GENETIC AND MOLECULAR ANALYSIS OF MILDEW DISEASE RESISTANCE IN GRAPEVINE

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To my wife Delsi and Family

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1. General introduction

1.1 The grape: Economical importance and geographical distribution

Grapevine is the fruit crop with the worldwide largest harvested area. In 2005 approximately 7,3 millions of hectares were covered by vine, leading to a grape production of almost 70 million tons (FAO, 2007).

The grapevines are members of the *Vitaceae* family. All cultivated grapes belong to the genus *Vitis* (2n = 38). More than 70 species of *Vitis* have been described. Based on their geographical dispersion, these species can be roughly connected to three main grape gene pools: Eurasian, American and Asian. The Eurasian gene pool is represented only by the species *Vitis vinifera* L., which can be subdivided into two, alternatively three, subspecies: *sylvestris* Gmel. (wild form); *caucasica* Vav. (wild form) and *sativa/vinifera* (cultivated form). All internationally acknowledged grape cultivars employed for wine production, such as `Cabernet Sauvignon´, `Chardonnay´ and `Riesling´, are members of the *V. vinifera* spp. *vinifera* and are commonly designated as grapevine or European grapevine. Furthermore, a considerable fraction of table grape cultivars are composed of a pure *V. vinifera* background. Because of its intrinsic potential, especially for wine production, cultivars of *V. vinifera* were spread throughout the world, where the environmental conditions allow its cultivation.

Contrarily to the Eurasian gene pool, several grape wild species can be found within the American and Asian gene pools. Although these species are an essential source of agronomically relevant characters, such as resistance to biotic and abiotic stresses, the wine produced from such grapes is of inferior quality, as compared to European grapevine. The wine resulting from wild species is characterized by atypical flavors, usually unpleasant to the wine consumer. The production of wine from American or Asian grapes is even legally prohibited in the European Union.

1.2 Diseases: Powdery and downy mildew

The grapevine mildew diseases (powdery and downy mildew) are native to North America and were introduced inadvertently into Europe in about 1851 and 1875, respectively. All *V. vinifera* cultivars are more or less susceptible to these two diseases. Powdery and downy mildew are the two economically most important diseases in viticulture worldwide. Epidemics of these two obligatory biotrophic pathogens result in losses in both wine quality and yield. To protect these cultivars against the attack of these pathogens enormous amounts of fungicides -widely exceeding the amounts used in other crops- are applied in vineyards every year. To exemplify, in 1999 more than 100.000 tones of Active Ingredient of fungicides were applied in vineyards in the European Union (European Union, 2002). A great portion of this fungicide amount is used to control the mildew diseases. This chemical control is costly and energy demanding and can result in risks for the environment as well as for human and animal health. Therefore, the breeding of resistant grapevine cultivars seems to be the best alternative to prevent losses caused by these pathogens.

The grapevine powdery mildew, caused by *Erysiphe necator* [syn. *Uncinula necator* (Schw.) Burr.] (anamorph *Oidium tuckeri*) is a biotrophic fungus from the *Ascomycete* family. In temperate climate the pathogen is characterized by sexual and asexual reproductive cycles. The sexual phase occurs when environmental conditions disfavor mycelial growth. The pathogen overwinters with specific structures known as cleistothecia. In the cleistothecia ascospores are sexually generated in the ascus and released when environmental conditions are favorable (spring), initiating primary infections. In contact with grapevine tissues the ascospores germinate forming the germ tube. This enlarges at the distal extremity forming a structure known as primary appressorium. The development of an infection peg from the middle of a mature appressorium allows the pathogen to overcome the cuticle and epidermal cell wall and penetrate the intracellular space. Within the cell the infection peg enlarges to form the haustorium, a structure responsible for the nutrient uptake from the plant cells. Hence forth, an intimate contact between the pathogen and the plant cell cytoplasm is established. If the colonization of the plant cell is successful, the pathogen initiates the germination of primary hyphae, followed by the formation of secondary hyphae and so on, until the pathogen is able to develop its reproductive structures (conidiophores), which produce conidia (asexual reproduction).

The mycelium grows only on the surface of grape tissues. Mature conidia are spread initiating a secondary disease cycle. The symptoms of powdery mildew are characterized by the presence of white to grayish spots or patches of talcum-like powder, mainly visible on the upper site of grape leaf blades. In a later stage, infected leaves may become yellow/brown and distorted, falling prematurely. Young infected berries have disturbed growth and may burst.

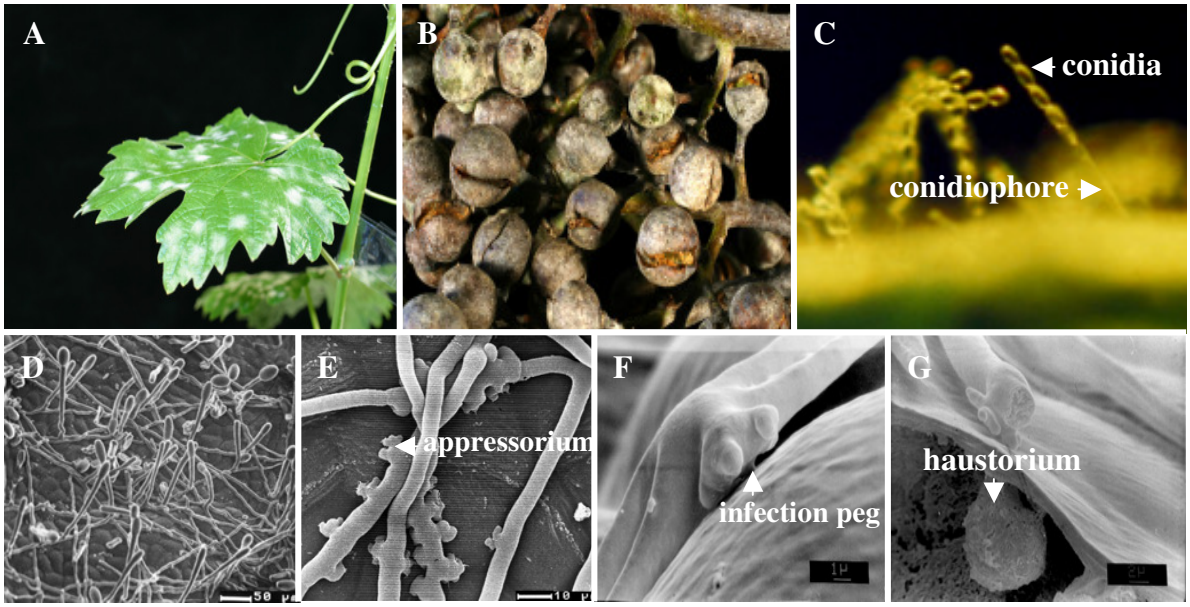

Figure 1. Symptoms and infection/reproductive structures of grapevine powdery mildew. A) Typical white spots of talcum-like powder on the upper site of a grape leaf blade; B) Berries burst caused by early and strong infection; C) Asexual reproductive structure; D) Mycelium grows only on the upper site of leaf surface; E) Appressoria; F) Appressorium showing the infection peg; G) Haustorium developed in the intercellular space. Source: Archive of the Institute for Grapevine Breeding Geilweilerhof.

Plasmopara viticola (Berk. and Curtis) Berl. et de Toni, the causal agent of grape downy mildew, is a member of the *Oomycetes*. This pathogen is commonly erroneously designed as a fungus, but instead of chitin as in fungi, the cellular wall of oomycete pathogens is primarily composed of cellulose. As powdery mildew this pathogen also undergoes the sexual and asexual life cycles in temperate climates. In this case, the sexual reproduction

occurs with the formation of overwintering oospores in dead leaf lesions and shoots. When the climatic conditions are adequate (raining periods in spring) the oospores germinate and produce macrosporangia. Inside the macrosporangia zoospores are formed. The sporangia containing the zoospores are released by wind or water drops falling sporadically on grapevine tissues. The bi-flagellated zoospores move within a water film preferentially towards stomata, where they attach (encysted zoospores) and germinate penetrating it. The pathogen colonizes the intercellular space, extracting nutrients through intracellular haustoria. As result of successful colonization sporangiophores grow out of the stomata, forming sporangia. The asexual zoospores contained inside these sporangia start secondary infections. The pathogen attacks leaves, fruits and vines of grape plants. The symptoms of downy mildew infection originally appear as translucent oilspots on the adaxial leaf surface, which develop into circular yellow spots. Under conditions of high humidity masses of sporangiophores containing the sporangia can be observed at the lower leaf surface as white/gray mold (mildew), exactly beneath the yellow spots. Later, infected leaf areas are killed and become brown, causing premature defoliation under strong attack.

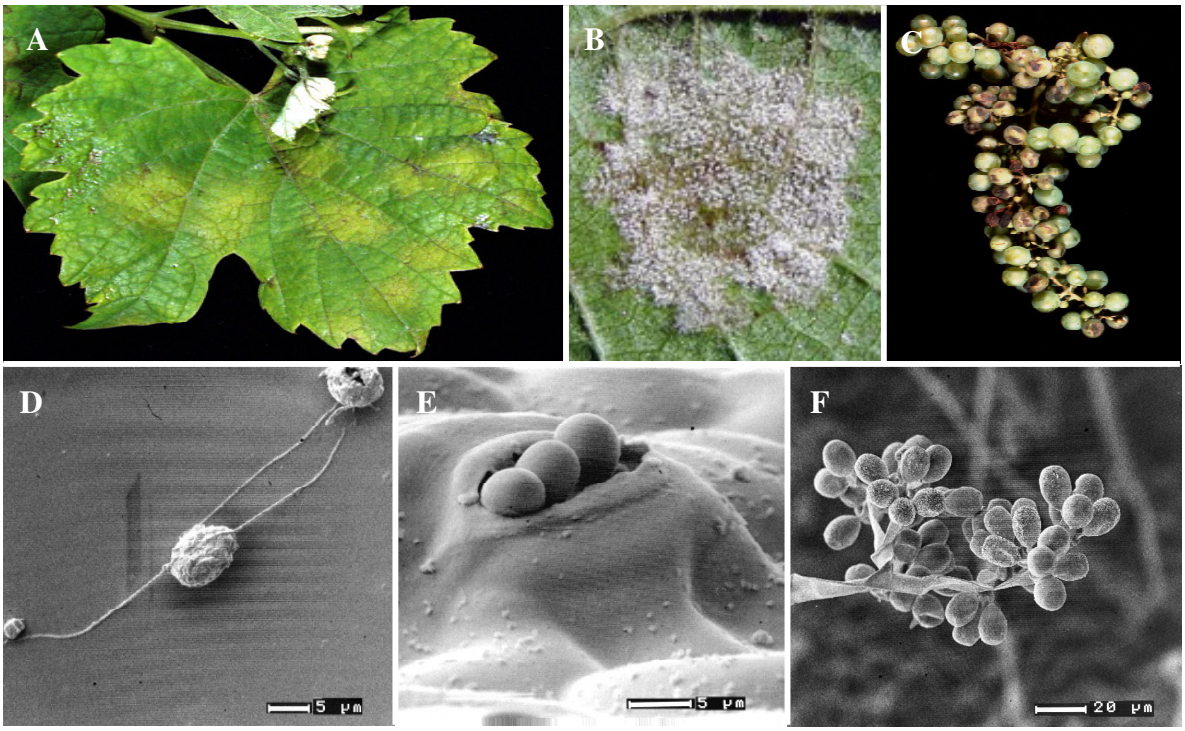

Figure 2. Symptoms on leaf and berries and reproductive structures of grapevine downy mildew. A) Typical circular yellow spots visualized in the upper leaf surface; B)

Sporangiophores and sporangia grown in the lower leaf surface; C) Infected berries; D) Bi-flagellated Zoospore; E) Sporangiophore growing out of a stomata; E) Sporangia attached to a sporangiophore. Source: Archive of the Institute for Grapevine Breeding Geilweilerhof.

The sexual phase of both pathogens is extremely important. During the meiosis spores with a new genetic background are generated. In special cases these new pathogen variants can overcome the resistance conferred by specific resistance genes.

1.3 Traditional grapevine breeding: breeding for disease resistance

Grapevine resistance breeding to mildew pathogens started in Europe at the end of the 19th century. Because no resistance source against these two diseases was available in European grapevine, highly resistant wild species growing in North America were introduced into Europe. These American grapes were then crossed to European grapevine cultivars. The idea was to combine the resistance present in North American grapes with the wine quality of the European grape cultivars. However, this strategy showed some practical limitations. As already mentioned, the wine quality of North American grapes is very low. In consequence, the wine produced from the first generation of resistant hybrids derived from these crosses is not suitable for consumption. To restore the wine quality several generations of backcrosses with European cultivars are required. At each generation new hybrids are selected based on their resistance level and potential for wine production, as well as other agronomically relevant characters, and used in further crosses. Considering the perennial nature of grapevine, this selection process is very tedious and time consuming. Therefore, many resistance breeding programs initiated at the end of the 19th and beginning of the 20th centuries were stopped. Alternatively, chemical products were developed to allow the protection of the European grapevine against the mildew diseases. Nevertheless, some resistance breeding programs survived during all these years. One example is the continuous effort of the Institute for Grapevine Breeding Geilweilerhof. As fruit of the hard work during all these years, several resistant cultivars with high wine quality have been released to the market. The most successful of them is the cv. `Regent´.

An important aspect to be considered in resistance breeding is the durability of the resistance trait introduced into a new cultivar. The transfer of single dominant resistance genes into elite cultivars is a strategy usually applied. The resistance conferred by such genes can be broken by the pathogen in a couple of years, varying from pathogen to pathogen. One approach applied to improve the stability of resistance is the combination of several diverse resistance genes into one single newly bred cultivar. This gene pyramiding should render it more difficult for the pathogen to overcome the resistance. Durable resistance is especially important in perennial crops, such as grapevine, that are planted for a productivity period of about 30 years. The identification of different sources of resistance is thus essential for the successful breeding of new grapevine cultivars with stable resistance.

The resistance breeding to biotic and abiotic stresses in grapevine has received a new impulse in the last years, motivated mainly by three factors: i) the laws are getting more and more restrictive in relation to the use of phytochemical products for disease control; ii) climatic changes and, iii) the advances achieved in the field of grapevine genetics and genomics, which should contribute significantly to improve the breeding efficiency in a near future.

1.4 Grapevine resistance breeding supported by molecular markers

The traditional breeding process can be accelerated through the use of molecular marker techniques. These molecular techniques in breeding programs may permit the better characterization of breeding material, informed choice of cross partners and marker-assisted selection (MAS) of the progeny. The use of MAS in breeding programs is advantageous in many aspects. Considering the resistance breeding to diseases three main advantages can be highlighted: i) selection of resistant genotypes without the requirement of pathogen infections; ii) selection of resistant genotypes in juvenile stages and iii) monitoring the introgression of various resistance sources (genes) for a particular or multiple diseases into a single elite grapevine cultivar (gene pyramidization). The MAS is especially important for gene pyramidization. Many resistance genes have a dominant effect on the phenotype. The plants that possess such genes are completely resistant to a specific disease. In this case, the introgression of additional resistance genes cannot be

distinguished by phenotypic scoring. The use of molecular markers linked to different resistance genes is therefore very helpful. These markers may also be used to identify plants homozygous to these resistance genes. The complete offspring obtained by the use of such a plant as cross partner would be resistant, and thus a bigger population would be available to select for other characters of agronomical importance, such as wine quality. The MAS in grapevine is expected to render the breeding for disease resistance more efficient, reducing the time required to breed a new resistant grapevine cultivar by a factor of two. But before molecular markers can be applied efficiently for selection purposes, their association with genes (alleles) or traits of interest must be firmly established.

1.5 Grapevine genetics and genomics

The relatively recent advances achieved in plant genetics and genomics also stimulated the research in grapevine. Although this investigation started a little later when compared to many other crops or model plants, a surprisingly great progress was obtained in these fields during the last decade. This progress was specially stimulated by the foundation of the International Grape Genome Program (IGGP) (http://www.vitaceae.org/index.php/International_Grape_Genome_Program). The multinational collaborative grapevine research resulted in rapid generation of relevant genetic and genomic informations. The development of microsatellite markers and the use of them in genetic mapping (e.g Di Gaspero et al., 2007; Doligez et al., 2006; Riaz et al., 2004) and phylogenetic analysis (e.g. This et al., 2004), the construction of BAC (bacterial artificial chromosome)-libraries (e.g. Adam-Blondon et al., 2005) and the expansion of EST (Expressed Sequence Tags)-libraries (e.g. Peng et al., 2007; Salmaso et a., 2004) are only some of these advances accomplished. However, the apogee of grapevine genomics was arrived with the publication of the draft genome sequence of the highly homozygous clone of *V. vinifera* cv. Pinot Noir (PN40024) in September of this year (Jaillon, 2007). Grapevine is the fourth flowering plant, the second woody species and the first fruit crop of which the whole genome was sequenced. Importantly, all the genomic information is freely accessible under the website: http://www.genoscope.cns.fr/externe/English/Projets/Projet_ML/index.html. The availability of the grapevine genome sequence will also accelerate the resistance research.

Although the genomic sequence was obtained from a genotype susceptible to the main grapevine diseases, this genome sequence will allow the identification of genes or gene clusters homologous to well characterized resistance genes from other plants. Quantitative trait loci (QTL) analysis already permitted the detection of QTLs conferring resistance to diseases (e.g. Fischer et al., 2004). These QTLs can now be anchored to the genome sequence supporting the selection of resistance candidate genes. Sequencing and functional analysis of these genes in a larger spectrum of genotypes (e.g. resistant genotypes) can rapidly identify alleles associated with the resistance. The design of allele-specific markers can then be employed to monitor the transfer of the corresponding resistance alleles into European grapevine backgrounds by conventional crosses. Alternatively, these genes may be transferred to elite cultivars by means of genetic transformation. However, the idea of grapevine transformation for commercial production still provokes hard discussions.

However, it can be expected that only the investigation of allelic diversity of the candidate genes present in the *V. vinifera* genome will not be sufficient to access all the genes involved in resistance. Some genes may exclusively have evolved in the wild species originally growing in America or Asia or been lost during domestication of grapevine. Therefore, complementary strategies have to be employed to explore this genetic diversity, leading to the identification of different sources of resistance.

1.6 Strategies and objectives

The present PhD thesis was developed at the Institute of Grapevine Breeding Geilweilerhof (IRZ). The Institute has an internationally recognized tradition in grapevine breeding for resistance to diseases. A Department of Molecular Genetics was created in 1992 to support the breeding process. Within this department the present project was realized.

The central goal of this thesis was the genetic and molecular analysis of mildew diseases resistance in grapevine. This investigation aimed at the generation of new basic knowledge about the interaction between grapevine and mildew diseases and the development of molecular tools to be employed in the breeding process. `Regent´ was the essential grapevine cultivar employed in all investigations. This cultivar was bred at IRZ and combines good level of resistance to mildew diseases with high wine quality. The

investigations performed during the doctorate involved different aspects of the interaction between grapevine and mildew diseases as follows.

Initially, a genetic map derived from the cross between the resistant cv. `Regent´ and susceptible cv. `Lemberger´ was constructed. A previous map had already been generated from this mapping population (Fischer et al., 2004). However, this genetic map was predominantly based on dominant molecular markers. The major objective of the present investigation was the integration of co-dominant microsatellite markers into this genetic map to render it more informative. The resulting genetic map was then employed for the localization of quantitative trait loci affecting mildew disease resistances and leaf morphology in grapevine (Welter et al., 2007). Additionally, one SCAR marker correlated with resistance to grapevine powdery mildew was integrated in the genetic map. This SCAR marker and microsatellite markers linked with the resistance QTLs localized in the `Regent´ x `Lemberger´ genetic map were tested for their potential use in marker assisted selection (MAS) (see annex 1, Eibach et al., 2007). Complementarily, functional and structural resistance candidate genes (CG) were localized using the same mapping population. The functional CG were selected from an EST- (expressed sequence tags) library constructed of `Regent´. The structural genes are RGAs (resistance gene analogs) originally developed by Di Gaspero and Cipriani (2002 and 2003). This investigation aimed the identification of candidate genes linked to the resistance QTLs.

Transcriptional analysis was approached in order to detect powdery mildew-responsive genes in grapevine. Initially, differentially expressed genes were accessed by microarray technology and subsequently, a subset of the induced genes were selected and their expression was analyzed in more detail by means of quantitative Real Time PCR (qRT-PCR). To get insights about the differential gene activity between an incompatible and compatible host x pathogen interaction, the resistant cv. `Regent´ and susceptible cv. `Chardonnay´ were evaluated.

A grapevine resistance gene analog family, coding for a CC-NBS-LRR protein was identified, isolated and *in silico* characterized. Differential display analysis performed before this thesis had identified transcripts exclusively expressed in a resistant grape (`Gloire de Montpellier´) after infection with downy mildew. Three of these fragments were closely related to each other and shared high homology to characterized resistance

genes from other plants. The objective of the present investigation was firstly the isolation of the full gene sequence, followed by the *in silico* characterization and genetic mapping of the isolated resistance genes. The full gene sequence was isolated from a BAC (Bacterial Artificial Chromosome)-library constructed from genomic DNA of cv. `Regent´ (published in Welter et al., 2007).

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Genetic mapping and localization of quantitative trait loci affecting fungal disease resistance and leaf morphology in grapevine (*Vitis vinifera* L)

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Abstract The aim of this study was the improvement of a genetic map of the F_1 population from the cross between the fungus-resistant grapevine cv. ''Regent'' and the susceptible cv. ''Lemberger'' and its use to localize factors affecting pathogen resistance and leaf morphology. To construct an integrated map combining the information from both parental meiotic recombination frequencies co-dominant microsatellite markers were employed. Resistance gene analog (RGA)-derived and sequence characterized amplified regions (SCAR) markers correlated with powdery and downy mildew resistance were additionally mapped. The new integrated map contains 398 markers aligned along 19 linkage groups, covers a total length of 1,631 cM and shows an average distance between markers of 4.67 cM. One hundred and twenty-two microsatellite markers were newly mapped. This genetic map was used to localize QTLs (quantitative trait loci) conferring resistance to powdery and downy mildew pathogens transmitted from ''Regent''. Factors influencing specific leaf morphology traits were identified in addition. A major QTL for powdery mildew resistance and one major and one minor QTL for downy mildew resistance were detected. Some RGA-derived markers are found co-located in the region covered by the major QTL for resistance to downy mildew hinting at their putative functional relevance. Furthermore, 27 QTLs affecting leaf morphology descriptors were identified. This map is an important tool for grapevine breeding and resistance research.

Keywords *Erysiphe* (syn. *Uncinula) necator* · Genetic mapping · Leaf morphology · Pathogen resistance · *Plasmopora viticola* · QTL analysis · Resistance gene analogs

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Introduction

Development of genetic maps as tools for grapevine breeding was retarded as compared to other crops due to high heterozygosity levels and inbreeding depression. The first genetic map for grapevine was published in 1995 (Lodhi et al. 1995). Nine genetic mapping studies have been published meanwhile (Dalbó et al. 2000; Doligez et al. 2002; Grando et al.

2003; Fischer et al. 2004; Doucleff et al. 2004; Adam-Blondon et al. 2004; Riaz et al. 2004; Doligez et al. 2006; Lowe and Walker 2006). The first maps were mainly based on dominant random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers (Dalbó et al. 2000; Doligez et al. 2002; Grando et al. 2003; Fischer et al. 2004; Doucleff et al. 2004). These kinds of markers allow a rapid generation of maps but do not easily permit the transfer of information between different genotypes and comparison between maps (Adam-Blondon et al. 2004). To generate a set of codominant markers for grapevine genetics, 21 research groups formed an international consortium to identify microsatellite-based markers for grapevine (VMC, *Vitis* Microsatellite Consortium, coordinated by AGROGENE, Moissy Cremayel, France). More than 350 VMC-markers were developed and usedfor construction of the internationally approved reference linkage map from the cross of ''Riesling'' × ''Cabernet Sauvignon'' (Riaz et al. 2004). Two additional sets of microsatellite-based markers for grapes were recently developed in Italy (UDV, Di Gaspero et al. 2005) and in France (VVI, Merdinoglu et al. 2005). A second microsatellite-based linkage map using the VMC set together with the VVI markers yielded the most elaborate published microsatellite-based map for grapevine currently available (Adam-Blondon et al. 2004).

The use of PCR primers with 20–30 nucleotides in length flanking the microsatellite loci usually leads to amplification of a unique, specific locus. The conservation of microsatellite-flanking sequences within cultivars and closely related species frequently allows one to transfer results between different mapping studies. It enables one to compare the linear order of markers between maps obtained from different *Vitis vinifera* cultivars or *Vitis* species genotypes. It permits one to search for coinciding location of important traits evaluated in different mapping studies using genotypes with divergent genetic backgrounds. This approach can elucidate main factors involved in selection processes.

