hypersensitive PCD is highly effective at trapping biotrophs, but in this case PM was able to escape and sporulate on 'Kishmish vatkana' leaves, though to a significantly lower extent than that in symptomatic controls. Third, browning of cells contacted by the appressoria occurred also in the susceptible genotype 'Nimrang' during the late stages of PM infection as well as in another PM susceptible genotype 'Cabernet Sauvignon' (Fung et al. 2007). Therefore, we cannot exclude the possibility that the development of the brown colour might have been the consequence of such cell wall-associated biochemical processes as polyphenol oxidation, oxidative cross-linking of polymers, or deposition of callose or tannins. Another potential explanation is that the observed phenotype was the result of a delayed PCD, which was executed too late for trapping the pathogen, but in time for slowing down its growth.

Resistance to powdery mildews in other plants may result from the exclusive effect of a single defence reaction or the synergistic actions of different mechanisms for host interference with fungal growth. A series of recent studies demonstrated that there are at least two distinct lines of defense against powdery mildews: a pre-invasion, or pathogen associated molecular pattern-triggered immunity, which prevents the ingress of the pathogen or the onset of the pathogenic process; and an effector-triggered immunity, which halts further invasion if the first line of defense is circumvented by the pathogenic effectors (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). For instance, loss-of-function *mlo* mutants in barley and *Arabidopsis thaliana* resist attempts of invasion of the adapted PMs *Blumeria graminis* f. sp. *hordei* and *Golovinomyces orontii*, respectively. In both plants, both powdery mildews require targeting the plant native MLO protein for host cell entry (Büschges et al. 1997; Collins et al. 2003). Nevertheless, in single mutant lines, functional redundancy in a small family of MLO proteins led to incomplete resistance, characterised by a diminished rate of host cell entry and a reduced production of conidiophores (Consonni et al. 2006). Cell entry by *E. necator* in 'Kishmish vatkana' was not significantly hampered when compared to a symptomatic control. Hence, the mechanism of resistance in 'Kishmish vatkana' is clearly at the level of the post-invasion response. In barley, an additional line of post-invasion resistance against adapted PMs, like *Blumeria graminis* f. sp. *hordei*, is triggered by pathogen effectors in plant hosts that possess the appropriate NBS-LRR *Mla* alleles. This post-invasion response is usually characterised by an early and effective termination of pathogen growth through PCD. However, in some instances the pathogen may partially escape, as various *Mla* alleles induce resistance responses of different amplitude, negatively correlated with the timely onset of the PCD (Boyd et al. 1995). The execution of an effective hypersensitive response also requires intact signalling pathways downstream of the NBS-LRR proteins. For instance, mutations that impaired the lipase-like proteins PAD4 and SAG101, and consequently the salicylic acid-mediated signal transduction, suppressed PCD in *A. thaliana* and allowed PM to sporulate (Feys et al. 2005). Further investigations are required for understanding which mechanism is operating the restriction of PM growth observed in 'Kishmish vatkana'.

Conclusion

The resistance of 'Kishmish vatkana' to powdery mildew is of great viticultural significance. PM parasitism of grapes is an increasingly pressing problem for the grape and wine industry. The disease is currently controlled by the application of inorganic and organic fungicides. However, the antimicrobial activity of some of these chemicals is partially overcome by the occurrence of resistant strains of the pathogen (Miller and Gubler 2004). Public perception that fungicides adversely impact the environment and pose risks to human health if misused, puts further pressure on viticulturists to reduce the use of chemicals (Epstein and Bassien 2003). In the near future, the exploitation of natural resources may contribute to more sustainable viticultural practices. In this context, *REN1* resistance proved to be a second line of defence that is activated when *E. necator* has invaded the epidermal cells. The resistant phenotypes were free of substantial PM infection in multi-year greenhouse and field trials and hence exploitable for practical breeding purposes. The occurrence of a fungal resistance gene, like *REN1*, naturally embedded into a pure *V. vinifera* genetic background opens the door to new opportunities in conventional breeding. Although molecular evidence has yet to be presented, there is little doubt that the stenospermocarpic 'Kishmish vatkana' is a cultivated variety of *V. vinifera*. All known forms of stenospermocarpy in grapes are strictly peculiar to *V. vinifera* subsp. *sativa* and the centre of origin of this trait is believed to be Iran and Turkey

(Negrul 1936). Uzbekistan, the country where 'Kishmish vatkana' is currently cultivated, is within the geographic region to which stenospermocarpic grapes could have spread from Iran and the Black Sea region. Fruit quality, agronomic features, and stenospermocarpy, as well as the resistance to PM of 'Kishmish vatkana', conform with the parental ideotype for both wine and table grape breeding programs. Thus, *REN1* may be recombined into novel genetic backgrounds within one generation and without compromising the purity of the *V. vinifera* genome. This feature is of particular interest for a crop such as grapevine, for which only pure *V. vinifera* varieties are admitted for cultivation in most European countries. Progeny genotyping with SSR markers that were linked to fungal resistance genes have been successfully applied to the pyramidisation of resistance alleles from different sources, a method which appears to outperform the use of phenotypic evaluation in large-scale breeding (Eibach et al. 2007). The demonstration of the monogenic model of inheritance and the information about the *REN1*-linked SSR markers available in this paper will further facilitate marker-assisted breeding.

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