Considering the grapevine genome sequencing programs operating currently in France and Italy genetic maps saturated with transferable markers will be instrumental to utilize the genomic information from model genotypes to target genomic regions for comparative analysis in non-model genotypes contributing important traits for breeding such as resistance to pathogens.

In highly heterozygous crops like grapevine a strategy termed ''double pseudo-testcross'' is employed to generate genetic maps (Grattapaglia and Sederoff 1994). This approach yields two separated genetic maps, one for each parent. The implementation of codominant markers like microsatellites allows their combination into one single integrated map. Furthermore, a consensus map can be generated by integrating information from different mapping populations (Doligez et al. 2006). The use of microsatellite-based markers is an important strategy to achieve these aims.

The principal aim of the present study was the construction of an integrated grapevine map by introduction of microsatellite markers into the previously elaborated map (Fischer et al. 2004) derived from the cross between ''Regent'' and ''Lemberger''. Additionally, 14 RGA (resistance gene analogs)-based markers and three SCAR (sequence characterized amplified regions)-markers correlated with resistance to powdery and downy mildew (Akkurt et al. 2006; Akkurt et al., in prep.) were analyzed. The resulting map was employed for QTL analysis of powdery and downy mildew resistance and leaf morphology traits. The latter have been investigated as a first example of localizing morphogenetic regulatory factors in grapevine.

Material and methods

Mapping population

The mapping population consisted of 144 F_1 plants obtained by the cross of the red wine cultivars ''Regent'' and ''Lemberger.'' ''Regent'' was bred at the Institute for Grapevine Breeding Geilweilerhof and shows resistance to both powdery (*Erysiphenecator*) and downy mildew (*Plasmopora viticola*) (Anonymous 2000). ''Lemberger'' is a traditional fungus-susceptible *Vitis vinifera* cultivar. The population segregates also for other agronomical and morphological traits. Young leaves of the population (second and third insertion from the apices) were collected at the start of the vegetation cycle and stored after shock freezing with liquid nitrogen at −70°C until DNA extraction. DNA extraction was

performed according to the protocol described by Thomas et al. (1993).

Phenotypic evaluation of resistance traits

The mapping population was scored for resistance to powdery and downy mildew in five (1999, 2000, 2003, 2004 and 2005), respectively, four (1999, 2000, 2003, 2004) growing seasons. The evaluations were performed at the Institute for Grapevine Breeding Geilweilerhof, omitting any fungicide protection, under natural field infection conditions. Additionally, a replicate of the mapping population was evaluated in 1999 for downy mildew resistance after experimental infection under greenhouse conditions at INRA Colmar. The degree of resistance to powdery and downy mildew was evaluated independently on leaves and berries or clusters, as described by Fischer et al. (2004).

Phenotypic determination of leaf morphology characteristics

From 129 genotypes of the mapping population 10 adult leaves from the middle third of several shoots were collected in summer 2005, pressed and dried. Eighteen ampelometric leaf characteristics (Genres 081 2001; OIV 2007) (Table 1) were recorded by using a digitiser tablet (SummaSketch II Professional Plus) (Fig. 1). Mean values were calculated for each leaf characteristic. The following ratios were calculated: length of vein N3/length of vein N1 (OIV 603/OIV 601), length petiole sinus to upper leaf sinus/length of vein N2 (OIV 605/OIV 602), length of petiole sinus to lower leaf sinus/length of vein N3 (OIV 606/OIV 603), length of tooth of N2/width of tooth of N2 (OIV 612/OIV 613), length of tooth of N4 / width of tooth of N4 (OIV 614/OIV 615) and length of vein N5/length of vein N1 (OIV 611/OIV 601).

Table 1 List of leaf morphology characteristics scored as OIV (Organization Internationale de la Vigne et du Vin, International Vine and Wine Organization, Paris; OIV 2007) descriptors

OIV code	Morphological traits of mature leaves
601	Length of vein N1
602	Length of vein N2
603	Length of vein N3
604	Length of vein N4
605	Length petiole sinus to upper leaf sinus
606	Length petiole sinus to lower leaf sinus
607	Angle between N1 and N2 measured at the first ramification
608	Angle between N2 and N3 measured at the first ramification
609	Angle between N3 and N4
610	Angle between N3 and the tangent between petiole point and the tooth tip of N5
611	Length of vein N5
612	Length of tooth N2
613	Width of tooth N2
614	Length of tooth N4
615	Width of tooth N4
617	Length between the tooth tip of N2 and the tooth tip of the first secondary vein of N2
618	Opening/overlapping of petiole sinus
665*	Vein N3, length petiole sinus to vein N4

* Descriptor 665 is not an official OIV descriptor but has been developed by Dettweiler (1987)

Genotyping

The earlier genetic maps of ''Regent'' and ''Lemberger'' were based mostly on randomly amplified polymorphism DNA (RAPD) and amplified fragment-length polymorphism (AFLP) markers as described by Fischer et al. (2004). These maps were improved and combined by newly mapping 122 microsatellite loci, three sequence characterized amplified regions (SCARs) and 12 resistance gene analogs (RGA)-derived markers.

The primer pairs flanking microsatellite loci originated from different marker sets: VVS (Thomas and Scott 1993), VVMD (Bowers et al. 1996; 1999), VrZAG (Sefc et al. 1999), VMC (*Vitis* Microsatellite Consortium), UDV (Di Gaspero et al. 2005) and VVI (Merdinoglu et al. 2005). Primers developed by the Institute for Grapevine Breeding Geilweilerhof within the VMC are listed in Table 2. All ''forward'' primers were labeled at their 5′-ends with fluorescent dyes (Ned, Hex or Fam) and the PCR products were analyzed by capillary electrophoresis using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California/USA). Two hundred and twelve primer pairs were first tested for segregating polymorphism using the genitors and 14 randomly picked

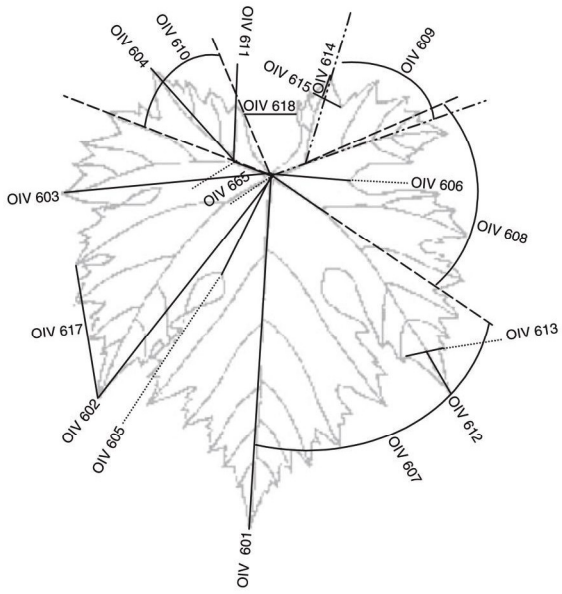

Fig. 1 Schematic representation of leaf ampelometric measures according to OIV 2007 as described in Table 1

genotypes from the progeny. After this screening, informative primer pairs were combined into multiplex applications for PCR considering the fluorescence labels, annealing temperatures and lengths of the amplified products. The markers were amplified in standard reactions of 10 µl final volume containing 8.0 ng template DNA, 1× NH_4*Taq* buffer (Invitek, Berlin/Germany), 1.5 mM or 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (Sigma, Taufkirchen/Germany), 0.2 µM of each primer and 0.3 U *Taq* DNA Polymerase (Invitek, Berlin/Germany). Amplification was performed in GeneAmp PCR System 9700 thermocyclers (Applied Biosystems, Foster City, California/USA), using the following program: 94°C for 3 min; 30 cycles of 94°C for 1 min, 48–65°C annealing for 1 min (depending on the primer pair sequences) and 72°C for 2 min and finally 72°C for 20 min.

A set of RGA-derived primers developed by Di Gaspero and Cipriani (2002, 2003) was tested for polymorphisms segregating in the present cross according to the original PCR protocol. Four of them showed clearly scorable polymorphic bands and were used to genotype the mapping population. The SSCP (single-strand conformational polymorphism) analysis was performed as described (Schneider et al. 1999). The three SCAR-markers employed are converted RAPD markers correlated with resistance traits (Akkurt et al. 2007; Akkurt et al. in prep.).

Genetic mapping and QTL analysis

The ''double pseudo-testcross strategy'' (Grattapaglia and Sederoff 1994) was used for the construction of the genetic map. The implementation of codominant microsatellite-derived markers allowed the combination of both parental maps into one map.

Marker segregation was tested with regard to the goodness-of-fit to the expected ratio using the x^2 test. Markers showing distorted segregation were originally included in the map calculation. After a preliminary mapping, individual distorted markers located in regions surrounded by non-distorted markers were excluded and the map was re-calculated.

The genotypic information was subjected to genetic mapping through linkage and recombination analysis with JoinMap 3.0 software (Van Ooijen and Voorrips 2001), applying the Kosambi function for the estimation of map distances (Kosambi 1944). LOD (logarithm of the odds) score thresholds equal or greater than 6.0 were used to determine linkage groups. The maximal recombination fraction permitted was 0.4.

Putative QTLs were primarily identified by interval mapping (Lander and Botstein 1989; Young 1996). Subsequently, molecular markers coinciding or closely flanking the LOD maxima of QTLs were used as co-factors in multiple QTL analysis (restricted MQM and full MQM mapping). The linkage group specific and genome wide significance thresholds of QTL LOD scores were determined by permutation tests (1.000 permutations, $P \geq 0.05$) of the quantitative trait data (Churchill and Doerge, 1994). All these calculations employed MapQTL 4.0 software (Van Ooijen et al. 2000).

Results

The integrated map

The combination of new microsatellite marker information with previously generated, mostly dominant marker data, allowed the construction of an integrated map for the cultivars ''Regent'' and ''Lemberger''. In total, 398 markers were aligned

Table 2 Primer sequences flanking microsatellite loci developed by the Institute for Grapevine Breeding Geilweilerhof within the VMC consortium

Primer name		Primer sequence (5′ > 3′)	Accn[a]
VMC1a7	Forward	ACGACCGGCAGAACAACAGT	BV681752
	Reverse	GGGCCAAACCTCTAAAAGCA	
VMC1a12	Forward	ATGTAATTACCGGTCATGAGTT	BV681753
	Reverse	TTCTTGTTTTGCCTATCTATCC	
VMC1b11	Forward	CTTTGAAAATTCCTTCCGGGTT0	BV681754
	Reverse	TATTCAAAGCCACCCGTTCTCT	
VMC1b12	Forward	AGGTGCTCCAGCCAGTCAG	–
	Reverse	CCCCTAATGCTCCGTGTTC	
VMC1c10	Forward	CACAGCTGTTCCAAGTCCCA	BV681755
	Reverse	ACAAGCCTTCCGCCACTCTC	
VMC1d10	Forward	CAGGTGTCCAGGACATATAAGG	BV681756
	Reverse	TTGGTTGGAATCTTGTAGAGGG	
VMC1d11	Forward	CTGCATGCTCATTGTACTATCA	BV681757
	Reverse	AGTGTCTTCTCGTCTTAAAACCT	
VMC1e8	Forward	CAGCGAGCTCTTGATTTATTGT	BV681758
	Reverse	GATCATAGCTTCAACGGCTTTT	
VMCe11	Forward	GGGGTCCAATGTGGACTTTATC	BV681759
	Reverse	CCATGAACAACAAACATGGCTT	
VMC1e12	Forward	GTGTGACCTTATGCAACACCAA	BV681760
	Reverse	GCTACCACATGCAGACAGGTTAGT	
VMC1f10	Forward	CATACAAGGAATTTACCCCCA	BV681761
	Reverse	ACCTCTTGTGCTGTCTAACCA	
VMC1f12	Forward	AAACCTTTCTGATGGTATCTAA	BV681762
	Reverse	GCTCATTGTAACATCAAAACTT	
VMC1g7	Forward	GGGTCCACATAGGTAGGAGATT	BV681763
	Reverse	AGCCCATAAAGGCCTTAAAAAC	
VMC1h9.1	Forward	ACAAGCTCCTACCGGTTCCAA	BV681764
	Reverse	TTCTGTGGCAATGGGGTAGTTC	
VMC1h11.1	Forward	TGGGTTACTTCAGGAGACAAAA	–
	Reverse	ACAACATAATTGGCCTCCACAT	
VMC_NG4b9	Forward	CTGGGGAGCATATACACATACCAG	BV681765
	Reverse	CTCTCTCTTCCCGATAGCCACC	
VMC_NG4c8	Forward	CGAGAATCACCGGCGAA	BV681766
	Reverse	TGCAGCGCGGAGCA	
VMC_NG4c10	Forward	AAGCAATGAACACAACATTCTCC	BV681767
	Reverse	CTAAGTTTCTATGACACTTTCCTCCA	
VMC_NG4d10.1	Forward	AGGGGGAGACGCACGAA	BV681768
	Reverse	GCGCAGCCTTTGCCAGA	
VMC_NG4e9	Forward	AGAGACAGGGAGAGGAGAGT	BV681769
	Reverse	TGGGAAATGCAAACAGAG	
VMC_NG4e10.1	Forward	AATGCAGCAGCGCCAGATG	BV681770
	Reverse	GCAGGCTGCTGCTGTTTTG	

Table 2 continued

Primer name		Primer sequence (5′ > 3′)	Accn[a]
VMC_NG4f9.2	Forward	GGGGAGAGTGGAGTGGAGT	BV681770
	Reverse	TCCTCCATGTCCCTCTGCT	
VMC_NG4h9	Forward	GATCTGCCCGCAAATACCG	BV681772
	Reverse	GGGACGAGGACGTGAGTGT	

[a] NCBI GenBank accession number

along 19 linkage groups (LG), covering 1,631 cM with an average distance between markers of 4.67 cM (Table 3; Fig. 2). The LGs were numbered following the nomenclature of the International Grape Genome Program (IGGP) (Riaz et al. 2004; Adam-Blondon et al. 2004). In this presentation 122 microsatellites markers were mapped. Seventy-six (60%) of these were heterozygous in both ''Regent'' and ''Lemberger,'' and 67 (53%) of them could be mapped as co-dominant markers. The nine remaining markers were analyzed as dominant markers due to the presence of ''null'' alleles. ''Regent'' and ''Lemberger'' were heterozygous for 89% and 71 % of the mapped microsatellite markers, respectively (Fig. 3)

Distortion of segregation

Distortion of segregation is a commonly observed phenomenon in genetic mapping studies. Markers showing distorted segregation were hence included in the mapping as described in the methods section. Clusters of distorted markers (two or more) were identified in ten linkage groups as indicated in Fig. 2. Microsatellite markers located in five of these regions have also been found to be distorted in the map constructed from ''Syrah'' × ''Grenache'' (Adam-Blondon et al. 2004). Interestingly, one of these regions co-locates with the major QTL for resistance to powdery mildew (LG-15).

Table 3 Summary of the information generated for the integrated map ''Regent'' × ''Lemberger''

LGs[a]	Length (cM)	No. of markers	Microsatellite markers	RGA/SCAR markers	Average distance
1	94	26	7	stkVr001	3.62
2	90	14	6	–	6.43
3	58	13	7	–	4.46
4	79	15	7	–	5.27
5	88	28	11	–	3.14
6	93	13	6	–	7.15
7	84	20	5	rgVrip158	4.20
8	80	25	9	–	3.20
9	42	5	1	–	8.40
10	66	8	6	–	8.25
11	100	22	7	–	4.55
12	118	33	7	stkVa011	3.58
13	77	21	7	–	3.67
14	119	18	6	–	6.61
15	76	25	4	ScORA7, ScORN3R	3.04
16	86	31	5	–	2.77
17	90	23	4	–	3.91
18	102	33	9	rgVamu137, ScPRA14	3.10
19	89	25	8	–	3.56
Total	1631	398	122	–	4.67

[a] Linkage groups numbered according to the International Grape Genome Program (IGGP) nomenclature

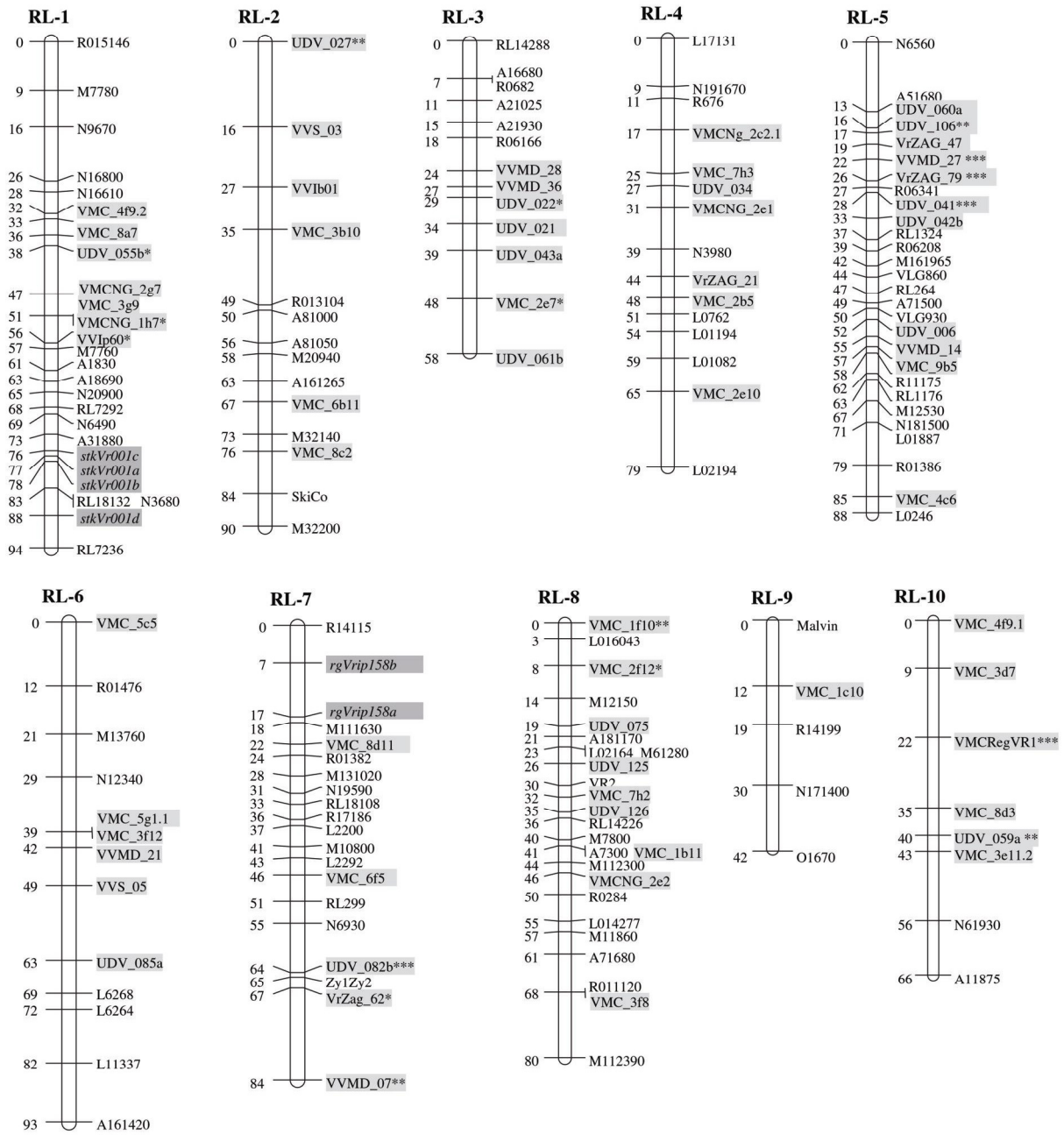

Fig. 2 Integrated ''Regent'' × ''Lemberger'' genetic map. Linkage groups are numbered according to the International Grape Genome Program (IGGP) nomenclature. The genetic map was constructed employing JoinMap 3.0 software with LOD ≥ 6.0 for linkage. The cumulative distance between markers in cM, calculated with the Kosambi function, is indicated on the left of the linkage groups. Microsatellite loci and RGA-derived markers or ScOR markers are represented with light grey and grey boxes, respectively. Distorted microsatellite markers are indicated by asterisk(s) according the level of distortion (*$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$)

To promote a better understanding of the events involved in the genetically distorted regions, the x^2 test was applied to test the goodness-of-fit of the observed gametic (test 1) and zygotic (test 2) segregation with regard to the their expected segregation (methodology adapted from Lorieux et al.

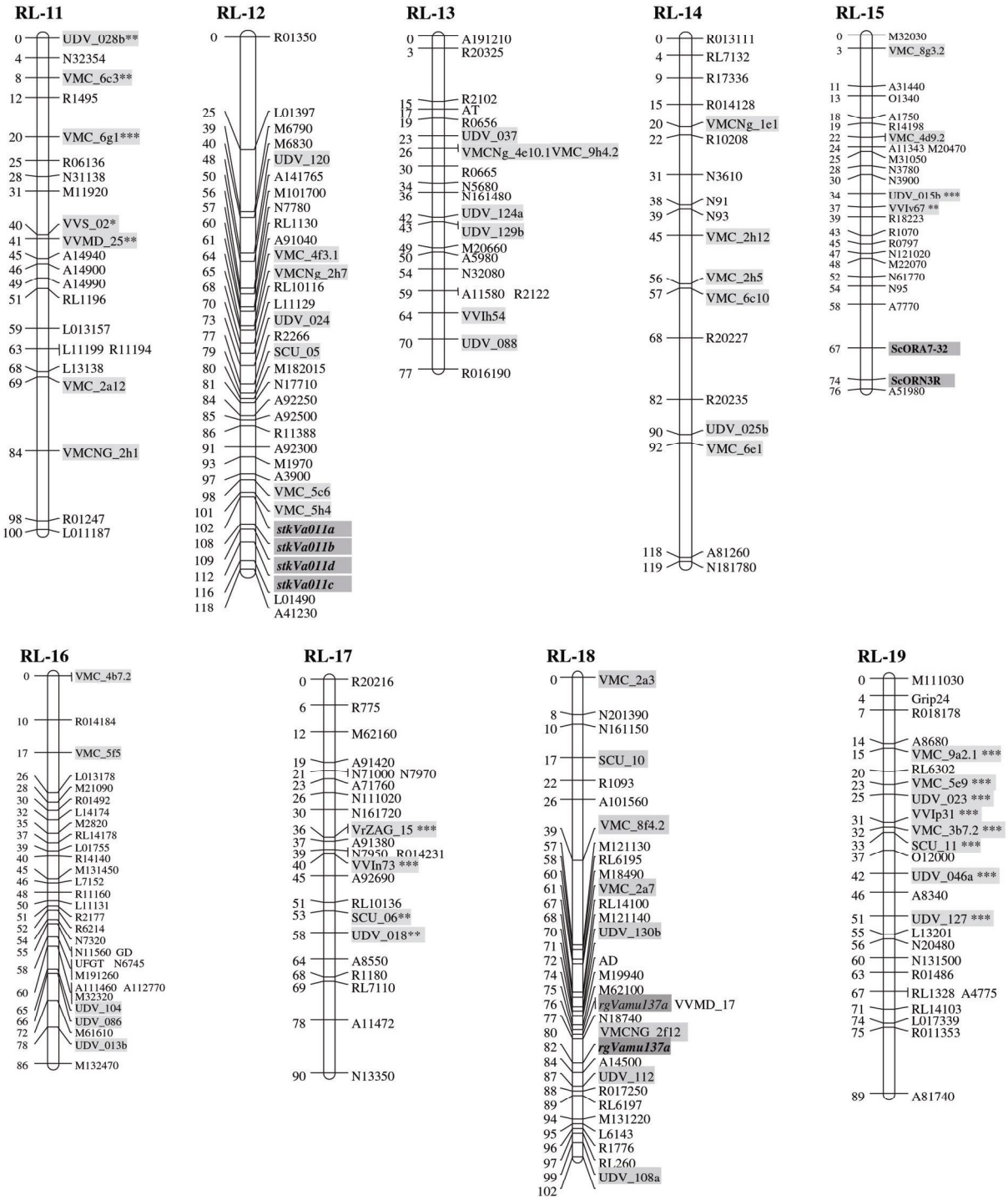

Fig. 2 continued

1995). For this analysis only fully informative microsatellite markers (heterozygous in both parents) mapped to distorted regions were used. Distorted markers located in seven linkage groups were analyzed. The gametic segregation was tested independently for each parent, considering that an F_1

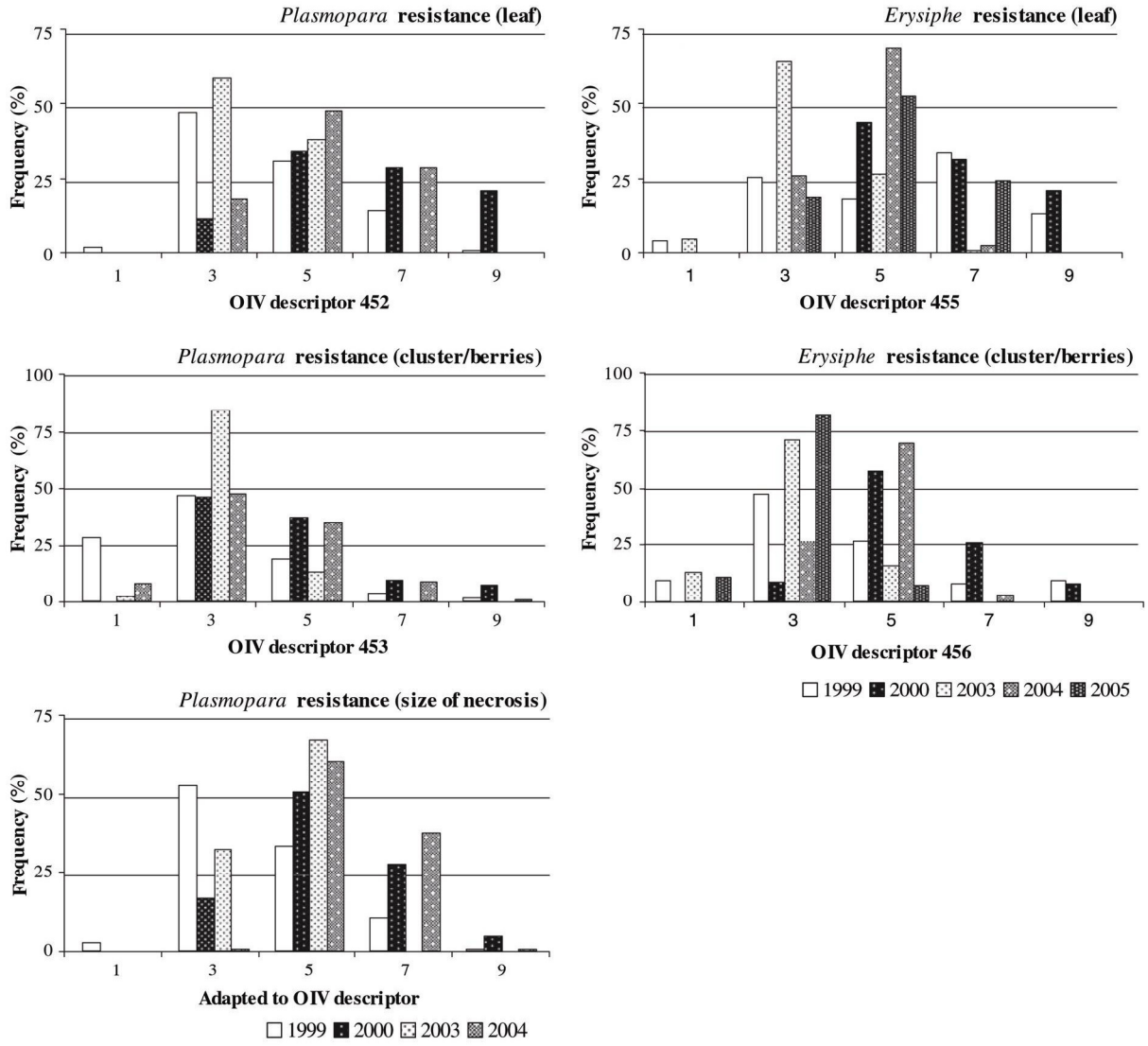

Fig. 3 Frequency distribution (%) of the mapping population plants based on the level of resistance expressed to *Plasmopara viticola* and *Erysiphe* (syn. *Uncinula*) *necator* in different years. The level of resistance was evaluated under field conditions employing the OIV classification

population was used for the map construction. A region was considered to be under the gametic selection if only test 1 was significant, while the zygotic selection was assumed when tests 1 and 2 or only test 2 were significant ($P \leq 0.05$).

The x^2 tests showed that different phenomena were involved in the deviation of segregation ratios (data not shown). The microsatellite markers located on the distorted region of LG-01, 05 and 10 were significant only for the test 1 in ''Lemberger,'' suggesting a gametic selection in favor of one of the paternal alleles (pollen). Otherwise, marker VVIn73, located on LG-17, showed a gametic selection in favor of one of the maternal alleles (''Regent''). The microsatellite markers located in the distorted region of LG-15 and LG-19 present gametic selection in both parents. Finally, marker VMC_6 g1, located in the distorted region of LG-11, was significantly distorted in both tests, suggesting zygotic selection.

Fungal disease resistance QTLs

Use of interval mapping allowed the identification of QTLs conferring resistance to powdery mildew,

respectively, downy mildew. The molecular markers with the highest LOD-value present in these regions were used in the second step in either restricted MQM (for downy mildew) or full MQM (for powdery mildew) mapping to increase the resolution of QTL analysis. Nevertheless only one major QTL conferring resistance to powdery mildew could be detected. This QTL, located on LG 15, shows significant effects on both the resistance of leaves (1999, 2000 and 2005) and berries (1999, 2000) and explains up to 56.8% resp. 64.5% of the phenotypic variance. In the years 2003 and 2004 no significant QTL could be found.

In contrast, one QTL showing major and one showing minor effects on the resistance to downy mildew were identified. The major QTL is located on LG 18 and was detected in three different scores of the trait: leaf resistance, berry/cluster resistance and lesion size. This QTL was very stable over the years, showing significant effects in all years evaluated, except for berry resistance data scored in 2004 (Table 4). The QTL seems to be spread over a large region in this linkage group. The markers with the maximal LOD value explains up to 37.3% of the phenotypic variance, detected in 2000 for leaf resistance (Table 4). The position of the QTL peak (maximal LOD value) identified by interval mapping fluctuated over the years. The selection of the molecular marker with the greatest LOD value as co-factor during the restricted MQM mapping did not

Table 4 Description of the quantitative trait loci (QTLs) detected for resistance to *Plasmopara viticola* and *Erysiphe* (syn. *Uncinula) necator* in different years

Trait	Year	LG [a]	QTL LOD max [b]	LOD threshold specific LG[c]	LOD threshold genome wide [c]	Map position LOD max	Confidence Interval [d]	Flanking marker [e]	% Var. expl.
P. viticola leaf resistance (OIV-452)	1999	18	15.27	3.3	4.9	71.2 cM	66.8-93.5	M21300	34.9
		4	5.80	2.8	4.9	25.1 cM	22.3-30.8	VMC7h3	15.2
	1999*	18	10.62	3.3	5.4	71.2 cM	66.8–101.8	M21300	31.3
		4	4.75	2.9	5.4	25.1 cM	22.3–35.8	VMC7h3	9.0
	2000	18	17.56	3.3	5	86.8 cM	66.8–101.8	UDV112	37.3
	2004	18	6.47	3.4	4.6	74.0 cM	66.4–88.2	M19940	15.6
		4	5.38	3	4.6	30.8 cM	22.3–38.8	VMCNg2e1	12.5
P. viticola leaf resistance (size of necrose)	1999	18	12.04	3.4	5.6	71.2 cM	66.8–101.8	M21300	21.9
		4	6.82	2.9	5.6	25.1 cM	22.3–35.8	VMC7h3	10.4
	1999*	18	11.23	3.4	5.6	71.2 cM	66.8–84.2	M21300	36.1
		4	5.61	2.9	5.6	30.8 cM	22.3–44.4	VMCNg2e1	22.6
	2000	18	14.53	3.4	4.6	84.2 cM	66.8–101.8	A14500	37.2
	2004	18	5.22	3.3	–	84.2 cM	66.8–96.8	A14500	17.7
P. viticola berry resistance (OIV-453)	1999	18	9.04	3.2	5.1	93.5 cM	66.8–101.8	M131220	29.8
	1999*	18	10.0	3.3	5	93.5 cM	75.7–101.8	M131220	33.4
	2000	18	10.86	3.3	7.7	84.2 cM	66.8–101.8	A14500	30.2
E. necator leaf resistance (OIV-455)	1999	15	20.1	3.1	6.5	47.1 cM	44.5–47.1	M121020	56.8
	2000	15	16.98	–	–	43.5 cM	38.6–43.5	R1070	42.1
	2005	15	3.84	3.1	4.5	66.7 cM	58.1–71.7	ScORA7	13.7
E. necator berry resistance (OIV-456)	1999	15	13.92	3	6.4	51.7 cM	48.1–51.7	N61770	64.5
	2000	15	7.44	3	5	43.5 cM	43.5	R1070	21.9

[a] Linkage groups named as the International Grape Genome Program (IGGP) nomenclature

[b] Maximum LOD score obtained by restricted MQM (*P. viticola*) and full MQM (*E. necator*) mapping

[c] Calculated by permutation test at $P \leq 0.05$

[d] The confidence interval was determined by dividing the highest LOD score of the QTL by two

[e] Nearest molecular markers to the peaks of the QTLs

* Phenotypical evaluation performed under greenhouse conditions at INRA Colmar

reduce the extension covered by the QTL. More than one resistance factor seems to be located in this genomic region. The minor QTL is located on LG 4. Despite its small effect, the QTL could be detected in the 4 years evaluated, explaining up to 22.6% of the phenotypic variance (Table 4). The QTL located on LG 5 identified previously by Fischer et al. (2004) was significant only in 1999 in both field data and greenhouse assays (data not shown).

Position of RGA-derived markers

The RGA-STSs primer pairs used to screen the mapping population revealed rather complex patterns of bands in SSCP-analysis. Each segregating band was scored individually as a dominant marker. In total, 12 polymorphic bands amplified with four RGA-STSs primer pairs were recorded and mapped. The polymorphic bands obtained from one primer pair mapped in the same linkage group, generally closely linked to each other (Fig. 2). The RGA-derived markers were coded according to the original description by Di Gaspero and Cipriani (2003), followed by a letter specifying the scored band. Interestingly, two markers amplified with primer pair ''rgVamu137'' mapped on LG 18, in the region covered by the major QTL for resistance to downy mildew. Other RGA-derived markers were found on LGs 1, 7 and 12.

Leaf morphology QTLs

Leaf morphology traits are discriminant parameters in ampelography contributing to the identification of unknown cultivars (Genres 081 2001). Their variation is most likely caused by subtle differences in morphogenetic factors shaping the developing leaves. Selected leaf traits serve as internationally approved grapevine descriptors (OIV 2007) and their variation is considered to be cultivar-specific (Dettweiler 1987). Hence they must rely predominantly on genetic factors determining their phenotypic expression.

The ''Regent'' × ''Lemberger'' progeny shows considerable variation in leaf morphology. This population was chosen as a model to study the genetics of morphogenetic factors operating in grapevine. A selection of 18 internationally acknowledged and well defined leaf characteristics (Table 1) scored in summer 2005 and six different calculated trait ratios were analyzed in QTL mapping. For 13 out of 18 traits and four out of six calculated trait ratios QTLs exceeding the genome wide LOD significance thresholds could be identified (Table 5). The QTLs were found dispersed all around the genome on 12 of the 19 LGs corresponding to the grapevine chromosomes. Most LGs carried one or two different leaf trait QTLs with the noticeable exception of LG-01 that appears to carry factors affecting eight different morphological traits. Two of these, OIV 605 and 606, as well as the related trait ratio OIV 605/OIV 602 even exhibit their LOD maximum in the very same position at 13.6 cM. Most of these traits concern leaf sinus formation and thus may be regulated by a common factor located at this genetic position. The leaf trait with the most dispersed QTLs was OIV 607 (the angle between leaf veins N1 and N2 at first ramification) affected by QTLs on five different linkage groups (LG-01, 06, 12, 13 and 15) with maximal LOD scores of five to eight.

Discussion

The Integrated ''Regent'' × ''Lemberger'' map

The mapping population used in the present investigation has been previously employed for mapping studies (Fischer et al. 2004). In contrast to the present study, two separate maps of the parental genotypes had been obtained and could only partially be integrated, due to the predominant application of dominant molecular markers (RAPD and AFLP) at that time. To render this map more informative, microsatellite markers and resistance-related markers (RGA-derived markers and SCAR markers) were additionally mapped in this new investigation. The codominant microsatellite markers easily permitted an integration of the parental maps (Fig. 2). The combination of various types of molecular markers (Microsatellites, AFLP, RAPD, SCAR, CAPS, RGA-based markers) detailed the genetic map with a high density of markers, improving its usefulness for QTL detection and future map-based cloning approaches.

Several of the microsatellite loci used in this investigation have also been mapped in other studies (Riaz et al. 2004; Adam-Blondon et al. 2004; Di Gaspero et al. submitted). A congruence of the linear

Table 5 Description of the quantitative trait loci (QTLs) detected for morphological leaf traits

OIV code	LG[a]	QTL LOD max[b]	LOD threshold threshold specific LG[c]	LOD threshold genome LG[c]	Map position LOD max	2- LOD support interval	Flanking marker[d]	% Var. expl.
601	LG-05	4.63	3.3	4.4	27.2 cM	21.9–28.5 cM	R06	
603/601	LG-11	5.51	3.0	4.6	7.1 cM	2.9–14.8 cM	N32	
605	LG-01	16.4	3.1	4.8	13.6 cM	8.6–20.8 cM	N96	
605/602	LG-01	22.5	3.2	7.5	13.6 cM	8.6–20.8 cM	N96	
606	LG-01	10.65	3.2	4.6	13.6 cM	8.6–20.8 cM	N96	
606	LG-15	5.2	3.0	4.6	71.7 cM	63.1–76.3 cM	ScO	
606/603	LG-01	14.16	3.4	5.0	56.2 cM	51.5–56.9 cM	VVI	
607	LG-01	7.79	3.0	4.4	20.8 cM	5.0–26.5 cM	N96	
607	LG-06	8.04	2.8	4.4	38.6 cM	28.8–39.4 cM	VM(	
607	LG-12	5.05	3.1	4.4	85.1 cM	81.2–86.3 cM	A92	
607	LG-13	6.62	3.0	4.4	41.0 cM	36.0–42.7 cM	UDV	
607	LG-15	5.08	3.1	4.4	24.0 cM	18.9–28.0 cM	A11	
608	LG-05	5.23	3.2	4.4	54.7 cM	50.1–56.7 cM	VVN	
608	LG-12	4.54	3.1	4.4	85.1 cM	78.9–97.4 cM	A92	
608	LG-16	5.13	3.1	4.4	31.8 cM	28.1–35.3 cM	L14	
609	LG-01	6.58	3.1	4.6	33.0 cM	31.6–37.0 cM	VM(	
610	LG-01	6.22	3.2	4.7	35.9 cM	31.6–37.9 cM	VM(	
612	LG-02	7.87	3.0	4.6	58.1 cM	54.8–62.9 cM	M20	
613	LG-06	5.77	3.0	4.5	54.2 cM	46.6–69.0 cM	UDV	
613	LG-07	4.67	3.0	4.5	33.2 cM	28.4–36.1 cM	RL1	
614	LG-02	7.05	2.8	5.5	58.1 cM	54.8–62.9 cM	M20	
614	LG-11	7.95	5.4	5.5	51.8 cM	51.4–54.1 cM	A14	
614/615	LG-02	8.13	3.0	4.6	89.8 cM	84.3–89.8 cM	M32	
614/615	LG-08	7.67	3.1	4.6	54.5 cM	46.1–57.4 cM	L01	
617	LG-01	5.27	3.2	4.5	35.9 cM	31.6–37.9 cM	VM(	
618	LG-10	4.51	2.6	4.5	19.2 cM	5.0–32.5 cM	VM(	
665	LG-07	6.8	3.1	4.6	36.6 cM	33.2–41.0 cM	L22(	

[a] Linkage groups named as the International Grape Genome Program (IGGP) nomenclature

[b] Maximum LOD score obtained by MQM mapping

[c] Calculated by permutation test at $P \leq 0.05$

[d] Nearest molecular markers to the peaks of the QTLs

order of the commonly mapped microsatellite markers was generally observed when comparing the present map to the others. Few inversions occurred in microsatellite markers located close to each other. The map length observed is in the range of other genetic maps developed for grapevine.

Distorted marker segregation has been observed in genetic maps constructed from grapevine crosses (e.g., Adam-Blondon et al. 2004). In the case of ''Regent'' × ''Lemberger,'' one distorted region (LG-15) coincides with the major QTL for resistance to powdery mildew (see below). This fact may hint at the possibility that these regions were introgressed from *Vitis* wild species genotypes serving as resistance donors in the complex pedigree of ''Regent'' (Akkurt et al. 2007). The sequences of the two homologous chromosomal regions of the diploid grapes may differ considerably in these introgressed areas affecting meiotic synapsis and recombination.

QTL analysis

Powdery and downy mildew resistance factors

The integrated ''Regent'' × ''Lemberger'' map was employed for QTL analysis by interval mapping and subsequent MQM mapping. This allowed the identification of QTLs affecting the resistance to powdery and downy mildew in grapevine. For all the QTLs detected the positively linked marker allele is one from ''Regent''. ''Regent'' is a recently bred red grapevine cultivar that combines high wine quality and resistance to the two worldwide most important pathogens (powdery and downy mildew). Its resistance traits were introgressed by complex crossbreeding between susceptible traditional *Vitis vinifera* cultivars (high wine quality) and specific genotypes of wild *Vitis* species: These show inferior wine quality, but are the only known source of resistance to both pathogens. Different wild species could have been the donor of the resistances to both diseases presents in cv. ''Regent'' (Akkurt et al. 2007).

Both for powdery and downy mildew one QTL with major effect was detected and is in agreement with the earlier description of Fischer et al. (2004). The present investigation provides three additional years of phenotypic evaluations for both diseases as compared to the previous study. The resistance to powdery and downy mildew has now been scored in the population for at least 5 years. However, the data from 2003 did not allow to detect any QTL, probably due to the low natural infection pressure resulting from the exceptionally warm and dry climate Europe experienced in that summer. Disregarding the year 2003, the major resistance QTL to downy mildew located on LG-18 was detected reproducibly in all years evaluated, despite the variation on climatic conditions. Contrarily, the major QTL for powdery mildew on LG-15 could not be detected in 2004 and showed only a small effect in 2005. This is probably due to effects of varying climatic conditions.

For downy mildew resistance, one supplementary QTL with minor effects was identified on LG-04. The presence of a major QTL accompanied by minor QTLs appears to be a common phenomenon in plant genetics of resistance (e.g., Nair et al. 2005; Calenge et al. 2005a; George et al. 2003). In contrast, no minor QTLs were identified for powdery mildew, although the resistance response revealed a continues phenotypical distribution. Hypothetically, diverse factors such as the limited size of the population, epistatic interactions and environmentally caused variation could limit the power of QTL analysis, preventing the identification of additional regions with a small effect on the phenotype. A unique major QTL responsible for the resistance to powdery mildew was also identified in some other crops such as wheat (Jakobson et al 2006) and mungbean (Humphry et al. 2003).

The identification of (a) genomic region(s) affecting the resistance to pathogens through QTL analysis is only the first step to isolate and characterize the resistance factors. QTL analysis allows only a rough localization of the resistance factors, requiring further efforts of physical mapping and genetic fine analysis for map-based cloning approaches. Several studies demonstrated the high efficiency of RGA-based markers for the identification of markers closely linked to resistance loci (e.g., Xu et al. 2005; Calenge et al. 2005b; Donald et al. 2002). Therefore, RGA-STS (sequence-tagged sites) primers developed for grapevine (Di Gaspero and Cipriani 2002; 2003) were tested. Two RGA-based markers amplified by primer pair ''rgVamu137'' are found located within the support interval of the major QTL for resistance to downy mildew. These primers had been designed to amplify a Toll-interleukin-type receptor nucleotide

binding site (TIR-NBS-LRR) RGA class, which is thought to play an important role in pathogen recognition and signal transduction. Their co-location with the major resistance QTL could hint at a putative functional role to be studied further.

A greater number of RGA-derived markers were mapped in the two mapping populations ''Chardonnay'' × ''Bianca'' and ''Cabernet Sauvignon'' × hybrid 20/3 (Di Gaspero et al. submitted). Several RGA-derived markers used in that study were mapped in a cluster on LG-18. The RGA marker ''rgVrip064'' was found associated to downy mildew resistance in some resistant genotypes (Di Gaspero and Cipriani 2002) and is located in the same region (Di Gaspero et al. submitted). Based on the position of commonly mapped microsatellite markers it was confirmed that this is the same region covered by the major QTL for downy mildew resistance as identified here in ''Regent.''

This study permitted the identification of genomic regions associated with powdery and downy mildew resistance. RGA-derived markers within the support interval of the QTL with major effect on the resistance to downy mildew were detected as well as microsatellite loci in close linkage to resistance QTLs. These markers can now be tested for their correlation to resistance in different genetic backgrounds (e.g., Akkurt et al. 2007). Such validated markers will serve in marker-assisted selection procedures to accelerate breeding as well as in germplasm characterization. In addition, they can be employed for positional cloning of the genomic regions of interest to study possible candidate genes for resistance and understand the cellular pathways involved.

Leaf morphology traits

Grapevine leaf morphology traits have high discriminant power for the differentiation of the comprehensive variety of cultivars present (estimated ca. 8,000–10,000 worldwide). They have proven very useful for ampelographic examination of cultivars. These traits should be controlled by genetic determinants influencing the developmental patterns of leaf morphogenesis. As a first approach to address morphogenetic regulation in grapevine, the segregation of leaf characteristics was studied in the ''Regent'' by ''Lemberger'' progeny. In total, 18 different ampelometric traits clearly defined as OIV descriptors were measured (OIV 2007). Ratios of OIV 603 to OIV 601, 605/602, 606/603, 611/601, 612/613 and 614/615 were calculated in addition, as they are environmentally more stable scores than the individual ampelometric measures. All leaf characteristics were processed through QTL analysis.

In total, 27 statistically significant QTLs affecting leaf morphology were identified in the ''Regent'' × ''Lemberger'' map (Table 5). The morphology of leaf teeth and the angles of leaf veins seem to be determined by many different loci dispersed around the genome. Leaf angles are strongly correlated with the opening or overlapping of the leaf sinus (Dettweiler 1987). There seems to be an accumulation of morphogenetic factors, particularly those affecting the depth of the leaf sinus, on LG-01.

To our knowledge, this is the first report on the analysis of morphogenetic factors in the genome of grapevine.

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Development of SCAR markers linked to powdery mildew (*Uncinula necator*) resistance in grapevine (*Vitis vinifera* L. and *Vitis* sp.)

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Abstract Sequence-characterized amplified regions markers (SCARs) were developed from six randomly amplified polymorphic DNA (RAPD) markers linked to the major QTL region for powdery mildew (*Uncinula necator*) resistance in a test population derived from the cross of grapevine cultivars "Regent" (resistant) × "Lemberger"(susceptible). RAPD products were cloned and sequenced. Primer pairs with at least 21 nucleotides primer length were designed. All pairs were tested in the F1 progeny of "Regent" × "Lemberger". The SCAR primers resulted in the amplification of specific bands of expected sizes and were tested in additional genetic resources of resistant and susceptible germplasm. All SCAR primer pairs resulted in the amplification of specific fragments. Two of the SCAR markers named ScORA7-760 and ScORN3-R produced amplification products predominantly in resistant individuals and were found to correlate to disease resistance. ScORA7-760, in particular, is suitable for marker-assisted selection for powdery mildew resistance and to facilitate pyramiding powdery mildew resistance genes from various sources.

Keywords Grapevine · Marker-assisted selection · Molecular marker · Powdery mildew resistance · *Uncinula necator* · *Vitis* sp

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Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest and economically most important cultivated plants of the world. Grapevines are threatened by pathogens (viruses, bacteria, fungi, and insects) and abiotic stresses (drought, winter cold, etc.). Fungal infections damage fruit and wine quality, so viticulture typically requires substantial fungicide application. This is expensive and laborious and has environmental risk. The best way to solve these problems is the development of naturally resistant cultivars with high-quality fruit. This aim requires the introgression of resistance characteristics from wild species of *Vitis* with low fruit quality into the gene pool of *V. vinifera* in order to obtain high-quality cultivars. In classical crossbreeding the hereditary causes of individual traits and performance characteristics are combined based on empiric

evaluations. However, crossbreeding is time-consuming and expensive. Development of a new cultivar usually takes 25–30 years from the original cross to the introduction into the market. In this situation, molecular investigations will lead to considerable acceleration. The genetic mapping of molecular markers, labeling of certain traits at DNA level, and studies on their transmission and distribution in germplasm collections are important tools for early trait inheritance diagnosis and successful breeding programs.

To obtain these tools, at first genetic maps of grapevine were developed by linkage/recombination studies using molecular markers produced with various techniques. The investigation of populations originating from the cross of "Regent" (resistant to powdery mildew and downy mildew) and "Lemberger" (susceptible) (Fischer et al. 2004), as well as from the cross of Gf.Ga-47-42 (a fungus-resistant white grapevine breeding line) and "Villard blanc" (a resistant French hybrid) (Zyprian et al. 2005; Zyprian et al. in preparation) yielded detailed genetic maps. The segregation of quantitatively described powdery mildew resistance was scored in the progenies according to OIV (Organisation Internationale de la Vigne et du Vin, International Wine Organization, Paris) descriptors 455 (resistance of leaves) and 456 (resistance of clusters/berries) over several years of field observations. These data have been correlated in QTL analysis with molecular marker profiles of individual plants. One region with factors of resistance against *Uncinula necator* (powdery mildew) has been identified in the genetic map of the cultivar "Regent" (Fischer et al. 2004). To make this knowledge easily applicable for breeding purposes, six randomly amplified polymorphic DNA (RAPDs) markers from this major QTL region were now converted into sequence characterized amplified region (SCAR) markers, which are experimentally more stable than RAPDs and simple to score.

After conversion it is necessary to examine and validate the markers by mapping and testing in genetically different but partially related families for the maintenance of their correlation with the resistance characteristics. The current communication describes these investigations.

Material and methods

Plant material and DNA extractions

For the development of SCAR markers 152 genotyped individuals from the segregating population of "Regent" × "Lemberger" were used. For testing a different segregant family, 10 resistant and 10 susceptible individuals from 150 progeny plants of Gf.Ga-47-42 × "Villard blanc" were selected. Both populations are maintained in the fields of Geilweilerhof. Source genotypes contained in the pedigrees of "Regent," Gf.Ga-47-42 and "Villard blanc" were sampled from the germplasm collection at Geilweilerhof.

DNA was isolated from young, healthy leaves following the protocol of Thomas et al. (1993) or using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

RAPD markers for conversion

Six RAPD markers linked to the major QTL region for powdery mildew resistance (Fischer et al. 2004) were chosen for conversion. The 10-mer RAPD primers A7, M2, M3, N3, N9, and N12 (Operon Technologies, Alameda, CA and Roth, Karlsruhe, Germany) were used in standard 50 µl PCR reaction mixtures according to Williams et al. (1993). Resulting amplification products were separated by electrophoresis on 1.5% agarose gels in 0.5 × TBE buffer (44.5 mM Tris-borate, 1.25 mM EDTA, pH 8.3) at 8 V/cm. Banding patterns were visualized under UV light (λ = 312 nm) after staining with Ethidium bromide (0.5 µg ml^{-1}) and documented with a Polaroid digital camera.

Cloning and sequencing of RAPD products

Selected RAPD marker bands were excised from 1.5% agarose gels with a sterile cutter and the DNA was purified using the QIAquick Gel Extraction kit (QIAGEN, Hilden, Germany). These fragments were ligated into the pDrive Cloning Vector (QIAGEN) and transformed into *Escherichia coli* strain DH5α using the protocol of Sambrook et al. (1989). Each 180 µl transformation mixture was spread on Luria Bertani solid

medium (LB, 10 g Trypton, 5 g NaCl, 5 g yeast extract, 14 g agar l^{-1}) containing 30 μg ml^{-1} Kanamycin, 80 μg ml^{-1} X-Gal, 40 μg ml^{-1} IPTG and cultured at 37 °C. White colonies were grown overnight in 5 ml of LB liquid medium supplemented with 30 μg ml^{-1} Kanamycin and used for plasmid DNA preparations according to Birnboim and Doly (1979). The size of DNA inserts was determined after *Eco*RI (Boehringer Mannheim, Germany) restriction digestion by separating fragments in 1.5% agarose gels. Several positive clones of each selected fragment were sequenced (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Weiterstadt, Germany) and analyzed on an automated laser-based capillary electrophoresis detection system (3100 Genetic Analyzer, ABI Prism; Applied Biosystems, Weiterstadt, Germany). The sequences were edited and compiled in DNasis v. 2.5 (Hitachi Software Engineering Ltd., Tokyo, Japan) and analyzed further using software from the HUSAR (Heidelberg Unix Sequence Analysis Resources, http://genome.dkfz-heidelberg.de) software package.

Design of SCAR markers and their amplification

From each sequenced RAPD marker sequence oligonucleotides to be tested as SCAR primers were designed. Nucleotides up to an overall length of 21–26 b were attached to the 3′-end of the original RAPD primer. The newly developed primer pairs were tested for their amplification of corresponding markers. PCR reactions were performed in a total volume of 25 μl containing 0.1 mM of each dNTP, 0.4 μM of each primer, 0.5 U of *Taq* DNA polymerase (Roche, formerly Boehringer-Mannheim, Germany) and approximately 20 ng of genomic DNA in 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3). Temperature profiles were run in GeneAmp PCR System 9,700 thermocyclers (*PE* Biosystem, Weiterstadt, Germany) and consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing for 1 min and a synthesis step at 72 °C for 2 min; ending with a final extension at 72 °C for 10 min. Amplification products were separated by electrophoresis on 1.5% agarose gels in 0.5 × TBE buffer, stained with Ethidium bromide (0.5 μg ml^{-1}), visualized under UV light (λ = 312 nm) and documented with a Polaroid digital camera.

Marker confirmation by hybridization

PCR products to be analyzed for their homology with the original marker fragments were run on agarose gels and blotted according to Southern (1975) onto positively charged nylon membranes for hybridization with PCR-labeled probes using nonradioactive Digoxigenin-labeling and chemiluminescence detection chemistry according to the DIG Application Manual (Roche Molecular Biochemicals, Mannheim, Germany).

QTL analysis

QTL interval mapping (Lander and Botstein 1989) was performed using MapQTL 4.0 (Van Ooijen et al. 2000) with the genetic map from "Regent" × "Lemberger" (Welter et al. submitted) in combination with the field evaluation data for resistance to powdery mildew as described in Fischer et al. (2004).

Results

Identification of RAPD markers linked to powdery mildew resistance and conversion into SCAR markers

Genetic mapping and QTL analysis in the population of "Regent" × "Lemberger" segregating for resistance to the mildew fungi originating from "Regent" had identified one region for resistance to *U. necator* (the causal agent of powdery mildew) on "Regent" linkage group 16 (Fischer et al. 2004). This QTL extends over less than 10 cM (delimiting its extension by the drop of significance by two LOD scores from the peak maximum) but is covered predominantly by RAPD- and AFLP markers that are difficult to transfer for analysis of genetic material different from the original mapping population. To make markers from this powdery mildew resistance

region available as molecular tools for breeding purposes (characterization of genetic resources and development of marker-assisted selection), a selection of RAPD markers originally produced with dekamer oligos from that region were cloned from amplifications with "Regent" genomic DNA and DNA from the resistant individual #32 of the progeny. The marker fragments were sequenced to design longer primer pairs for the amplification of SCAR markers under highly stringent conditions at annealing temperatures of at least 52 °C. In total nine pairs were developed as SCAR markers (Table 1).

Test and mapping of SCAR markers to the major QTL for powdery mildew resistance

All the newly developed SCAR markers were tested for maintenance of the location of their PCR products in linkage to the powdery mildew resistance QTL in "Regent" by genetic mapping. The primer pairs of ScORM3-R1, ScORM3-R2, ScORM3-32, ScORN12-32, ScORN9-R, and ScORA7-760 showed amplification products of expected sizes according to the original RAPD product sequence. Some of the markers (ScORN3-R, ScORN3-32, and ScORM2-R2) produced multiple bands. PCR products of ScORA7-760 and ScORN3-R mapped back to the original linkage group containing the major QTL to *U. necator* resistance at high statistical stringency of linkage (LOD ≥ 6.0). The two markers ScORM2-R2 and ScORN12-32 were found associated to the same group at lower LOD values but were not exactly positioned. In agreement with international nomenclature rules developed by the International Grape Genome Program (IGGP, www.vitaceae.org), this linkage group has now been designated LG15 (Fig. 1) and mean while has been detailed by the inclusion of further markers (Welter et al. submitted). Positional shifts observed for ScOR markers as compared to the original RAPD markers are probably due to the higher experimental stringency applicable to SCAR markers and more reliably generated PCR products.

Table 1 Sequence-specific oligonucleotide primers developed from RAPD marker sequences and their annealing temperatures

Marker[a]	Primer	Sequence 5′–3′	Annealing temperature (°C)
ScORA7-760	A7-760 forward	GAA ACG GGT GTG AGG CAA AGG TGG	56
	A7-760 reverse	GGC CAT TAG GAA ATC AAC ATT AC	
ScORN3-R	N3Reg forward	GAA AGA AAA AAT GGT CAT CTC CTT GC	52
	N3Reg reverse	GAA GTA TGT TCA TCA TGG CTT TGT AG	
ScORM2-R2	M2R2 forward	ACA ACG CCT CAA AAA TCA AAA TCC	52
	M2R2 reverse	GAG AAG ATA TTT AGT TTG GAG ATC	
ScORN12-32	N12-32 forward	GTA CTT GTA TGC TTC TAG CTG G	56
	N12-32 reverse	CAC AGA CAC CAA ACG TAT TCA A	
ScORN3-32	N3-32 forward	GGT ACT CCC CAT TAA CGA CAG C	57
	N3-32 reverse	GTA CTC CCC CCA ACA TAG CCA T	
ScORN9-R	N9Reg forward	GCA AAA GGC TTT GGT CCA ATA CCT A	58
	N9Reg reverse	GCT ATG GGG AAC AGT TGC CAC CC	
ScORM3-R1	M3R1 forward	GGG ATG AGA TGA TCT TTT CTG	53
	M3R1 reverse	GAT AAA TAC CGA GTC AAC TGC	
ScORM3-R2	M3R2 forward	GGA TGA GCC GAA GAC TAC ATG	53
	M3R2 reverse	CAT GGA TGC TAG TTC TGC CTT AC	
ScORM3-32	M3-32 forward	GGG ATG AGG ATA TGT TCC AGT G	56
	M3-32 reverse	GGG ATG AGC AGT GTG TAT GGG	

ScORA7-760 has been developed from individual #32 but was named indicating the size of its relevant PCR product as part of the name

[a] SCAR markers designated as ScOR with appendix R indicate origin of the corresponding cloned RAPD product from the parent "Regent," while appendix -32 represents development from a sequence cloned from the *U. necator* resistant individual #32 of the progeny)

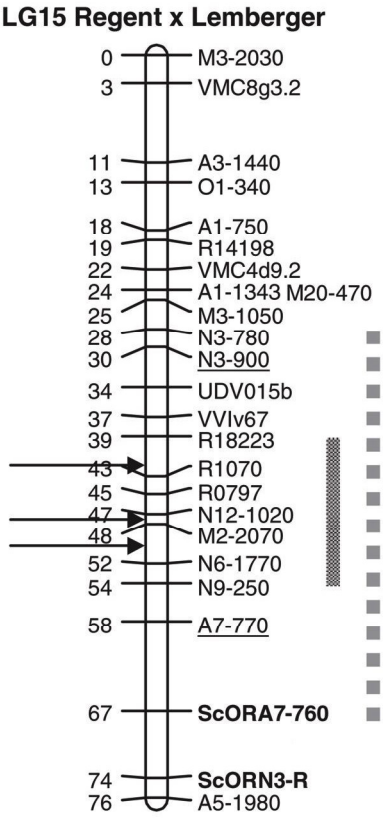

Fig. 1 SCAR markers mapped back to linkage group 15 carrying the major QTL for *U. necator* resistance in the "Regent" × "Lemberger" cross segregants (formerly "Regent" linkage group 16 as described in Fischer et al. 2004). Corresponding RAPD markers are underlined. The SCAR markers are highlighted by bold printing and flanking the QTL whose position is indicated as a dark bar as delimited statistically strictly by the decrease of the maximum LOD score by two (e.g., from LOD 10 at peak maximum to LOD 8). The dotted line shows the extension of the QTL when delimited in a more relaxed way by the decrease of the maximum LOD score by factor two (e.g., from LOD 20 at peak maximum to LOD 10). The arrows at left pinpoint to the LOD peak maxima positions observed in several years of field evaluation on *U. necator* resistance. This map has been newly calculated by inclusion of SSR markers in addition to SCARs (Welter et al. submitted) and hence slightly deviates from the map published earlier (Fischer et al. 2004)

QTL analysis with newly developed mapped SCAR markers

Data from 3 years of field observations were consulted for QTL analysis as described (Fischer et al. 2004). The correlation between developed SCAR markers and the phenotype of the resistance was investigated by repeating the interval mapping (Lander and Botstein 1989) of QTL (MapQTL 4.0; Van Ooijen et al. 2000) applying high statistical stringency (LOD ≥ 3). The SCAR markers ScORA7-760 and ScORN3-R showed a reliable correlation and flank the QTL peak for resistance to *U. necator*. The correlation of these markers was in the ranges between LOD 8.4 to 9 (ScORN3-R) and LOD 8.1 to 10.2 (ScORA7-760) for resistance of leaves as results from different years of phenotypic evaluation varied. The correlation with *U. necator* resistance of berries/clusters was somewhat reduced for both markers resulting in LOD ranges of 1.6–4.4 for ScORN3-R and 4.1–5.9 for ScORA7-760. The QTL data indicate, that the SCAR markers are reproducible and correlated with resistance to *U. necator*.

Validation of the powdery mildew-linked SCAR markers in a different segregant population and within genetic resources

Resistance breeding in grapevine currently relies on a number of particularly suited resistance donors originating from French and American hybrids generated during the nineteenth century. Their exact origin of resistance is sometimes difficult to trace back and unfortunately, not all the plant material is available any more for testing. For these reasons, it is very important to try to differentiate common and divergent sources of resistance in germplasm. This information is crucial for future pyramiding approaches in breeding for improved and durable resistance. In this context, the usefulness of SCAR markers was checked in different, but genetically related backgrounds. They were tested for their maintenance of correlation to *U. necator* resistance in a second population used for genetic mapping and segregating for fungal disease resistances. This is derived from the cross of Gf.Ga–47-42 x "Villard blanc" (Zyprian et al. 2005; Zyprian et al. in preparation). Gf.Ga–47-42 is a powdery mildew resistant breeding line generated by the cross of "Bacchus weiss" × "Seyval." "Villard blanc" is a resistant French hybrid from the cross of Seibel 6468 and Seibel 6905 ("Subereux"). These parental lines are related to each other as well as to

"Regent" to various degrees as evident from their pedigrees elaborated from literature (Fig. 2). Ten individuals with high-level powdery mildew resistance and 10 susceptible individuals were selected for profiling using the SCAR markers from "Regent." Two markers, ScORA7-760 and ScORN3-32 maintained their correlation to *U. necator* resistance in this genetic background, although to different extent. Marker ScORA7-760 kept its correlation with resistance at approximately 90% (Fig. 3), ScORN3-32 to roughly 70%. Marker homology was confirmed by using the original marker amplification products as probe in hybridization experiments.

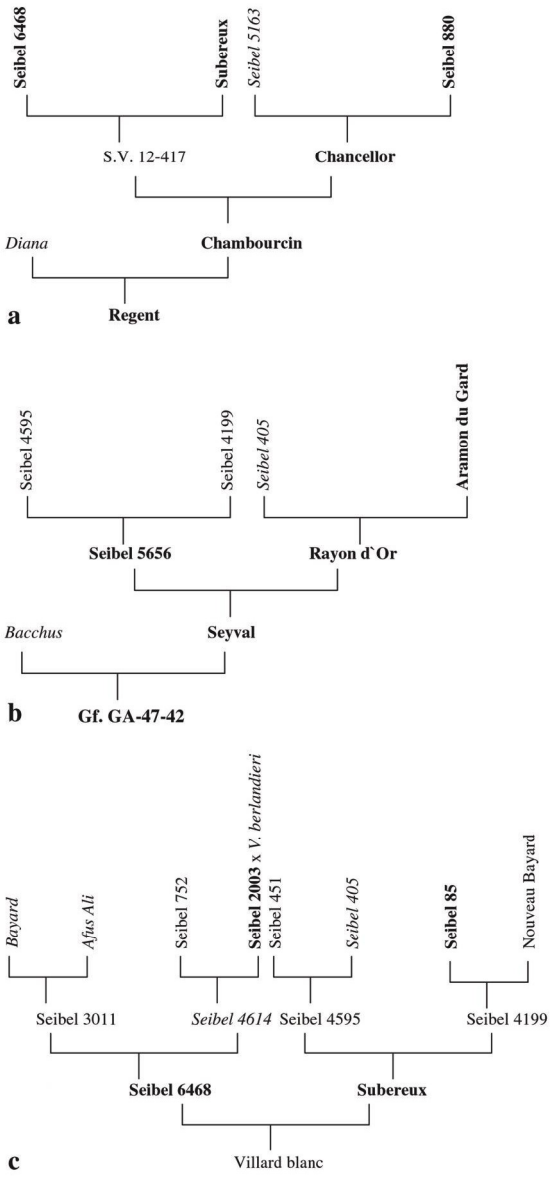

Fig. 2 Comparison of pedigrees three generations back for (**a**) the resistant high quality variety "Regent," (**b**) the resistant breeding line Gf.Ga-47-42, and (**c**) the resistant French hybrid "Villard blanc." Plants positive for the 760 bp band of ScORA7-760 are indicated by bold printed names. Grapevines typed in italics showed absence of this band. Other ancestors shown were unavailable for sampling. *Note*: "Subereux" is Seibel 6905 and a full sibling of Seibel 5656

Marker ScORA7-760 was further applied in PCR assays with source genotypes contained in the three pedigrees as deduced from the literature and as far as still available. Ancestors showing the characteristic 760 bp band linked to powdery mildew resistance in "Regent" are indicated in Fig. 2. The results show that "Regent" may have inherited this band from Seibel 6468, "Subereux" or Seibel 880. Seibel 6468, Subereux, and Seibel 880 have been evaluated as resistant to *U. necator* (R. Eibach, pers. comm.) In Gf.Ga–47-42 it may have been transmitted from Seibel 5656 or "Aramon du Gard," both described as resistant, while "Villard blanc" may have received it also from Seibel 6468 or "Subereux," derived in the "Subereux" branch from Seibel 85. The phenotype of Seibel 85 in respect to behaviour versus *U. necator* is not known at present.

Considering this data, it seems that ScORA7-760 possesses diagnostic value and can serve to indicate resistance factors of a specific type with useful probability in the characterization of genetic resources.

Marker ScORA7-760 thus appears particularly suited for marker-assisted selection (MAS) and the characterization, respectively, differentiation of genetic resources in grapevine resistance breeding.

Discussion

The aim of this work was the development of molecular markers associated with resistance to powdery mildew (*U. necator*) suitable for MAS and characterization of genetic resources in grapevine breeding programs.

RAPD polymorphisms used as the basis for marker development can be caused by differences

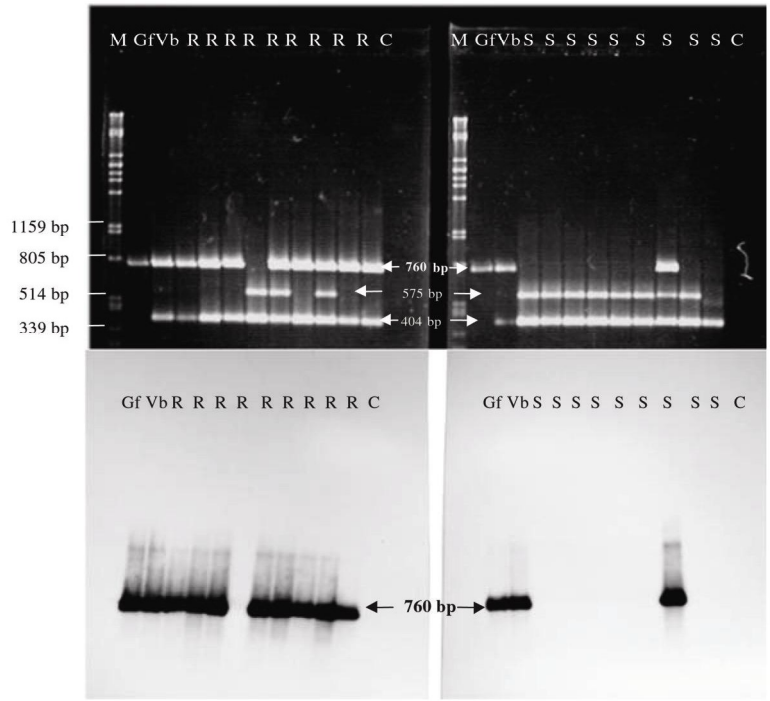

Fig. 3 Test of marker ScORA7-760 from the powdery mildew resistance region of "Regent" in selected progeny of the cross Gf.Ga-47-42 x "Villard blanc." Amplification products were resolved on 1.5 % agarose gels (upper part): M: size standard, lambda DNA cut with *Pst*I, Gf: Gf.Ga-47-42; Vb: "Villard blanc," R: resistant individuals; S: susceptible individuals; C: control without template DNA added to the PCR assay. The diagnostic band is the upper one with 760 bp in size. The lower part shows the results of Southern hybridization with the 760 bp amplification product of ScORA7-760 from "Regent"

in nucleotide sequences at the priming sites or by structural rearrangements within amplified sequences. The method uses extremely short primers and low annealing temperature, making likely that RAPD amplification can also be initiated from genomic sites that do not perfectly match the primer sequence (Paran and Michelmore 1993). Annealing and amplification efficiency become very sensitive to slight modifications of reaction conditions (Büscher et al. 1993) and spurious PCR inhibitors that may be present in DNA preparations, particularly from woody plants. These factors lead to problems of reproducibility and transferability of RAPD markers between different laboratories and between different genetic resources of grapevine breeding material. For this reason six-RAPD markers from the genetic map of "Regent" × "Lemberger" in correlation with *U. necator*-resistance on former linkage group 16 of "Regent" (Fischer et al. 2004) were converted into SCAR markers. Such markers are experimentally more stable and applicable in marker-assisted selection approaches (Adam-Blondon et al.1994; Mejia and Hinrichsen 2003). Paran and Michelmore (1993) were the first to describe SCAR markers developed in lettuce, which were associated with resistance to downy mildew, and Naqvi and Chattoo (1996) developed a SCAR marker for a dominant blast-resistance gene in rice. In grapevine, the first SCAR marker developed was for seedlessness, a trait of high relevance for table grape breeding (Lahogue et al. 1998). Currently, there is a plentitude of SCAR marker development for crops described in the scientific literature, especially for disease resistance traits (e.g., Julio et al. 2006).

In this study a major QTL region related to powdery mildew resistance in grapevine was used for the development of SCAR markers. Two markers from this region maintained their correlation with resistance after conversion as checked by QTL analysis and investigation in a second

resistance-segregating F1 population. In this context it is noteworthy to point out, that the amplification products of ScORN3-R and ScORN3-32 are not identical in sequence, although they both originated form the same RAPD primer N3 and are very similar in size. The possibility that both represent different alleles of the same locus is currently under investigation.

These studies serve to identify genomic regions responsible for important traits and to develop trait-linked markers as molecular tools for breeding purposes. Such efforts not only cover the development of marker-assisted selection procedures to avoid long-term phenotypic evaluation, but also concern the characterization and differentiation of genetic resources with potential contributions to resistance breeding programs. Fungus disease resistance can be introgressed from the gene pool of American or Asian *Vitis* wild species into the generally susceptible *V. vinifera* cultivars used for wine and table grape production. The wild species employed as resistance donors may possess divergent resistance mechanisms that are suitable to be combined in pyramiding approaches to ensure high level and long-lasting resistance in the newly bred resistant varieties. Hence it is necessary to distinguish different genetic resources and mechanisms that may be operating. Molecular markers linked to the traits, and to individual genes responsible for different resistance mechanisms as to be elaborated in the near future, should be of major advantage.

For this purpose it is required to develop experimentally reliable marker systems that are applicable to screen large numbers of individual genotypes. Complicated or high-technology analytical systems for markers will be difficult and too expensive to apply. To this end some of the *U. necator* resistance-linked markers from ''Regent'' were converted into SCAR markers and validated in different genetic backgrounds. Two markers seem to be useful, as they kept their correlation in a different resistance-segregating population. Especially ScORA7-760 produced its characteristic amplification product predominantly in resistant individuals. This marker was further tested in the collection of still available ancestral varieties of the three resistant grapevines ''Regent,'' Gf.Ga-47-42 and ''Villard blanc''. The pedigrees have been deduced from literature and not yet verified on a molecular basis in our hands.

Within this sample set, ScORA7-760 seems to keep its correlation to resistance at a useful level. As the genetic basis for resistance breeding is narrow, and resistance donors originating mostly from nineteenth century cross breeding have been used multiple times in the past, many breeding lines and cultivars are related to some extent. Markers like ScORA7-760 may be helpful to identify redundant sources of resistance. This knowledge based on molecular marker analysis enables the breeders for the first time to avoid redundancy in their choice of resistance donors and instead combine different sources of resistance with possibly varying and hence potentially accumulating resistance mechanisms in pyramiding approaches. Rigorous further testing of SCAR markers in a large variety of genetic resources needs to be performed to evaluate their diagnostic power in more detail.

Experimentally simple markers in linkage to fungal disease resistance in grapevine have so far been developed for monogenic *U. necator* resistance from *Muscadinia rotundifolia* (Barker et al. 2005), a grapevine species that is difficult to use in breeding procedures due to its increased chromosome number ($2n = 40$) as compared to *Vitis* ($2n = 38$) species. However, these markers may prove useful in combination with ScORA7-760 to follow pyramidization in the special cases of combining resistance from Muscadinia and American *Vitis sp*.

Plasmopara viticola (downy mildew) resistance also has been labeled in a wild species of grape native to China (*V. quinquangularis*) with a tightly linked RAPD marker that was converted into a SCAR (Luo et al. 2001). Our efforts to map this marker in ''Regent'' remained unsuccessful, but it may be useful for the characterization of Asian germplasm.

Recent improvement of the ''Regent'' genetic map by inclusion of a number of microsatellite-based SSR (simple sequence repeat) markers (Welter et al. submitted) has identified some of these in the vicinity of the *U. necator* resistance QTL (UDV015b and VVIv67 in Fig. 1). As they

are codominant markers correlation of specific allele sizes with resistance-carrying haplotypes can be established. These markers may be combined with ScORA7-760 to follow inheritance of the *U. necator* resistance QTL. Such studies are under way. However, SSR investigations require technically more elaborated analytical systems to determine the exact allele sizes with manual or automated sequencing equipment. SCAR markers have the advantage to be scorable on simple agarose gels making their use in screening high numbers of germplasm samples and progeny highly efficient.

Pedigree comparisons and analysis of ancestral lineages demonstrate a currently rather narrow genetic basis of grapevine resistance breeding. For future improvements it will be advisable to search, identify and exploit new resistance donors. Efforts to this end are under way.

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Integration of Microsatellite- and Functional Gene-Based Markers for the Improvement of a Grapevine Genetic Map[1]

Abstract

A mapping population derived from the cross between the grapevine (*Vitis vinifera*) cultivars 'Regent' and 'Lemberger' was used for the construction of a genetic map and the detection of putative QTLs involved in resistance to *Uncinula necator* and *Plasmopara viticola*. 'Regent' shows high field resistance against both diseases. Different types of molecular markers were used to genotype 144 F_1 descendants of the cross. The genotypic information obtained with this set of markers was analyzed by JoinMap 3.0 software for the construction of the genetic map. The map information was combined with phenotypic evaluations of five years for QTL analysis using MapQTL 4.0 software. Four hundred and thirty markers could be mapped employing the Kosambi function at a LOD score threshold of 7.0. The markers were distributed in 19 linkage groups with total coverage of 1,585 cM and mean distance between markers of 3.67 cM. Resistance factors to both diseases were detected: one major QTL for resistance to *U. necator* and one major and three minor QTLs against *P. viticola*. RGA- and functional gene-based markers co-locating with the two major QTLs were also identified. This provides first insights about the functional role of those regions. The genetic and molecular information of this map is also being used for physical mapping of the regions involved in the resistance to *U. necator* and *P. viticola*. Additionally, the present map is combined with two other maps, for the generation of a consensus map (see Zyprian et al., this conference).

Keywords: genetic map, QTL analysis, *Uncinula necator*, *Plasmopara viticola*, resistance candidate genes, *Vitis*

[1] Welter LJ, Akkurt M, Salakhutdinov I, Göktürk-Baydar N, Eibach R, Töpfer R and Zyprian E (2008) Acta Horticulturae. In press.

Introduction

The construction of genetic maps is a well established strategy to elucidate grapevine genetics and to improve breeding. Several genetic maps have been published in the last decade (Lodhi et al., 1995; Dalbó et al., 2000; Doligez et al., 2002; Grando et al, 2003; Adam-Blondon et al., 2004; Doucleff et al., 2004; Fischer et al., 2004; Riaz et al., 2004; Doligez et al. 2006). The use of mapping populations segregating for different characters (qualitative or quantitative), e.g resistance to pathogens, allowed the detection of genomic regions correlating with the desired traits (Krivanek et al. 2006; Fischer et al. 2004; Donald et al. 2002). Genetic maps are additionally useful for the construction of physical maps (Barker et al. 2005), allowing the cloning of important genes (map-based cloning).

The development of a large number of microsatellite markers contributed significantly to grapevine genetic mapping studies. Microsatellites are co-dominant, highly polymorphic and reproducible markers. In addition, they are well conserved in *Vitis* species. These characteristics allow not only the construction of genetic maps of both parental types (as in the case of dominant markers), but also the integration of both parental maps into a consensus (Adam-Blondon et al., 2004; Riaz et al., 2004). In the same way, the genetic information obtained from different crosses can be combined (Doligez et al. 2006). Genetic maps based on commonly placed microsatellite markers can be directly compared.

Considering the advantage of microsatellite markers we decided to use them to improve a previously constructed genetic map, based mainly on dominant markers (Fischer et al. 2004). The implementation of microsatellite markers permitted the construction of a consensus map of the parents ‘Regent’ and ‘Lemberger’. This consensus map was then used to detect factors influencing the quantitative resistances to *Uncinula necator* and *Plasmopara viticola*, present in the cv. ‘Regent’. Additionally, resistance candidate genes selected from an EST-(expressed sequence tag) library and some RGA-based markers (Di Gaspero and Cipriani 2003) were analyzed for co-location with resistance factors to the two diseases investigated.

Materials and methods

Plant Material

The population employed for the construction of the present map consisted of 144 F_1 plants derived from the cross between 'Regent' and 'Lemberger'. ‘Regent’ shows resistance to *U. necator* and *P. viticola* (Anonymous, 2000).

Phenotypic Evaluation

The mapping population was scored for the level of resistance to *U. necator* and *P. viticola* during five growing seasons (1999, 2000, 2003, 2004 and 2005), according to the OIV (Organisation Internationale de la Vigne et du Vin/International Wine Organisation) classification.

Molecular Genotyping

Vitis microsatellite markers derived from different sources were used to screen for polymorphisms. The polymorphic markers were organized for multiplex PCR and applied to genotype the entire mapping population. The forward primer of all marker-flanking primer pairs were labeled with fluorescent dyes and the PCR products were analyzed by capillary electrophoresis using the ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, California/USA).

Additionally, RGA-derived markers developed by Di Gaspero and Cipriani (2002 and 2003 and G. Di Gaspero, pers. comm) and candidate genes for resistance selected from a ‘Regent’ EST (expressed sequenced tag)-library (Salmaso et al. 2004) were analyzed. The SSCP (Single-Strand Conformational Polymorphism) method was applied to search for informative polymorphism as described by Schneider et al. (1999).

Data Processing

The genotypic information was subjected to linkage and recombination analysis with JoinMap 3.0 software (Van Ooijen and Voorrips, 2001), using the Kosambi function (Kosambi, 1944) and LOD (logarithm of the odds) score thresholds equal or greater than 6.0.

QTL interval mapping of phenotypic characteristics was performed using MapQTL 4.0 (Van Ooijen et al. 2000). QTLs reproducibly detected through at least three years of investigation with a LOD score greater or equal to 3.0 were considered to be consistent.

Results and discussion

One hundred and twenty one microsatellite markers were used to genotype the mapping population. The information obtained using these markers was combined with the markers previously analyzed by Fischer et al. (2004) for the construction of a new consensus map of the cross 'Regent' x 'Lemberger'. The resulting consensus map contains 432 markers aligned along 19 linkage groups and covering a total map distance of 1585 cM. The average distance between markers is 3.67 cM (Table 1).

Genomic regions correlated with resistance to both diseases studied were observed. For *U. necator* only one major QTL, located on linkage group (LG) 15 could be detected. Otherwise, one major and three minor QTLs for resistance to *P. viticola* were identified. The major QTL is located on LG 18 and minor QTLs were identified on LGs 4, 5 and 12, respectively.

In total, 21 functional gene markers derived from the 16 resistance candidate genes investigated (Table 1) and 13 RGA-markers derived from five RGA-primer pairs tested, could be mapped (Table 1). Interestingly, some RGA- and functional gene-derived markers are co-located with the major QTLs for resistance to *U. necator* and *P. viticola*. For example, the three markers amplified with RGA primer pair “rgVamu137” and two markers amplified with the primer pair “IIIb08” are located approximately in the middle of

Table 1. Summary of the information from the 'Regent' x 'Lemberger' consensus map and QTL analysis

LG[a]	Length (cM)	No. total of markers	Microsatellite markers	Average distance	Resistance QTLs to
1	77	30	7	2,56	
2	82	18	6	4,56	
3	54	14	7	3,86	
4	92	17	7	5,41	
5	88	28	11	3,14	*P. viticola*
6	93	13	6	7,15	
7	77	20	4	3,85	
8	106	23	8	4,61	
9	42	5	1	8,40	
10	66	8	6	8,25	
11	87	19	7	4,58	
12	80	31	7	2,58	*P. viticola*
13	77	21	7	3,67	
14	116	21	7	5,52	
15	76	29	4	2,62	*U. necator*
16	86	31	5	2,77	
17	90	24	4	3,75	
18	106	51	9	2,02	*P. viticola*
19	90	29	8	3,10	
Total	**1585**	**432**	**121**	**3,67**	

the major QTL for resistance to *P. viticola*. The primer pair “rgVamu137” was designed to amplify a Toll-interleukin-type receptor nucleotide-binding site (TIR-NBS-LRR) RGA class (Di Gaspero and Cipriani, 2002). The marker IIIb08 was designed to amplify a putative receptor protein kinase gene. Both gene classes are known to play an important role in pathogen recognition and/or signal transduction.

This work allowed not only the detection of genomic regions correlated with resistance to *U. necator* and *P. viticola*, but also the identification of resistance candidate genes co-located with the genetic regions covered by the two major resistance QTLs to both diseases. It provides first insights about the functional role of those QTLs. As expected for biotrophic pathogens, the two QTLs with the largest effect on resistance to *U. necator* and *P. viticola* could be involved in pathogen recognition, signal transduction and/or downstream activation of defense responses. Additionally, this map is being used to

support the physical mapping of regions correlated with resistance as delimited in the QTL analysis.

Table 2. Putative function and localization in the consensus map of 'Regent' x 'Lemberger' of RGA-derived markers (Di Gaspero and Cipriani, 2002 and 2003) and "functional" markers developed from a 'Regent' EST-library

Markers	Putative function	Linkage group
01. Ia01	(+)-delta cadinene synthase Isoenzyme A	18
02. Ia06	Myb-related protein precursor	8
03. Id04	Motility protein B	3
04. Ih05	Olee1-like protein precursor	8
05. IIa05	Probable glycerate dehydrogenase	2
06. IIb05	Dicyanin (*L. esculentum*)	11
07. IIb12	Calcium dependent protein kinase	7
08. IIf04	Putative GTP-binding protein	12
09. IIIb08	Receptor protein kinase-like protein	18
10. IIIc03	DNA binding protein EREBP-3	19
11. IIIc08	Response regulator 9	1
12. IVb08	Snakin-1 (active against plant pathogens)	18
13. IVe09	Putative phosphoinositide kinase	1
14. IVh09	Putative phosphoesterase	15
15. DRP206	Disease resistance response protein 206	4
16. RGA	Resistance-gene-analogue (RGA)-like protein	14
17. stkVr001	RGA (RLK)	1
18. rgVrip158	RGA (NonTIRb-NBS)	7
19. stkVa011	RGA (RLK)	12
20. rgVamu137	RGA (TIR-NBS)	18
21. GLP1-1A	RGA (CC-NBS-LRR)	15

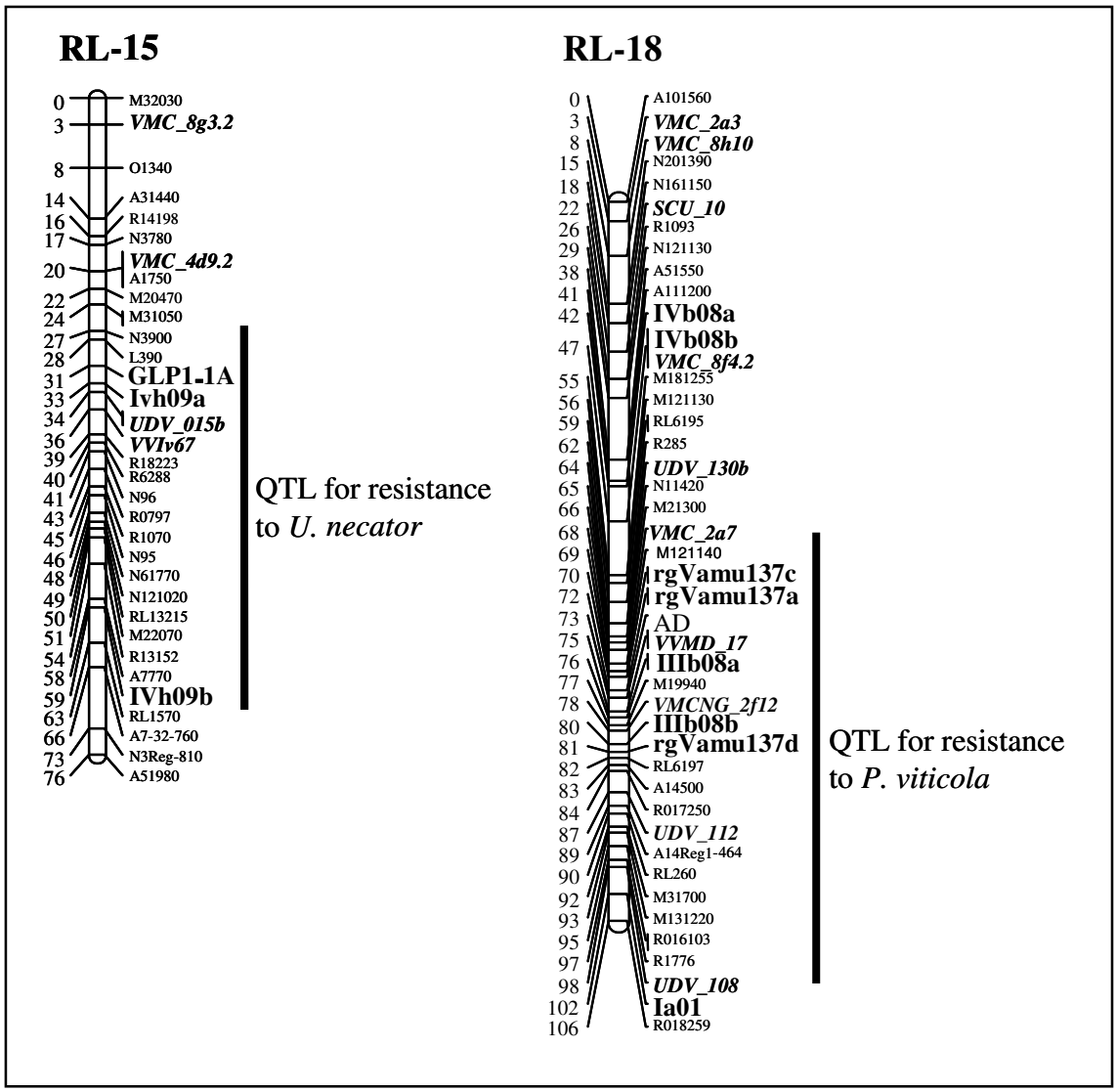

Fig 1. Linkage groups showing the major QTLs for resistance to *P. viticola* and *U. necator*. RGA- and functional-derived markers are highlighted.

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Untersuchungen potentieller Mehltauresistenzgene der Weinreben[1]

Pflanzen werden von einer Vielzahl an Schaderregern bedroht. Um diese erkennen und sich vor ihnen schützen zu können, haben sie im Laufe der Evolution verschiedene Abwehrmechanismen entwickelt. Sie besitzen z.B. quasi `Sensoren´ auf der Oberfläche ihrer Zellen, die eine Erkennung verschiedener Krankheitserreger ermöglichen und sofort eine Abwehrreaktion einleiten. Zusätzlich zu solchen allgemeinen Erkennungsstrukturen besitzen Pflanzen hochspezifische Resistenz-Proteine, die genau erkennen können, welcher Parasit angreift. Man geht davon aus, dass diese Rezeptor-Proteine mit Signal-Molekülen von Pathogenen direkt oder indirekt in Wechselwirkung treten können und nach der Erkennung ein Signal weiterleiten, was zu einer massiven "Abwehrreaktion" führt. In der Regel führt die Abwehrreaktion zum Absterben der angegriffenen Zellen. Sichtbar wird dieser Vorgang durch Ausbildung einer kleinen, eng umgrenzten Nekrose.

Bis 2005 konnten bereits 62 Resistenzgene aus unterschiedlichen Pflanzenarten isoliert werden, welche die Bildung solcher Resistenz-Proteine kodieren. Diese Resistenzgene führen zur Resistenz gegenüber biotrophen (d.h. sich von lebenden Zellen ernährenden) mikrobiellen Angreifern, wie Pilzen, Bakterien oder Viren. Ungefähr zwei Drittel dieser Gene kodieren für Proteine, die spezielle Strukturkomponenten wie eine NBS (Nucleotide Binding Site, Nukleotidbindestelle) und eine LRR (Leucine Rich Repeat, Leucin-reiche wiederholte Aminosäureabfolge) Domäne besitzen (Abb. 1). Schon früh wurde erkannt, dass die aus unterschiedlichen Pflanzenarten isolierten Resistenz-Proteine in den Bereichen ihrer speziellen Strukturkomponenten (Domänen) Ähnlichkeiten untereinander aufweisen, v.a. im NBS-Anteil. Mit molekulargenetischen Werkzeugen ergab sich dadurch die Möglichkeit, speziell zu `konservierten´ Bereichen im NBS-Abschnitt vergleichende Untersuchungen an Resistenzgenkandidaten in unterschiedlichen Pflanzen vorzunehmen. Die konservierten Domänen kommen nur in Resistenzgenen vor, deshalb bezeichnet man die vervielfältigten Sequenzen als "Resistenzgenanaloga" (RGA). Mit dieser Strategie konnte eine beträchtliche Anzahl an Resistenzgenanaloga in unterschiedlichen Pflanzenarten identifiziert werden. In weiteren Untersuchungen hat man feststellen

[1] Welter L; Eibach R, Töpfer R and Zyprian E (2008) Deutsches Weinbau Jahrbuch, Verlag Eugen Ulmer, p. 66-71.

können, dass diese RGA häufig in der Nähe von Resisistenzgenen liegen oder sogar echte Bestandteile von ihnen sind.

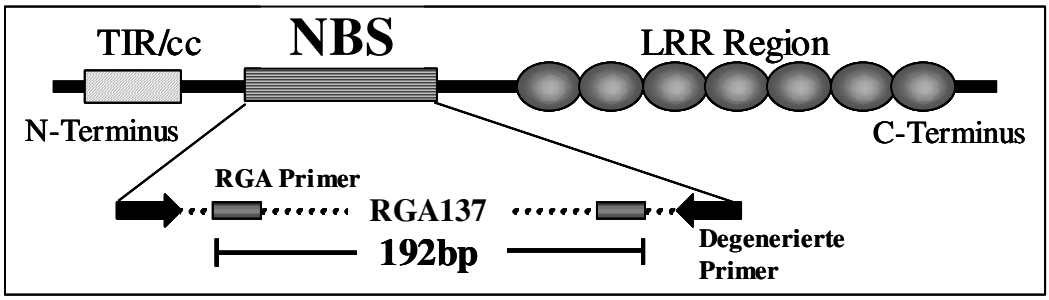

Abb. 1. Schematische Darstellung eines NBS-LRR Proteins, kodiert von einer bestimmten Klasse von Resistenzgenen; die typischen konservierten Domänen (TIR/CC, NBS und LRR) sind gezeigt. Herausvergrößert ist der NBS-Bereich, der für die Entwicklung der RGA-Marker benutzt wurde. Position und Ausdehnung des Genausschnittes, welcher im Fall des Markers RGA-137 erfasst wird, sind gezeigt. Nach Di Gaspero und Cipriani (2003), verändert.

Speziell für die Weinrebe haben Kollegen aus Italien (Universität Udine) zahlreiche RGA-"Marker" entwickelt (Di Gaspero and Cipriani, 2002 und 2003) und untersucht. Somit kennt man einen Teil der DNA-Sequenz der vermutlichen Resistenzgene, kann jedoch noch keine Aussage über die wahre Funktion dieser Gene machen. Falls ein RGA eine Funktion in der Pathogenabwehr hat, ist nicht bekannt, gegen welchen Schaderreger es wirkt. Als erster Schritt erfolgt daher häufig die genetische Kartierung der RGA-Marker in einer Kreuzungsnachkommenschaft, um festzustellen, ob diese in der Nachbarschaft zu bekannten Resistenzfaktoren liegen, d.h. mit diesen genetisch "gekoppelt" sind.

Wie bereits früher im Deutschen Weinbaujahrbuch berichtet, arbeitet das Institut für Rebenzüchtung Geilweilerhof seit längerer Zeit in der Aufklärung der biologischen Grundlagen der Resistenz, hauptsächlich gegenüber dem Echten und dem Falschen Mehltau (*Erysiphe necator* und *Plasmopara viticola*) bei Weinreben. So wurden gezielt Elterntypen ausgewählt (davon mindestens einer mit Resistenz gegenüber dem Echten und Falschen Mehltau) und miteinander gekreuzt. Die entstandenen Nachkommenschaften

wurden im Freiland über mehrere Jahren auf ihre Resistenzeigenschaften hin bewertet. Parallel zur Erfassung der Resistenzdaten wurden die Nachkommenschaften bezüglich der Vererbung der Resistenz mit molekularen Markern untersucht und genetische Karten konstruiert (Zyprian et al., 2004; 2006). Mit Hilfe biostatistischer Methoden wurden die Informationen aus den genetischen Karten mit den Resistenzdaten kombiniert und die Lage der Resistenzfaktoren gegen den Echten und Falschen Mehltau auf den Chromosomen in den genetischen Karten lokalisiert (Deutsches Weinbau Jahrbuch 2004 und 2006). Im Ergebnis sind damit relativ eng begrenzte Regionen auf einzelnen Chromosomen erfasst in denen jedoch immer noch dutzende bis hunderte Gene liegen. Der direkteste Weg zur Identifizierung einzelner Resistenzgene wäre die Sequenzierung des entsprechenden Genombereiches, was allerdings sehr Zeit- und kostenaufwändig wäre. Um gezielter vorzugehen, kann man Kandidatengene für Resistenz z. B. RGAs auswählen, diese nun in der genetischen Karte lokalisieren, und auf ihre gemeinsame Vererbung mit Resistenzfaktoren hin untersuchen.

Zu diesem Zweck wurden RGA-Marker aus der Arbeit von Di Gaspero und Cipriani (2003) ausgewählt und in den beiden Nachkommenschaften aus den Kreuzungen von `Regent´ x `Lemberger´ und `Gf.Ga-47-42´ x `Villard blanc´ genetisch kartiert. Die RGA-Marker wurden eingesetzt, um sie in beiden Kreuzungsfamilien in ihrer Vererbung zu verfolgen. Anhand der so erarbeiteten Informationen konnten die RGA-Marker in den genetischen Karten lokalisiert werden. Als Beispiel werden im Folgenden nur die mit der Resistenz gegen den Falschen Mehltau gemeinsam vererbten RGA-Marker diskutiert. Resistenzfaktoren gegen den Falschen Mehltau wurden sowohl in der Kreuzung `Regent´ x `Lemberger´ als auch `Gf.Ga-47-42´ x `Villard blanc´ in den genetischen Karten der Elternpaare identifiziert. In beiden Studien wurde ein Hauptresistenzfaktor auf Chromosom 18 lokalisiert (Abb. 2). Genau in diesem Bereich konnten interessanterweise vier Resistenzgenanaloga kartiert werden, nämlich RGA104, RGA108, RGA132 und RGA137. Zusätzlich liegen in diesem Chromosomenbereich noch zwei weitere Kandidatengene, Ia01 und IIIb08, welche andere Resistenzgentypen kodieren. Die Weinrebe besitzt einen doppelten Chromosomensatz (Diploidie) und daher jeweils zwei Kopien der auf den einzelnen Chromosomen liegenden Gene. Diese beiden Kopien können unterschiedlich sein (Heterozygotie).

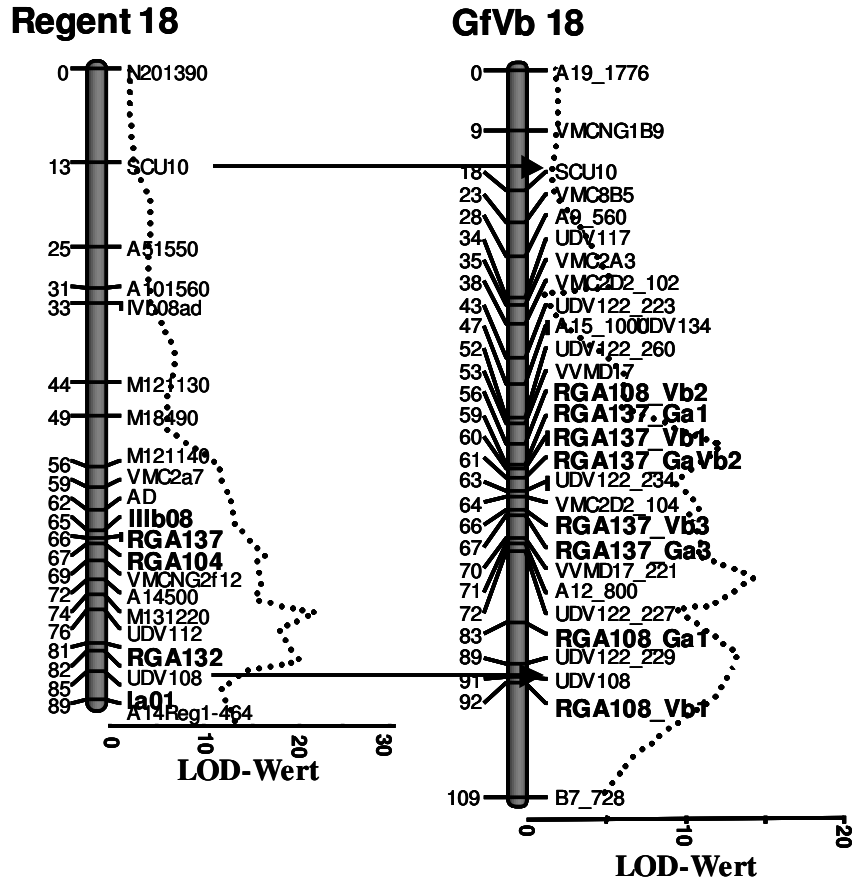

Abb.2. Wie im Text erwähnt, wurden genetische Karten aus Kreuzungsnachkommenschaften zwischen `Regent´ x `Lemberger´ und Gf.Ga-47-42 x `Villard blanc´erstellt. Hier ist die Kopplungsgruppe 18, jeweils aus der genetischen Karte von `Regent´ und aus der integrierten Karte von Gf.Ga-47-42 x `Villard blanc´, dargestellt. In beiden Karten wurden auf dieser Kopplungsgruppe (dem Chromosom 18) Resistenzfaktoren gegenüber dem Falschen Mehltau in mehreren Beobachtungsjahren gefunden. Die gepunktete Linie zeigt den Verlauf der Korrelationskurve (LOD Wert) mit Mehltauresistenz (je höher die Werte, desto signifikanter) für die Resistenzdaten aus dem Jahr 1999. Normalerweise betrachtet man einen LOD-Wert von drei als Schwellenwert für eine genetische Bedeutung der Chromosomenregion in der Ausprägung der Resistenz. Die fett gedruckten Marker sind Resistenzkandidatengene einschließlich der RGA (Resistenzgenanaloge). Man sieht, dass viele von diesen im Bereich der Resistenzfaktoren identifiziert werden konnten. Somit läßt sich feststellen, dass in beiden genetischen Karten wichtige genetische Faktoren der Resistenz gegen den Falschen Mehltau in ungefähr der gleichen Position auf Chromosom 18 gefunden wurden.

Man findet bei der Weinrebe häufig unterschiedliche Formen (Allele) des gleichen Gens. Auch im Fall des Genbereiches von RGA137 kann man unterschiedliche Allele auf den beiden Chromosomen finden. Wie die analytische Auftrennung der verschiedenen Allele des Markers RGA137 (Abb. 3) zeigt, sind bei `Regent´ und `Lemberger´ jeweils die beiden verschiedenen Allele als zwei `Banden´ zu sehen. Untersucht man die Nachkommenschaft, so läßt sich erkennen, dass jedes einzelne Individuen je ein Allel von `Regent´ und eines von `Lemberger´ ererbt hat. Mit Hilfe der Auftrennung lässt sich die Herkunft der beiden Allele jedes Individuums bestimmen. Durch die Kombination allelischer Information mit Resistenzboniturdaten lässt sich das mit Resistenz gekoppelte Allel identifizieren.

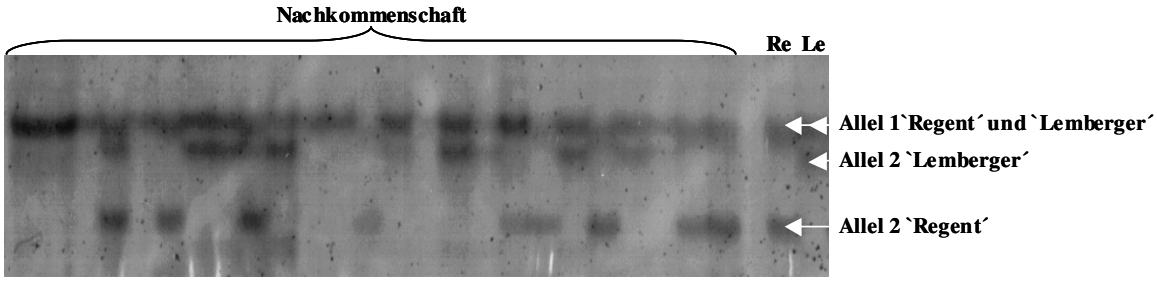

Abb.3. Ein spezielles analytisches System (MDE-Gel), in welchem sich die mit dem Marker RGA-137 amplifizierten Allele (Banden) von `Regent´ (Re) und `Lemberger´ (Le) auftrennen lassen. Deutlich wird hier auch, wie diese Allele in der Kreuzungsfamilie vererbt werden. Jedes Individuum der Nachkommenschaft erbt ein Allel von `Regent´ und eines von `Lemberger´. Die Banden werden sichtbar, indem man die durch PCR amplifizierten DNA Stückchen durch Gelelektrophorese auftrennt und mit Silbernitrat anfärbt. Die MDE-Gele haben die Fähigkeit, DNA-Fragmente nach ihrer Konformation (räumlichen Struktur) zu trennen. Diese räumliche Molekülanordnung wird durch Nukleotidaustausche beeinflusst, die somit indirekt nachgewiesen werden können.

Nehmen wir die `Regent´-Allele des Markers RGA 137 als Beispiel: Es wurden zunächst zwei Gruppen von Nachkommen gebildet: die erste hat das Allel 1 von `Regent´ und die zweite das Allel 2 von `Regent´ geerbt. Anschließend wurden diese beiden Gruppen nach den Resistenzeigenschaften jeweils in die fünf Klassen eingeteilt. Diese repräsentieren die

Boniturnoten (1, 3, 5, 7 und 9), mit denen jedes Individuum der Nachkommenschaft, basierend auf der Ausprägung der Symptome gegen die natürliche Infektion mit dem Falschen Mehltau, beurteilt wurde. Die Boniturnoten erlauben eine stufenweise Klassifizierung von völlig gesund gebliebenen Pflanzen (1) bis hin zu stark geschädigten (9). Individuen, welche das Allel 1 von `Regent´ besitzen, sind im Durchschnitt resistenter als solche, welche das Allel 2 geerbt haben. Das zeigt, dass Allel 1 mit der Resistenz `gekoppelt´ ist (d.h. häufig zusammen mit Resistenz vererbt wird). Hingegen scheinen die beiden Allele von `Lemberger´ keine Kopplung mit der Resistenz aufzuweisen, was durchaus der Erwartung entspricht, da `Lemberger´ anfällig gegen den Falschen Mehltau ist. Bei der zweiten Kreuzungsnachkommenschaft sieht das Bild ähnlich aus. Das Allel 1 von `Villard blanc´ scheint mit der Resistenz gekoppelt zu sein. Hingegen scheinen die beiden Allele von `Gf.Ga-47-42´ auf die Resistenz kaum Einfluß zu haben.

Die gemeinsame Vererbung von RGA-Markern und Resistenzfaktoren deutet darauf hin, dass diese Genklasse bei der Resistenz gegenüber dem Falschen Mehltau eine wichtige Rolle spielen könnte. Um diese Marker genauer zu analysieren, werden die unterschiedlichen Allele dieses Markers zur Zeit sequenziert. Durch Sequenzierung läßt sich die exakte Nukleotidreihenfolge (Die Abfolge der DNA-Bausteine A: Adenin, C: Cytosin, G: Guanin und T: Thymin) des Genbereiches feststellen und somit sehr präzise identifizieren, wo die feinen Unterschiede zwischen den verschiedenen Allele der untersuchten Sorten liegen. Ergebnisse der Sequenzierung des RGA137-Markers werden hier als Beispiel dargestellt.

Vergleicht man die erhaltenen acht RGA-137 Sequenzen miteinander (Abb. 4), so fällt zunächst auf, dass die acht Sequenzen untereinander sehr ähnlich sind. Insgesamt ist der Austausch einzelner DNA-Bausteine nur an sieben Positionen erkennbar. Das bedeutet, dass dieser Bereich sehr konserviert ist und die NBS-Domäne im vom Gen kodierten Protein eine Funktion in der Zelle hat, die wichtig ist und kaum durch Mutation verändert werden kann. Anhand der Sequenzvergleiche sucht man nach Mutationen, die einen direkten Zusammenhang mit der Resistenzantwort haben könnten. Das Allel 1 von `Regent´ und `Villard blanc´ sind beide mit Resistenz gekoppelt (Abb. 4) und in der Abfolge ihrer DNA Bausteine identisch (Abb. 5). Allerdings ist auch der untersuchte Teilbereich des Alleles 1 von `Lemberger´ in der Bausteinabfolge identisch, wobei dieses Allel auf die Resistenz keinen Einfluss besitzt. Dies bedeutet nicht, dass dieses vermutliche

Gen nichts mit der Resistenz zu tun hat, sondern dass die entscheidenden Unterschiede zwischen den resistenten bzw. anfälligen Allele in einem anderen Bereich des Resistenzgenanalogons liegen.

Abb. 4. DNA-Sequenzvergleich der RGA137 Allele der Rebsorten `Regent´, `Lemberger´, `Villard blanc´ und der Zuchtlinie Gf.Ga.-47-42. Bereiche in denen die acht Sequenzen Unterschiede zeigen, sind mit einer Umrandung hervorgehoben. Da die Weinrebe diploid und weitgehend heterozygot ist, konnte man zwei unterschiedliche Allele pro Sorte identifizieren (1 und 2). Allerdings wird auch deutlich, dass die Allele 1 von `Regent´, `Villard blanc´ (mit Resistenz gekoppelt) und `Lemberger´ (nicht mit Resistenz gekoppelt) in diesem speziellen Bereich die identische Sequenz haben.

Sehr interessant ist unter diesem Gesichtspunkt der Bereich der LRR-Domäne, der eine wichtige Rolle in der Erkennung des Schaderregers spielt. Untersuchungen an anderen Pflanzen haben gezeigt, dass kleine Veränderungen in diesem Bereich dazu führen können, dass das Pathogen nicht mehr erkannt werden kann, die Abwehrreaktion nicht in Gang gesetzt und somit die Pflanze anfällig wird. Deswegen ist es wichtig als nächsten Schritt das vollständige RGA zu isolieren,Varianten zu untersuchen und mit der Resistenzantwort zu verbinden. Letztendlich wird man diese Gene in ihrer Funktion genau studieren müssen. Dazu gibt es verschiedene Möglichkeiten. Eine davon wäre, das Gen in eine anfällige Sorte einzubringen und zu prüfen, ob diese dadurch resistenter würde. Dies wäre der schlüssigste Nachweis, dass dieses Gen an der Resistenzausprägung entscheidend beteiligt ist.

Das Ziel der Züchtung ist es letztendlich, verschiedene gewünschte Eigenschaften miteinander in neuen Rebsorten zu kombinieren, so z.B. Weinqualität mit hoher Widerstandsfähigkeit gegenüber Schaderregern, um einen nachhaltigen Weinbau für die Zukunft zu ermöglichen. Das Problem bei der Weinrebe ist, dass die Züchtung neuer Sorten ein langwieriger Prozess ist, da diese Pflanze einen langen Lebenzyklus hat. Deswegen ist es erforderlich, molekulare Marker zu entwickeln, die mit den gewünschten Eigenschaften genetisch gekoppelt sind. Die Verfügbarkeit solcher Marker macht den Selektionsprozess schneller und präziser, da man die gewünschten Individuen auf Grund des Besitzes der diagnostischen Marker schon früh im Zuchtgang auswählen könnte. Daher sind die hier beschriebenen RGA Marker ausgesprochen interessant und wertvoll für die züchterische Arbeit.

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Zyprian, E., Eibach, R. und Töpfer, R. 2006. Eine neue genetische Karte der Weinrebe aus der Kreuzung Gf.Ga-47-42 x `Villard blanc´. Deutsches Weinbaujahrbuch 2006, p.151-158, Verlag Eugen Ulmer, Stuttgart.

Differential gene expression in the early interaction between powdery mildew (*Erysiphe necator*) and resistant vs. susceptible grapevines [1]

Summary

Erysiphe necator Schwein., the causal agent of grapevine powdery mildew, is one of the most devastating pathogens in viticulture. Very little is known about the mechanisms involved in successful defense against this ascomycete fungus. Differential microarray hybridizations were therefore used to identify powdery mildew-responsive genes in a resistant vs. a susceptible grapevine cultivar. A subset of 27 genes detected as induced by the microarray hybridization was further investigated by quantitative real time PCR (qRT-PCR). These genes were chosen to comprise different functional classes, such as transcription factors, pathogenesis-related proteins and enzymes involved in the secondary metabolism. Their expression was studied in the resistant cv. `Regent´ compared with susceptible cv. `Chardonnay´ 10 hours post-infection with powdery mildew. The great majority of the genes evaluated were more stronger induced in `Regent´ as compared to `Chardonnay´. From the 27 genes tested, 23 genes were up-regulated (>2,0-fold) in the resistant cultivar. The annotation of these genes suggests a parallel activation of complementary defense pathways. Transcription factors found induced belong to four different families (WRKY, Myb, Ethylene-responsive factor and CZF1/ZFAR1). These have been shown to interact with specific *cis*-acting elements present in the promoter regions of pathogenesis-related genes. PR- (pathogenesis related protein) 10 and PR-5 were the most strongly activated genes, showing 50- resp. 15-fold induction. In addition, the phenylpropanoid pathway was up-regulated. Phenylalanine ammonia-lyase (PAL) was 12-fold induced. Defense related genes and pathways were clearly activated in the incompatible interaction, but only weakly in the compatible interaction, suggesting specific pathogen recognition and signalling triggering downstream defense reactions in the resistant cv. `Regent´.

Key words: grapevine, powdery mildew (*Erysiphe necator*), transcriptome analysis, defense response

[1] Welter LJ, Glissant D, Delrot S, Töpfer R and Zyprian E. Submitted to Molecular Plant Pathology.

Introduction

The establishment of monocultures accompanied by the development of elite cultivars ("green revolution") shares significant part of the considerable increase of productivity in modern agriculture. However, it also resulted in a severe reduction of genetic diversity. This standardization of cultivation favors epidemic outbreaks of plant diseases. Frequently, plant infections can only be prevented by repeated application of chemicals, which protect the cultures from pathogen damage. However, such chemical control is expensive and energy consuming and may cause serious risks for the environment as well as for human and animal health.

The utilization of the *innate* resistance of plants seems to be the best alternative for pathogen control. Elucidation of the molecular mechanisms involved in host/pathogen interactions can lead to the development of novel, environmentally safe strategies of plant protection, most adequate in the modern vision of sustainable agriculture. The investigation of various host/pathogen interaction models has contributed greatly to the elucidation of the mechanisms involved in plant defense. This information can be exploited to facilitate the investigation of resistance mechanisms in other agronomically important host/pathogen interactions, such as grapevine and powdery mildew.

The plant immune system can essentially be divided into two partially independent branches (reviewed by Jones and Dangl, 2006). The first branch, PAMP-triggered immunity (PTI), functions primarily through the extracellular recognition of microbial- or pathogen-associated molecular patterns (MAMS or PAMPS) by transmembrane pattern recognition receptors (PRRs), exposing a more or less general barrier to invaders. The second branch, effector-triggered immunity (ETI), resides in the specific recognition of a pathogen effector by an R gene (e.g. NBS-LRR), resulting in a strong defense response and frequently in hypersensitive cell death. As usually no transmembrane domain is found in the R genes, effector recognition seems to occur inside the cell. The amplitude of defense is significantly higher in ETI as compared to PTI. Besides local responses, PTI and ETI activate long-distance defense reactions, such as systemic acquired resistance (SAR). The ETI reaction seems to be only effective against biotrophic and hemibiotrophic pathogens. Recognition of the pathogen by the plant initiates a thorough transcriptional

reprogramming, resulting in a temporally and spatially coordinated expression of a battery of pathogenesis-related genes. Failure of this recognition process in general leads to susceptibility.

Erysiphe necator Schwein. (syn. *Uncinula necator*), the causal agent of grape powdery mildew, is an obligate biotrophic pathogen from the ascomycete family. Powdery mildew fungi interact specifically with their host. Microscopical investigations monitored the pathogen growth during the infection process in grapevine (Heintz, 1986; Heintz and Blaich, 1990; Rumbolz et al., 2000). After deposition of spores (conidia) on grapevine tissues, they germinate forming a germ tube. This enlarges at its distal extremity to form a structure called primary appressorium. An infection peg developed from the centre of a mature appressorium allows the pathogen to penetrate the cuticle and epidermal cell wall and intrude into the intracellular space. Once arrived within the cell the infection peg enlarges to form the haustorium, the functional structure for nutrient assimilation of the fungus from the plant cells. From this point on, an intimate contact between the pathogen and the plant cell cytoplasm is established. If colonization of the plant cell is successful, the pathogen initiates the germination of primary hyphae, followed by the development of secondary hyphae and so on, until the pathogen has propagated enough to be able to develop its reproductive structures (conidiophores) producing new conidia on the leaf surface. At maturity these are spread and promote a secondary disease cycle.

The pathogen *Erysiphe necator* originates from North America and was introduced into Europe during the middle of the 19th century. The European grapevine *Vitis vinifera* is extremely susceptible to the disease. The fungus attacks both leaves and berries, leading to considerable losses in yield and wine quality. Cultivars grown for wine and table grape production generally belong to the species *V. vinifera* and these are spread worldwide, rendering powdery mildew one of the most significant pathogens in viticulture. Enormous quantities of phytochemicals are required to prevent epidemics and control the disease. Resistant genotypes can – with one exception of a table grape cultivar from Uzbekistan (Hoffmann et al., 2008) - only be found in American or Asian wild species of grapevine in populations that co-evolved with the pathogen. Such resistant genotypes have been used in breeding programs to develop hybrids and upon backcrosses with *V. vinifera* advanced quality cultivars resistant to the disease. Although traditional breeding has achieved first resistant high-quality grapevine cultivars, the molecular mechanisms involved in the

resistance responses remained largely unknown. Elucidation of these mechanisms is a crucial step for the development of novel strategies of pathogen control and the creation of molecular tools to rationalize resistance breeding.

The knowledge about resistance mechanisms in grapevine is very limited. A resistant genotype of the American *V. aestivalis* (`Norton´) seems to have augmented endogenous salicylic acid (SA) levels (Fung et al., 2008). In susceptible grapevine cultivars investigations have shown that application of various signal molecules induced a defense response, in general leading to enhanced disease resistance. Treatments with methyl jasmonate (MeJA) stimulated the production of SA and resulted in local and systemic induction of defense-related genes (Repka et al., 2001). Complementarily, MeJA-treated leaves reacted by increasing transcript levels of genes coding for pathogenesis-related proteins and enzymes involved in the phenylpropanoid pathway, resulting in enhanced tolerance to powdery mildew (Belhadj et al., 2006). The application of beta-Aminobutyric acid (BABA) induced resistance to *Plasmopara viticola* in grapevine, and further investigations suggested the involvement of the Jasmonic Acid (JA) pathway in this BABA-induced resistance (Hamiduzzaman et al., 2005). Benzothiadiazole (BTH) induced resistance against gray mold caused by *Botrytis cinerea.* In this case, resistance was associated with the increase of total polyphenolics in berry skins, in particular the proanthocyanidin fraction was found elevated by 36% (Iriti et al., 2004). The beta-1,3-glucan laminarin, derived from the brown algae *Laminaria digitata,* was shown to be an efficient elicitor of defense responses both in grapevine cells and plants and to effectively reduce *B. cinerea* and *P. viticola* development on infected grapevines (Aziz et al., 2003; Aziz et al., 2007). The results of these experiments suggest that downstream inducible resistance mechanism (e.g. PR-Proteins) are present in susceptible plants and can be activated by signal molecules resulting in enhanced resistance to diseases. Considering powdery mildew, susceptible *Vitis vinifera* plants are probably unable to recognize the pathogen and therefore inefficient in triggering a rapid defense response. The lack of co-evolution between *V. vinifera* and the biotrophic pathogen may explain this failure of the recognition process.

Knowledge on the molecular reactions during the efficient defense response of resistant grapevines to powdery mildew is yet scarce. Therefore, differential hybridizations on *Vitis* transcriptome microarrays were applied in this study to identify genes responsive to

infection with grapevine powdery mildew in a resistant as compared to a susceptible grapevine cultivar. A subset of the modulated genes was further evaluated during compatible and incompatible interactions by quantitative real time PCR (qRT-PCR) and mapped in the grapevine genome.

Experimental procedures

Powdery mildew inoculation

Young leaves carrying sporulating infections from susceptible grapevine plants growing in the greenhouse were used as donors of powdery mildew (*E. necator*) spores (conidia). Spores were carefully collected and transferred to surface sterilized detached leaves, placed in "Weck®"glass containers containing agar. Approximately every 14 days the sporulating mildew colonies were transferred to newly detached leaves. Spores obtained after at least five cycles of asexual reproduction were used to infect *in vitro* cultivated grapevine plants. Axenic plants of the varieties `Regent´ and `Chardonnay´ were grown in "Weck®"glass containers with 0.5 x MS medium (pH 6.2) (Murashige and Skoog, 1962; Duchefa M0233, Haarlem, The Netherlands) under controlled conditions [16h photoperiod (~42 µE m^{-2} s^{-1}) and 24^0C temperature)]. Three plants were raised per glass container. The plants were inoculated with powdery mildew conidia at the 6-8 leaves developmental stage. To this purpose sporulating detached leaves were sliced into small pieces under sterile conditions. Using tweezers the spores present in the small pieces of leaf were allowed to drop directly onto each leaf of the axenic test plants. Three to five leaves per plant growing in at least five glass containers per variety (15 plants in total) were inoculated with powdery mildew. As negative control non-inoculated plants from five glass vessels of both varieties were subjected to the same experimental conditions. Ten hours after the inoculation leaves from both treatments (inoculated and non-inoculated) were collected, frozen immediately in liquid nitrogen and stored at –70 until RNA preparation. Inoculated leaves from susceptible plants were observed during the 10 following hours under a binocular and a

microscope in order to monitor the development of the fungus. Two completely independent biological replications were performed.

RNA preparation

Total RNA was isolated using modifications of the protocol described by Chang et al. (1993). The leaves obtained from each treatment were bulked and ground with liquid nitrogen using a mortar and a pestle. About 100 mg of powder was mixed with 800 µl of 65^0C-pre-warmed extraction buffer [2% CTAB (Cetyltrimethylammonium bromide), 2% PVP (Polyvinylpyrrolidone) K30, 100mM Tris-HCl (pH 8,0), 25 mM EDTA (Ethylenediamine tetraacetic acid), 2.0 M NaCl, 0.5 g/l Spermidine and 2% ß-mercaptoethanol (added just before use)] and incubated for 10 min. at 65^0C. An equal volume of Chloroform:Isoamyl alcohol (24:1) was added to the buffer-tissue homogenate, mixed by inverting during five min. and centrifuged at 8944 g for 10 min. at 4^0C. The inorganic phase was submitted to a second organic extraction as described above. The supernatant was collected and homogenized by addition of 0.25 volume of 10 M Lithium Chloride. The RNA was precipitated overnight at 4^0C and pelleted by centrifugation at 20937 g for 30 min. at 4^0C. The pellet was washed twice with 80% Ethanol and once with absolute Ethanol, dried at room temperature and resuspended in 30 µl of RNase-free-water. RNA quality and quantity were monitored by denaturating 1.5% agarose /1.0% formaldehyde gel electrophoresis and UV-visible spectrophotometry. The RNA was treated with DNase I (Qiagen) and purified immediately with RNeasy MinElute CleanUp kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. Again RNA quality and quantity were checked by agarose gel electrophoresis and spectrophotometry. High quality preparations of RNA were further used for cDNA synthesis.

Microarray hybridizations

Eighteen microgram of total RNA was used to synthesize the Cy3- and Cy5-labelled cDNA using the CyScribe Post-labeling Kit (Amersham Biosciences, UK). The purified CyDye labeled cDNA was analysed by UV-visible spectrophotometry to monitor cDNA yield and the amount of CyeDye incorporated into the cDNA products. The hybridizations were performed in the fully automated Tecan HS 4.800 Hybridization Station (TECAN,

Mannedorf/Zürich, Switzerland)) at constant temperature of 42ºC for 16 h. The fluorescently-labeled cDNA probes were hybridized to the Array-Ready Oligo Set™ for grapevine (Grape Genome Oligo Set Version 1.0) from Operon Biotechnologies (Alameda, CA, USA). Each slide contains 14,562 70mers representing 14,562 open reading frames (ORFs) from *Vitis vinifera*. The slides were scanned with GenePix 4000B scanner (Axon Instruments, now Molecular Devices, Toronto, Canada). The expression data was analysed by GenePix Pro software. Normalization of the data was performed assuming that the average expression does not change under the condition studied (average expression = 1). Transcripts showing at least 2-fold variation were considered as differentially expressed.

Primer design

A subset of 27 powdery mildew-induced genes detected in hybridization analysis was analysed by quantitative Real Time PCR (qRT-PCR). Nucleotide sequences for primer design were retrieved from public databases. These sequences were compared to the grape EST database stored at (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape) and sequences showing high similarity (possible members of gene family) were compared to each other. Primers were designed manually to attach specifically to the EST of interest and supported by Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) (T_m: 58-60^{o}C, length: 70-150 bases). The primer sequences are listed in table 1. All primers were applied in a preliminary amplification to test PCR efficiency and specificity. Dissociation curves performed for all amplificates analysed confirmed the absence of primer dimers. The amplified PCR products were separated in 2% agarose gels to verify them by fragment length.

Quantitative Real-time PCR analysis

Two step qRT-PCR was performed. In the first step, 1µg of total RNA was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer`s instructions. In the second step, the cDNA was amplified by qRT-PCR. PCR reactions were performed in 96-well plates with an ABI PRISM® 7500 sequence detection system using SYBR® Green as fluorescent reporter dye.

The genes were amplified in standard reactions of 25 µl, containing 12.5 µl Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 150-300 nM of each primer and about 25 ng cDNA of each sample. The thermal cycling conditions consisted in one initial polymerase activation step at 95^0C for 10 min., followed by 40 cycles at 95^0C for 15 s and 60^0C for one min. Raw fluorescence data were exported to Excel spreadsheets and used to estimate the PCR efficiency for each sample employing the LinRegPCR softwareV7.2 (Ramakers et al., 2003). To avoid subjective influence, the cycle threshold value (C_t) as start point and the raw fluorescence information of at least four PCR cycles were included in all samples to estimate PCR efficiency by linear regression. In every case the correlation factor (R) was greater than 0.99. The C_t value was defined by employing the 7500 Fast System SDS Software (Applied Biosystems, Foster City, CA, USA) with manual baseline (3-15) and threshold (0.2) settings. A mean PCR efficiency including all samples of each gene was calculated. Together with the C_t values of target and control samples the mean PCR efficiency was used to calculate the relative gene expression (infected against non infected) employing the REST 2005 software (V1.9.12) (Pfaffl et al., 2002). Beta-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes (Table 1).

Genetic mapping

At the time of this investigation the sequence quality of the two grapevine genome projects available was not yet sufficient to localize the genes evaluated here. Therefore, it was decided to genetically map some of the most interesting genes in a resistance segregating mapping population from the cross of `Regent´ x `Lemberger´ (Welter et al., 2007). The location of the genes, primer pairs used and expected amplicon lengths are listed in table 1. The SSCP (Single-Strand Conformational Polymorphism) method was applied to display segregating polymorphism as described by Schneider et al. (1999). Recent Blast analysis localized almost all the genes considered here in the genome sequence of the largely homozygous derivative of `Pinot Noir´ (PN40024) currently analyzed by a French-Italian consortium (Jaillon et al., 2007; http://www.cns.fr/externe/English/Projets/Projet_ML/ML.html).

Table 1. Gene bank accession number, predicted gene name, primer sequences and expected PCR-product length of the genes whose expression was evaluated by quantitative Real Time PCR during the interaction between grapevine and powdery mildew.

Gene Bank Accession	Predicted Gene Name[1]	Primer sequences (forward/reverse)[2] (qRT-PCR)	Product Length	Primer sequence[3] (Genetic mapping)	Product length
1. TC47186	WRKY DNA-binding protein, *Nicotiana tabacum*, locus BAB61053 , E=1e-18	ATTCAAGCACTAGTATGAACAGAGCAG **CCTTGTTGCCTTGGCATGA**	74	F: ACCTTTCACCCTGGAAATGTTG	307
2. TC46952	WRKY transcription factor NtEIG-D48, *Nicotiana tabacum*, locus BAB16432, E=2e-72	CGCTGGTCTCATCCTAGAATCTTC GAGCTGCTATTCTACTCCTGTTGCT	69	F: GGTTAGAGTTCCGGCAATAAGC	306
3. TC41321	WRKY-type DNA binding protein, *Solanum tuberosum*, locus BAC23031, E=3e-25	AGAGATGAGATCATTGCAGCTGAA **GTCACACCAAATCTTTGGATCTCA**	94	F: TGACCCAATCACCAAGTTATCATG	298
4. TC50306	Pathogenesis-related genes transcriptional activator PTI5, *Solanum lycopersicum*, locus O04681, E=3e-33	CACTGTTGCGGAATCACAAGA **GCCCATGGATACTTACTTTGATCA**	99	F: CTCGGACAATAGCCAAGAAGC	351
5. TC54435	Ethylene-responsive transcription factor 5, *Nicotiana tabacum*, locus Q40478 , E=3e-28	CACCGTTAACGCCGTTATCAC **TGTCTTTGGTAAGGTAAACTCCTTTGAT**	87	F: ATCTGATCCACCAGCGATATCTG	307
6. TC42111	Myb-related transcription factor VlMYBB1-2, *Vitis labrusca* x *Vitis vinifera*, locus BAC07544, E=1e-42	**CAGTCGCAGACGAATTACAGCTT** CTCCATGCCATCATCCATCA	80	R: TCTATCTTTTTGACCCGTCAATCAC	298
7. TC54167	CZF1/ZFAR1 transcription factor, *Arabidopsis thaliana*, locus NP181543, E=6e-41	GCAGTAGCAGCAGCAGTGATG ATACCTCCCCTATCGATGAAGCT	80	-	-
8. TC45048	Pathogenesis-related protein 10, *Vitis vinifera*, Locus CAC16166, 04e-85	GCACATCCCGATGCCTATTAAG **ACTTACTGAGACTGATAGATGCAATGAATA**	101	F: CTACACTGTGGTTGACGGAGATG	312
9. TC64228	Osmotin-like protein, *Vitis vinifera*, locus CAA71883, E=8e-97	CCCTGATGAGCTCCGAGCT **GGACACCTATCCTTGAAAAACTTGG**	129	F: CAATGCCAAGCCTATGGTACAC	312
10. TC46460	Beta-1,3-glucanase, Vitis vinifera, locus AAF44667, E= 0.0	TCGACTTAACACTTCTGGGCAACT CAGAAGCGGCGACTTATTGTCTA	119	-	-
11. TC38204	Phase-change related protein, *Quercus robur*, locus CAB72442, E: 1e-08	AGACCAGCATGGCTGTGTTGTA GACGGCATTCATCATACTCCATT	77	-	-

Table 1. Continued.

Gene Bank Accession	Predicted Gene Name[1]	Primer sequences (forward/reverse)[2] (qRT-PCR)	Product Length	Primer sequence[3] (Genetic mapping)	Product length
12. TC70328	Heat shock protein 70, *Nicotiana tabacum*, locus AAP04522, E:0,0	CTTCTGCTGGAGGTGCTGGT AGACTCACTCCATATTTCCAAACTTGA	117	-	-
13. TC50337	Leucine-rich repeat family protein, *Arabidopsis thaliana*, locus NP187250, E=1e-59	CACCACCTTCAGGAGCTTAAACA GGTTGAACTACAAGAGTTGAGCATAGA	89	-	-
14. CB914701	NBS-LRR type disease resistance protein, *Poncirus trifoliata*, locus AAN62351, E=1e-30	ATCCTGGAGAAAGGCGATCA TTGTAGCTGGACCGGCAGTT	80	-	-
15. TC39520	Serine/threonine kinase, *Persea americana*, locus AAL23677, E=4e-104	TGAAACAGGAAGCAAGGACATG GCTAACTCTTGCTTTGCTTCAGAA	99	-	-
16. TC39349	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, *Nicotiana tabacum*, LocusAAA34068, E=5e-101	TCCACACCTAATTCGGGCAAT TGCTCTTACTTCGGCCCTGAT	139	-	-
17. TC68756	5-enolpyruvylshikimate-3-phosphate synthase, *Arabidopsis thaliana*, locus NP182055, E: 0,0	CATTACATGCTCGGAGCACTGA ACTTCGCCTACTGATCCATTTCC	125	-	-
18. TC66528	Phenylalanin ammonia-lyase, *Vitis vinifera*, locus ABM67591, E=0,0	CTAAAAGTGGTGGATAGGGAACATG TCTTCTCATTTTCGCCATTGCT	130	-	-
19. TC70715	Cinnamate-4-hydroxylase, *Arabidopsis thaliana*, locus NP180607, E: 0,0	GGCAAGCACAAAGAGCACAGA AAGGACGTTGTCCTCGTTGATC	100	-	-
20. TC45576	Caffeic acid 3-O-methyltransferase, *Ipomoea nil*, locus BAE94403, E: 6e-165	CTGCTTGAGCCTCTAGCTTGGT **CTTCATACACAGTATAAAGCCACTTGTG**	99	F: AATTGTTGCTGAATGCATCCTTC	311
21. CF215866	Polyphenol oxidase, *Vitis vinifera*, locus AAB41022, E=5e-95	TGCTGACCCTAACTCACCACTCTAC ATTGCTGTCGGTGAGGTTGTAGT	97	-	-
22. TC52173	Glutathione S-transferase, *Vitis vinifera*, locus ABL84692, E=1e-117	GAAGGGGCACATGGGTCTTC **TAAGCAAACACTCCTCTTACATTACCA**	156	F: GACCTTTGGCAACTTCAGCATAG	312
23. TC57332	Molybdenum cofactor sulfurase family protein, *Arabidopsis thaliana*, locus NP174376, E=2e-104	TCCCAAATTTGCTCCTGGATT CATCCAATGATCCCTGAGATATCA	80	-	-

Table 1. Continued.

Gene Bank Accession	Predicted Gene Name[1]	Primer sequences (forward/reverse)[2] (qRT-PCR)	Product Length	Primer sequence[3] (Genetic mapping)	Product length
24. TC58552	Hydrolase, acting on ester bonds, protein, *Arabidopsis thaliana*, locus NP567701, E= 1e-52	CCTTTTGATGGTTTGCTTGGA **ACCTTCCCCTGTCCCTGGTA**	80	-	-
25. TC63583	DC1 domain-containing protein, *Arabidopsis thaliana*, locus NP_181316 , E=6e-46	**TTATGGGAGCGAAGAGTTCCA** AATTGCATGTGTGGCATCGA	80	R: ACATACTCAGGAAGTTTGGTGCATAG	294
26. TC64430	Unknown, low similarity to protein kinase domain containing protein	GAGGAAGAAGGTCCATCCCAAT **TGCAATGGGCTGCCTTAGA**	81	F: GCGACACACCGTAGCACAAC	292
27. TC63952	Calnexin homolog 1 precursor, *Arabidopsis thaliana*, locus P29402, E=3e-113	TTCGACCTCAGGATGCTACATG CTTCGGGTTCTTATGCTTTACGATA	131	-	-
28. AF196485	Beta Tubulin, *Arabidopsis thaliana*, locus NP_568437, E=6e-68	GGATGCCAAGAACATGATGTGT CACGGAACATCGCTGAAGCT	74	-	-
29. TC44917	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), *Arabidopsis thaliana*, locus NP_001077530, E=1e-80	ACAGCTCCCGTGTGATCGA TAAAAGCTCTGCCCTTGGAAAT	80	-	-

[1] The predicted gene name was defined based on blastx analysis performed in the NCBI database

[2] Primer pairs used in qRT-PCR analyses

[3] Primer employed in combination with one of the primers used in qRT-PCR analyses (as indicated in bold) to genetically map the corresponding genes

Results

An experiment was designed to identify genes whose expression is modulated by challenging axenic grapevine plants with the powdery mildew (*E. necator*) pathogen by transcriptional profiling. The focus of the investigation was laid on the early stages of interaction, in particular the time point at which the pathogen shows a fully developed appressorium and hence a first tight contact between pathogen and host cell is established. To determine when the pathogen arrives at this stage the progress of infection was followed microscopically during the initial phases of interaction (30 hpi) with a susceptible cultivar (`Müller-Thurgau´) under sterile conditions (Figure 1). At four hours post-infection (hpi) the majority of spores had germinated. Primary appressorium formation was observed for most spores at 5-6 hpi and apparently had "matured" at 8-10 hpi. The first primary hyphae were observed at 21 hpi. This stage represents successful colonization of the plant cell. At 30 hpi tertiary hyphae became visible. Based on these findings, 10 hpi was selected as the time point to collect samples for RNA preparation. It is supposed that at this time the appressorium is fully developed and the pathogen is trying to penetrate the cell, delivering signal molecules that may be recognized by the products of resistance genes in the resistant cultivar, initiating a strong defense response.

The samples collected at 10 hpi were at first evaluated on transcriptional level by microarray analysis. After data processing, a battery of differentially expressed genes was identified. However, the statistical significance of this data set was limited due to some data points inconsistent between the biological replications. Therefore, the expression of a subset of genes, detected as powdery mildew-induced in the microarray experiment, was evaluated in more detail by qRT-PCR. These genes were selected based on their homology to genes involved in the defense response in other plants. This set covers different gene classes, such as transcription factors, PR-proteins and enzymes involved in secondary metabolism. The expression levels of the selected genes are shown in Figure 2. Application of qRT-PCR analysis allowed an overall confirmation of results obtained by differential microarray hybridizations. The expression of the great majority of the genes evaluated was more strongly induced in the resistant cv. `Regent´ (incompatible interaction) than in the susceptible cv. `Chardonnay´ (compatible interaction). From the 27 genes investigated, 23 showed at least two-fold transcriptional induction in `Regent´ infected with powdery

mildew, as compared to the non-infected control. Two of the remaining genes showed an induction ratio greater than 1.5. Only four genes showed higher induction in `Chardonnay´ than in `Regent´. These encode a leucine-rich repeat protein (TC50337), a phase-change related protein (TC38204), a heat shock protein 70 (TC70328) and a DC1 domain-containing protein (TC63583).

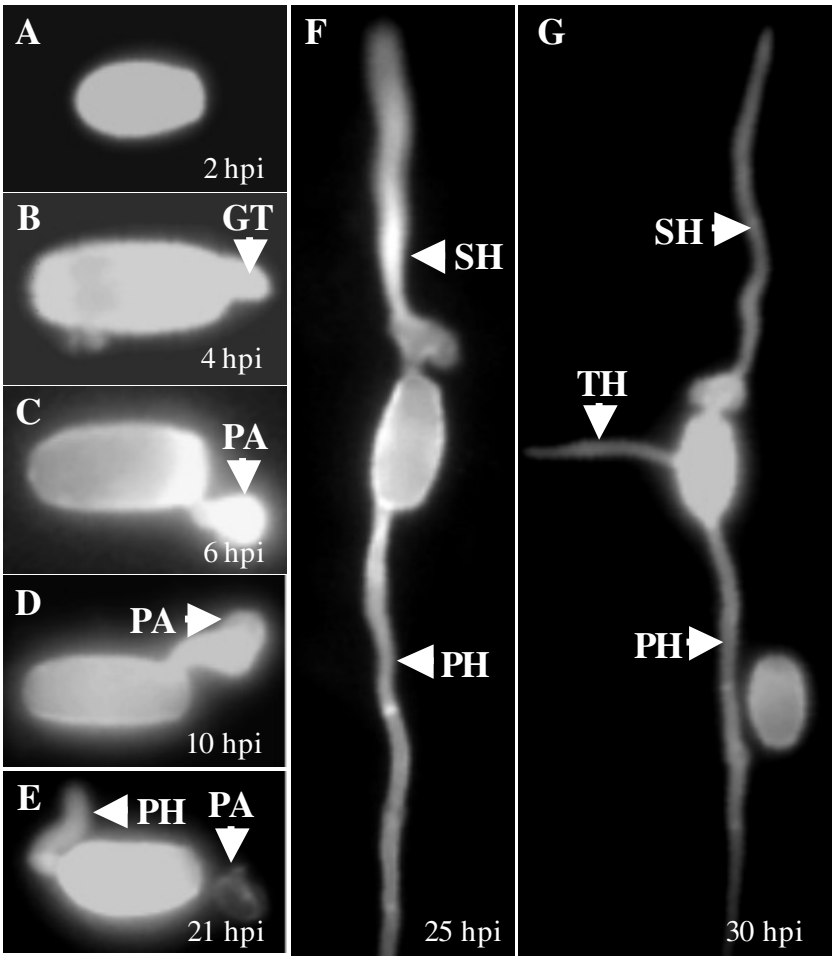

Figure 1. Fluorescence micrographs showing the initial developmental stages of grapevine powdery mildew (*Erysiphe necator*) on detached leaf surfaces of *Vitis vinifera* ssp. *vinifera* cv. `Müller-Thurgau´. A) Ungerminated conidium; B) Germinating conidium (GT: Germ tube); C) Initial differentiation of primary appressorium (PA); D) Mature primary appressorium (PA); E) Primary hypha (PH); F) Secondary hypha (SH) and G) Tertiary hypha (TH). Conidia were stained with Biofluor and are app. 40 µm in length.

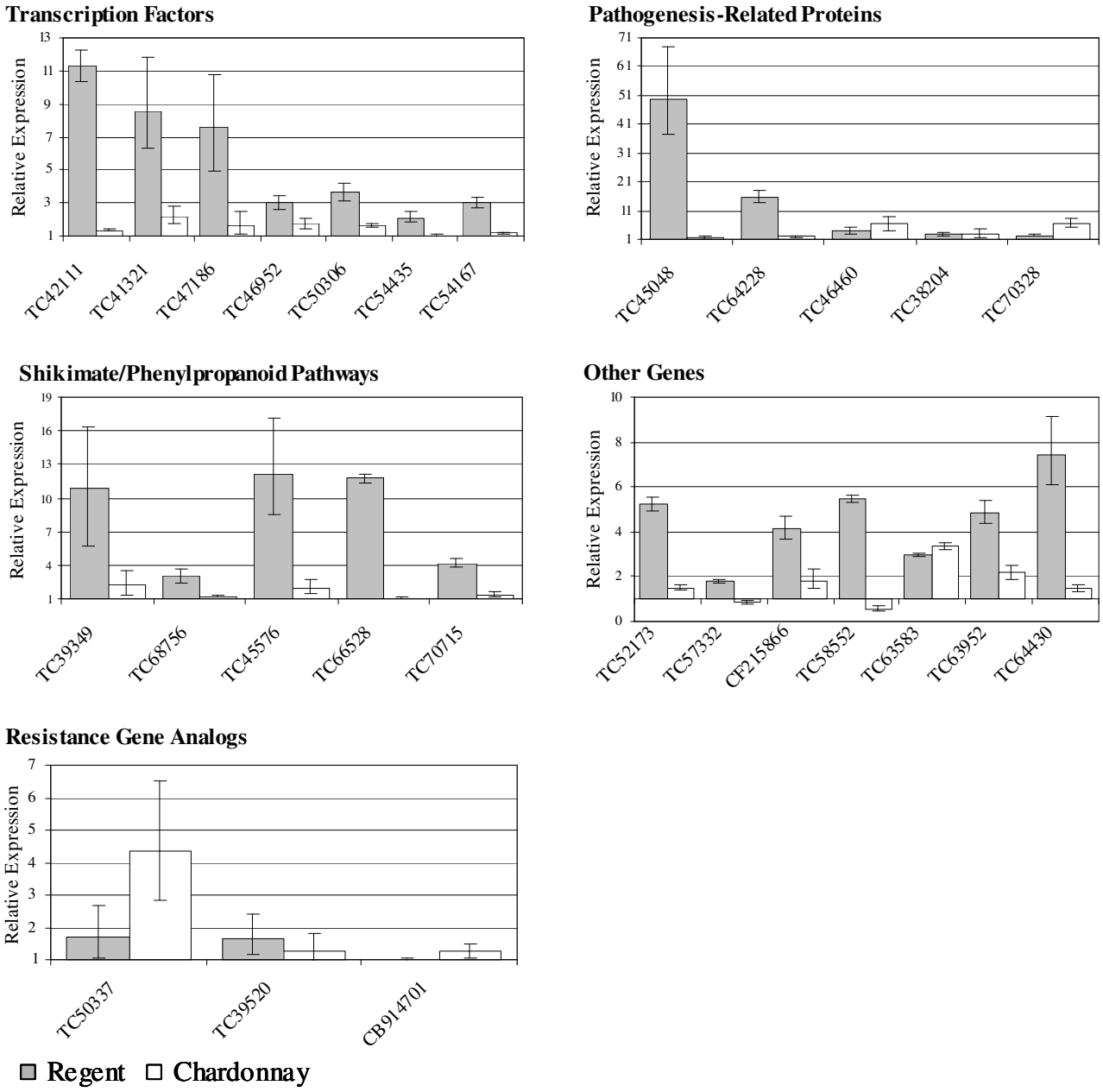

Figure 2. Relative gene expression (fold induction) determined by quantitative Real Time PCR after inoculation of the resistant cv. `Regent´ and susceptible cv. `Chardonnay´ with powdery mildew (*Erysiphe necator*) as compared to non-infected controls. The relative expression of each gene was determined based on the expression level in inoculated samples (10hpi) in relation to non-inoculated samples, employing the REST software. Beta-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes.

All the seven transcription factor genes tested were induced in the resistant cultivar in response to powdery mildew attack (Figure 2). The strongest induction (11.3 fold elevated expression) was observed for a Myb transcription factor that shows high homology to the VlMYBB1-2 gene isolated from grapevine (TC42111). The expression of the WRKY genes TC41321, TC47186 and TC46952 was increased 8.6-, 7.6- and 3.0-fold, respectively. Induction of expression was also confirmed for the three other transcription factor genes tested: TC50306 shares homology to the pathogenesis-related gene transcriptional activator PTI5 (*Solanum lycopersicum*) (3.6 fold induction), TC54435 shows similarity to the ethylene-responsive transcription factor 5 from *Nicotiana tabacum* (2.1 fold induction) and TC54167 is similar to the CZF1/ZFAR1 transcription factor from *Arabidopsis thaliana* (3.0 fold induction). These transcriptional factors were only weakly induced in the susceptible cultivar `Chardonnay´.

Three pathogenesis-related (PR) proteins were investigated. PR-10 and osmotin-like protein (PR-5) were strongly induced by challenging the resistant cultivar with powdery mildew. Induction ratios of 50- and 15-fold were observed for PR-10 resp. PR-5. PR-10 showed the highest transcriptional activity of all genes analysed. Both PR-5 and PR-10 genes were only weakly induced in the compatible interaction. In contrast, the expression of beta-1, 3-glucanase (PR-2) gene was induced to a higher level in `Chardonnay´ (6.3 fold) than in `Regent´ (3.9 fold).

Genes directly or indirectly involved in the secondary metabolism of the plant, participating in the aromatic amino acid biosynthesis and phenylpropanoid pathway, were found up-regulated in the microarray analysis. The expression of five of them, Phenylalanine ammonia-lyase (PAL), Cinnamate-4-hydroxylase (C4H), Caffeic acid 3-O-methyltransferase (COMT), 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase and 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase, were validated in the present investigation (Tables 1 and 2). PAL, C4H and COMT, enzymes involved in the phenylpropranoid pathway, show induced expression during the incompatible interaction (12, 5 and 12 fold, respectively). DAHP and EPSP synthases from the shikimate biosynthetic pathway seem to be co-expressed with the genes involved in the phenylpropranoid pathway, and exhibited a transcript accumulation increase of 10 and 5 fold, respectively. For all five genes, only weak induction was observed in the compatible interaction.

Three resistance (R) gene analogs were considered. Two of them seem to be weakly induced in the incompatible interaction. The first (TC50337) shares amino acid similarity to leucine-rich repeat family proteins, the second (TC39520) to serine/threonine kinases. The transcriptional induction of both genes was of approximately 1,7 fold in inoculated `Regent´ as compared to the control. Interestingly, the first one is more significantly induced in the incompatible interaction (4.3 fold). The third one, an NBS-LRR type disease resistance protein gene (CB914701), was not differentially expressed in both compatible and incompatible interactions.

Additional interesting genes were investigated. One transcript showing homology to the cell detoxification protein glutathione S-transferase showed 5.0 fold induced expression in the incompatible interaction. Another gene (Molybdenum cofactor sulfurase) involved in cellular detoxification was weakly induced in inoculated `Regent´. The expression of a polyphenol oxidase, the enzyme that catalyses the oxidation of hydroxyphenols to their corresponding quinone derivatives, was 4.0-fold induced during the incompatible interaction. Finally, the expression of four additional genes was analysed: TC5852 (a protein with hydrolase activity); TC63583 (a DC1 domain-containing protein); TC63952 (a Calnexin homolog 1 precursor) and TC64430 (a protein of unknown function that shows some similarity to protein kinase domain containing proteins). With the exception of TC63583, all genes were stronger induced during the incompatible interaction as compared to the compatible one (Fig.2).

The genomic position of 10 selected genes, especially transcription factors and strongly induced genes, was determined by genetic mapping. These genes and the linkage groups of their location are listed in table 2. Complementarily, these genes were positioned in the genome sequence of the homozygous derivative of `Pinot Noir´ - PN40024 (Jaillon et al., 2007) (Table 2). This *in silico* analysis confirmed the results obtained by genetic mapping in all cases but glutathione S-transferase, whose genomic scaffold of location was not yet anchored to a specific chromosome of PN40024. Due to the convergent results obtained in these two independent analyses, the remaining genes were also located by sequence comparison in the genome of PN40024 (Table 2). The positioning of these genes in the genome is particularly interesting to check a possible co-location with powdery mildew resistance QTLs known from previous investigations (Fischer et al., 2004; Welter et al., 2007). As shown in table 2, the genes are evenly spread throughout the grapevine genome

Table 2. Genomic localization of the genes evaluated by quantitative Real Time PCR (qRT-PCR).

Gene Bank Number	Gene name	Genetic mapping	Physical mapping
TC42111	Myb-related transcription factor	5	5
TC41321	WRKY1 transcription factor	1	1
TC47186	WRKY2 transcription factor	8	8
TC46952	WRKY3 transcription factor	7	7
TC50306	Ethylene-responsive transcription factor 1	10	10
TC54435	Ethylene-responsive transcription factor 5	16	16
TC54167	CZF1/ZFAR1 transcription factor	-	13
TC45048	Pathogenesis-related protein 10 (PR-10)	5	5
TC64228	Osmotin-like protein (PR-5)	2	2
TC46460	Beta-1,3-glucanase (PR-2)	-	5
TC38204	Phase-change related protein	-	19
TC70328	Heat shock protein 70	-	-
TC50337	Leucine-rich repeat protein	-	5
TC39520	Serine/threonine kinase	-	6
CB914701	NBS-LRR type disease resistance protein	-	9
TC39349	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	-	18
TC68756	5-enolpyruvylshikimate-3-phosphate synthase	-	15
TC45576	Caffeic acid 3-O-methyltransferase	Ungrouped	16
TC66528	Phenylalanine ammonia-lyase	-	16, 8
TC70715	Cinnamate-4-hydroxylase	-	6
TC52173	Glutathione S-transferase	19	Unmapped
TC57332	Molybdenum cofactor sulfurase family protein	-	1
CF215866	Polyphenol oxidase	-	Unmapped
TC58552	Hydrolase protein	-	8
TC63583	DC1 domain-containing protein	8	8
TC63952	Calnexin homolog 1 precursor	-	12
TC64430	Unknown, low similarity to protein kinase domain containing protein	18	18

and found dispersed over 14 of the 19 grapevine chromosomes. No direct co-location of powdery mildew-inducible genes and major QTLs conferring resistance to powdery mildew was detected. However, three genes are located in the region flanking a minor QTL conferring resistance to downy mildew detected on LG 5 (numbered according to Welter et al., 2007) of `Regent´ (Fischer et al., 2004). These genes are PR-10, PR-2 and the leucine-rich repeat (LRR) protein. The LRR protein is tightly linked to the microsatellite marker UDV-60, which is located within the confidence interval of the QTL. PR-10 (TC45048) is a member of a large gene family. This gene family is located app. 3.7 Mb upstream of UDV-60 and TC45048 mapped 17 cM from this microsatellite marker.

Discussion

The essential event triggering host resistance responses is the recognition of the pathogen by the plant. This recognition initiates a local and a systemic transcriptional re-programming, leading to rapid and coordinated defense responses, restricting or prohibiting the pathogen spread on the host tissues. This response is rather complex, involving the interaction of many gene products and signal molecules. As signal molecules, salicylic acid (SA) seems to play a primary role in the regulation of resistance to biotrophic pathogens (Thomma et al., 2001). Members of diverse transcription factor (TF) families are involved in the modulation of this `defense transcriptome´. Classical resistance reactions include the reinforcement of cell walls, the production of antimicrobial metabolites (phytoalexins) and the expression of pathogenesis-related (PR) proteins. The identification of responsive genes to pathogen infection is an essential step in the elucidation of the molecular mechanisms involved during the resistance process. Therefore, we decided to use the microarray technologies to identify genes differentially expressed by challenging resistant and susceptible grapevine with powdery mildew. The focus of the investigation was laid on the early stage of interaction, more precisely, the time point at which the pathogen showed a fully developed appressorium (10 hpi). At this time successful `powdery mildews´ have most likely intruded the host cell with the development of an haustorium. At this stage signal molecules deriving from the pathogen can reach the intracellular space and could then be recognized by intracellularly located R-genes, triggering effector immunity (Jones and Dangl, 2006). This experiment showed a

large set of grapevine powdery mildew-responsive genes, but these data could not be adequately assured by statistical analysis due to a problem with high background, resulting in missing data compromising the analysis. Therefore, the expression of a subset of genes found to be up-regulated in the microarray analysis was validated by quantitative Real Time PCR (qRT-PCR). These genes were selected based on their homology to genes involved in the defense response known in other plants.

The exact repetition of infection conditions allowed an overall confirmation of the microarray data. The expression of almost all genes analysed by qRT-PCR is induced in the incompatible interaction (`Regent´), whereas a significantly reduced induction was observed in the incompatible interaction (`Chardonnay´). The lower induction in the susceptible plant is probably related to failure in the recognition of the pathogen, resulting in only weak and/or retarded responses, insufficient to restrict the pathogen growth efficiently. The stronger response activated in the resistant cv. `Regent´ suggests either a PAMP-triggered immunity (PTI) or an effector triggered immunity (ETI) (Jones and Dangl, 2006). The `Regent´ response to the attack of powdery mildew resulted in the activation of: i) transcription factors, which have been described in other plants to modulate the expression of resistance related genes; ii) enzymes involved in secondary metabolism, including the phenylpropanoid pathway, and iii) pathogeneses-related (PR) proteins. The impact of these resistance components is discussed in the following section.

TFs play a fundamental role in the modulation of pathogenesis-related genes (Eulgem, 2005). Therefore, it is to be expected that these are early induced or co-expressed with pathogenesis-related genes. In the present analysis it was possible to confirm the induction of seven transcription factors after challenging the resistant cultivar with powdery mildew. They are members of four different transcription factor families: WRKY, ERF, MYB and CZF1/ZFAR1. Each family of TFs interacts with divergent *cis*-acting elements that are generally found in the promoter regions of pathogenesis-related genes, modulating the expression of these genes. WRKY TFs bind to W box sequences (C/TTGACC/T), found for example in the potato PR-10a protein (Després et al., 1995). W-boxes were found to be over-represented in the promoters of *Arabidopsis* genes that are up-regulated during R gene-mediated resistance, basal defense, elicitor responses, and systemic acquired resistance (Maleck et al., 2000). Their function is primarily associated with the plant defense process, causing activation or repression of gene transcription (Eulgem and

Somssich, 2007). One of the WRKY genes analysed here (TC41321) is closely related to the grapevine VvWRKY1. The over-expression of this gene in tobacco resulted in reduced susceptibility to different pathogens, including powdery mildew, strongly suggesting its involvement in plant defense (Marchive et al., 2007).

Ethylene responsive factors (ERF) bind to GCC-box elements found in the promoter regions of several PR genes (Endt et al., 2002). The Pti4, Pti5, and Pti6 proteins from tomato, members of the ERF family, were identified based on their interaction with the product of the *Pto* disease resistance gene, a Ser-Thr protein kinase (Zhou et al., 1997). Pti4, Pti5 and Pti6 activated defense response when expressed in *Arabidopsis* and congruently the plants expressing Pti4 showed increased resistance to the fungal pathogen *Erysiphe orontii* and tolerance to *Pseudomonas syringae* pv. *tomato* (pst) (Gu et al., 2002). Myb is a very large family of transcription factors that bind to several divergent *cis* element sequences. These TFs are often involved in the regulation of phenylpropanoid metabolism, which is responsible for the biosynthesis of phenolic components with a crucial role in plant defense (Endt et al., 2002).

The induction of TFs suggests their involvement in the downstream regulation of defense response. The presence of their corresponding *cis*-binding elements was evaluated in the promoter region of PR-10 and the five genes involved in the shikimate/phenylpropanoid pathways. This analysis was performed *in silico* using the grapevine genome sequence of PN40024. The *cis* elements were searched in the range of 2000bp upstream of the predicted initiator codon for protein synthesis employing the PLACE database (Higo et al., 1999). Their positions are recorded relative to the start codon. The typical box-W1 (TTGACC) was found in the promoter region of PR-10 at positions -549 and -1433 in the positive strand (+). This box was identified as the binding site of a WRKY transcription factor, which is responsible for the fungal elicitor response of parsley PcPR1, a member of the PR-10 proteins (Rushton et al., 1996). Grapevine VvWRKY1 binds specifically to the core motif TTGAC being part of the box-W1 (Marchive et al., 2007). Two additional TTGAC core motifs were found at positions -1433 and –1419 (+). The presence of W-boxes in the PR-10 promoter region suggests the involvement of WRKY TF in the regulation of expression. This function may be contributed by TC41321, which is closely related to VvWRKY1. Additionally, an ethylene-responsive enhancer element (ERE) motif (AWTTCAAA) was detected at positions -18 (+) and -1768 (+) and different Myb-binding

elements are present in the PR10 upstream region, such as the motif TAACAAA at position –1227 (+).

The predicted protein coded by Myb (TC42111) shares homology to the NtMYB2/LBM1 (BAA88221) and Myb1 (AAB41101) from *Nicotiana tabacum*, mainly throughout the first 117 amino acids, where two Myb DNA-binding domains are located (app. 80% amino acid identity). NtMYB2/LBM1 is responsive to wounding and treatment with fungal elicitor, and activates the PAL gene promoter by binding to the L and P boxes (Sugimoto et al., 2000). These two boxes were also detected in all of the five genes involved in the shikimate/phenylpropanoid pathways studied in the present investigation. This finding suggests the involvement of TC42111 in the regulation of shikimate/phenylpropanoid pathways as part of the grapevine defense response to powdery mildew infection. Tobacco Myb1 was induced by tobacco mosaic virus (TMV) and *Pseudomonas syringae* pv. *syringae* infection only in the resistant genotype and was shown to specifically bind to a Myb-binding consensus sequence found in the promoter of the PR-1a gene (Yang and Klessig, 1996). As mentioned, Myb-binding conserved motifs were present in the assumed PR-10 promoter region investigated here.

The expression of key genes of the phenylpropanoid pathway (PAL, C4H and COMT) was found induced to high levels in the resistant cultivar `Regent´. The phenylpropranoid pathway is an important branch of the plant secondary metabolism and leads to the biosynthesis of different components conferring the plant more resistant to pathogen attack. These components can function as phytoalexins that possess antimicrobial activity, or can act as signal molecules involved in local and systemic signalling for defense gene activation (via salicylic acid) and have structural functions as physical barriers against pathogen ingress (e.g. lignin biosynthesis). Several investigations provided evidence that phenylpropanoid products contribute to disease resistance. For instance, tobacco plants over-expressing L-phenylalanine ammonia-lyase (PAL), the key enzyme of the phenylpropanoid pathway, exhibit markedly reduced susceptibility to infection with the fungal pathogen *Cercospora nicotianae* (Shadle et al., 2003). Complementarily, transgenic tobacco plants showing a suppressed level of PAL appeared more susceptible to the same fungal pathogen as compared to the wild type (Maher et al., 1994). In grapevine the phenylpropanoid pathway contains branches for the production of the phytoalexin resveratrol and its derivatives. The transfer of a grapevine stilbene synthase gene, coding

for the enzyme responsible for the biosynthesis of resveratrol into a range of plant species resulted in overall increased disease resistance (Essenberg, 2001; Hain et al., 1993; Hipskind and Paiva, 2000 and Zhu et al., 2004). However, the expression of stilbene synthase was not evaluated in the present investigation. Also, the *de novo* biosynthesis of lignin and its deposition at the cell wall acts as a physical barrier prohibiting pathogen penetration into the intracellular space. In grapevine the formation of papillae at the penetration site was demonstrated to be at least partially successful in preventing powdery mildew penetration. These papillae contain phenolic compounds including lignin (Heintz and Blaich, 1990). As recently shown, both the phenylpropanoid and the shikimate pathways are involved in the synthesis of SA (reviewed by Métraux, 2002). In this study a gene coding for the key enzyme DAHP, component of the shikimate pathway, was found strongly induced. The shikimate pathway is responsible for the synthesis of aromatic amino acids and one of its products, the phenylalanine amino acid, is the precursor molecule of the phenylpropanoid pathway.

Diverse families of pathogenesis-related (PR) proteins have been found to be involved in the responsiveness to pathogen attack. Their functional role in the resistance process has been characterized for some of them. Particularly, genes for a member each of the PR-10 and PR-5 group were found strongly induced during the incompatible interaction in the present investigation. PR-10 showed the greatest transcriptional accumulation of all genes analysed (50-fold induction). The biological role played by the PR-10 protein family in plant defense is not completely understood. PR-10 proteins from different plant species display dissimilar biological activities, as reviewed by Liu and Ekramoddoullah (2006). To date, PR-10 protein with ribonuclease activity (Park et al., 2004), ligand-binding ability and enzymatic activities in the secondary metabolism (Koistinen et al., 2005) have been detected in different plant species and are linked to the resistance response. The strong induction of this protein in the resistant cv. `Regent´ suggests its importance for resistance. However, functional analyses have to be performed to gain evidence about the functional role of this protein family in the grapevine resistance response. Osmotin-like proteins, members of the PR-5 protein family, are induced by a variety of phytopathogens and abiotic stresses in many plants and share significant sequence similarity with thaumatin-like proteins (Zhang and Shih, 2007, Zhong and Shen, 2004, Zhu et al., 1995). Over-expression of an osmotin-like protein in tobacco resulted in increased tolerance to the late-

blight fungus *Phytophthora infestans* (Zhu et al., 1996). Hu and Reddy (1997) confirmed *in vitro* antifungal activity of the PR-5 protein against several fungal pathogens, in agreement with its involvement in plant defense. In contrast, only a weak transcript accumulation for the genes coding for beta-1,3-glucanase (PR-2), phase change-related protein and heat shock protein 70 was be observed in the compatible interaction studied here. These genes exhibited higher transcriptional induction in the compatible interaction (cv. `Chardonnay´).

Some additional genes showed altered expression levels when challenging the cv. `Regent´ with powdery mildew. Two genes showing similarity to resistance (R) genes seem to be weakly induced. These two genes, one coding for a leucine reach repeat protein and the other one for a serine/threonine kinase, could be involved in the pathogen recognition and signaling to downstream activation of defense responses. However, these genes were not co-located with the major QTL detected on Regent, which confers resistance to powdery mildew. Therefore these genes do not seem to play a major role in the defense response of `Regent´ to this fungus. Polyphenol oxidase (PPO) also is an important component of the `Regent´ defense response. This enzyme catalyzes the oxidation of hydroxyphenols to their quinone derivatives. Tomato plants over-expressing PPO exhibited a great increase in resistance to *Pseudomonas syringae* as compared to control plants (Li and Steffens, 2002). The transgenic lines showed 15-fold fewer lesions and a more than 100-fold reduction of the bacterial population in infected leaves. Complementarily, antisense down-regulation of polyphenol oxidase resulted in enhanced disease susceptibility. In the incompatible interaction, antisense PPO plants exhibited 100-fold increased bacterial growth and ten times more lesions/cm^2 than non-transformed plants (Thipyapong et al., 2004).

Although almost all genes evaluated were more strongly induced in the resistant cv. `Regent´, none of these genes was directly associated with the major QTL conferring resistance to powdery mildew, detected on LG-15 (Fischer et al., 2004; Welter et al., 2007). This finding suggests that these genes are ranked downstream of other higher level regulatory gene(s) probably involved in the initiation of the defense response. One possibility would be a crucial activity of some R-gene(s) present within the QTL region , responsible for pathogen recognition and triggering a strong defense response of genes evaluated in the present investigation. The genes functional at the first level of the defense process might not have been detected, e.g. because they are active at very early stages of

the host/pathogen interaction, they were not represented on the microarray slides, they are constitutively expressed or of low transcript abundance or were omitted from data analysis for any reason.

The present investigation identified powdery mildew-inducible genes in grapevine. Based on the transcriptional analysis it is possible to conclude that the resistant cv. `Regent´ responded faster and stronger to the powdery mildew attack as compared to the susceptible cv. `Chardonnay´. The response of `Regent´ involved the induction of transcription factors of different functional classes, which regulate complementary defense pathways, such as the expression of PR-proteins and branches of the secondary metabolism. The expression of PR-proteins and genes involved in the secondary metabolism were congruently more strongly induced in the resistant cultivar. In the present investigation only one time point was considered (10 hpi). Further analysis will focus on the more detailed kinetic patterns of expression of these genes.

These findings of clearly up-regulated defensive genes in the resistant cultivar `Regent´ including key regulators such as transcription factors after powdery mildew attack are quite in contrast to the results recently published from the resistant *V. aestivalis* genotype `Norton´. This grapevine showed only three differentially expressed genes upon challenge with powdery mildew in microarray hybridizations and showed elevated endogenous SA levels in the absence of the fungus (Fung et al., 2008). The resistance from *V. aestivalis* may therefore rely on a different mechanism as in `Regent´ with its complex resistance origin from `Chambourcin´ (Akkurt et al., 2007). The elaboration of resistance mechanisms needs more investigations on molecular and cellular level. However, for application in resistance breeding it will be most promising to combine different resistance mechanisms to produce stable and durable resistance in new quality cultivars for sustainable viticulture.

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Identification, isolation and characterization of a CC-NBS-LRR candidate disease resistance gene family in grapevine[1]

Abstract

Plasmopara viticola causes downy mildew of grapevine and is one of the most important pathogens in viticulture. Resistance to this oomycete is present in American and Asian wild species of grape, while traditional European *Vitis vinifera* cvs. used for wine and table grape production are susceptible. Modern breeding efforts aim to achieve resistance through introgression, but knowledge about the molecular mechanisms involved is still lacking. For this reason, the differential display approach was applied to detect grapevine genes involved in defense. Specific amounts of vital sporangia were applied to the lower leaf surface of *in vitro* plants of the resistant *V. riparia* selection `Gloire de Montpellier´ and susceptible cv. `Riesling´. Controls were treated with sterile water. Messenger RNAs extracted 12 hpi were subjected to differential display. Seven transcripts appeared specifically in the incompatible interaction. Sequencing showed that they build three classes. One of them, named VRP1, is represented by three transcripts of differing lengths and shows clear homology to resistance genes from other plant species. Northern hybridizations confirmed its elevated expression in the resistant `Gloire de Montpellier´ 12 hpi. The VRP1 genes were isolated from a BAC library of the resistant cv. `Regent´. Three copies were found within a BAC clone of 134,392 bp. The genes, referred to as VRP1-1, -2 and -3, encode proteins of 798, 811 resp. 813 amino acids. They all exhibit the typical structure of CC-NBS-LRR resistance genes. Linkage/recombination analysis located these resistance gene analogs on linkage group 10 of the grapevine genetic map.

Keywords: differential display, *Plasmopara viticola*, *Vitis sp.*, BAC-library, resistance gene

[1] Kortekamp A[*], Welter LJ[*], Vogt S, Knoll A, Schwander F, Töpfer R and Zyprian E (2007) Molecular Breeding. (Accepted with minor revisions, January 2008).

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Introduction

Plasmopara viticola [(Berk. and Curt) Berl. et De Toni], the causal agent of grapevine downy mildew disease, is one of the most devastating pathogens in viticulture. This biotrophic oomycete was accidentally introduced from America and first observed in Europe during the 19th century. In consequence, repeated use of fungicides during the vegetation period has become inevitable to protect the grower from the risk of severe losses, as the common European scion cultivars of grapevine (*Vitis vinifera* L. ssp. *vinifera*) are generally susceptible to the disease. The necessity of recurrent fungicide applications raises concerns about environmental safety, either for modern synthetic or older copper-based protectants.

Sources of resistance to downy mildew can be found in American and Asian wild species of grapevine. Such genetic resources have been used during decades as donor material for the traditional cross-breeding of resistant cultivars. A major problem however is the long time required to breed a new cultivar that joins resistance and high wine quality. Several generations of backcrossing to traditional grapevine cultivars are required to re-establish wine quality. Therefore, efforts are undertaken to use molecular tools to improve the knowledge about the interaction between the grapevine host and the *P. viticola* pathogen. Although some genetic and molecular aspects of the host/pathogen interaction have already been studied (e.g. Kortekamp, 2006; Hamiduzzaman et al., 2005; Kortekamp and Zyprian, 2003), the resistance mechanism is not yet understood at molecular level. Quantitative trait analysis identified QTLs with major and minor effects on the resistance to the pathogen (Fischer et al., 2004; Welter et al., 2007). Resistance gene analog (RGA)-markers co-located with the a major QTL for resistance to the pathogen were detected (Di Gaspero et al., 2007; Welter et al., 2007). QTL analysis is a widespread technique to detect resistance factors, but has limitations regarding the identification of genomic regions with minor effects on the phenotype. The investigation of gene expression after challenging the host plant with pathogen thus is an additional and complementary approach useful for the elucidation of genes involved in the resistance response.

In the present investigation a differential display approach was applied to identify differentially expressed mRNAs after challenging the plants with the pathogen. The resistant *Vitis riparia* selection `Gloire de Montpellier´ was used in comparison to the

susceptible *Vitis vinifera* ssp. *vinifera* cultivar `Riesling´. `Gloire de Montpellier´ exhibits a typical hypersensitivity resistance response upon infection with *P. viticola* (Kortekamp and Zyprian, 2003). This differential display analysis initiated the isolation, characterization and genetic mapping of a CC-NBS-LRR resistance candidate gene family in grapevine by full sequence analysis of a corresponding 134 kb BAC clone.

Materials and Methods

Plant/pathogen material and experimental infection

Axenic cultures of grapevine varieties `Riesling´ (*V. vinifera* ssp. *vinifera*) and `Gloire de Montpellier´ (*V. riparia*) were maintained on hormone-free modified LS medium (Linsmaier and Skoog, 1965; Blaich, 1977) at 26 to 28°C and 16 h light (40-50 µE/m^2) /day in glass containers (Weck® glasses). The plants were vegetatively propagated by cuttings and re-transferred onto fresh medium every 8 to 12 weeks.

Pathogen sporangia were harvested by collecting downy mildew symptom-carrying leaves from the field and incubating those in a moist chamber at room temperature over night. The sporangia were carefully collected and stored at -25°C until use and checked for their vitality by microscopy before application. A suspension of 40.000 vital sporangia/ml was applied in small droplets (35 to 40 µl) to the lower leaf surface. Controls were performed by applying the same amount of sterile water. Infected or control leaf tissue was cut out at different time intervals after the sporangia resp. water application by using a sterile 5 mm diameter cork borer. This material was shock-frozen in liquid nitrogen and stored at -70°C until further processing.

RNA and DNA preparation

Approximately 100 mg of tissue was used for RNA extraction according to Chang et al. (1993), with minor modifications. PolyA^+-RNA was prepared from the total RNA using the Oligotex™ system (Qiagen, Hilden, Germany). Preparation of genomic DNA from

grapevine was performed with 2 g fresh material (leaves, shoot axes or roots from in vitro-grown plants) as described by Thomas et al. (1993). Genomic DNA of the fungus was prepared from 30 - 50 mg of sporangia following the procedure of Lee and Taylor (1990) with minor modifications as described (Wielgoss and Kortekamp, 2006).

Differential display reverse transcription PCR (DDRT-PCR)

Anchor primers complementary to the 3´-poly A end of mRNA consisted of 11 dTs and two selective nucleotides at their 3´-extension ("Anchor primer kit", Roth, Karlsruhe, Germany). They were combined with random dekamer primers ("DD primer kit 150", Roth, Karlsruhe, Germany) in trial amplifications after reverse transcription using the "First strand cDNA synthesis kit" (Fermentas, St.Leon-Rot, Germany) according to the manufacturers instructions. PCR assays were run under standard conditions with 40 cycles using an annealing temperature of 30°C and 2.5 U Red*Taq* DNA polymerase (Sigma-Aldrich, Taufkirchen, Germany) in 50 µl assays. DDRT-PCR products were loaded onto denaturing 6% polyacrylamide gels and detected as described in the Promega Silver Sequence™ DNA Sequencing system (Promega, Madison, WI, USA).

Table 1. Primer combinations used and lengths of the differential displayed (DD) fragments detected

DD-Fragment	Primer combination	Length of amplified fragment (bp)
1 (VRP1)	dT_{11}AG + AGA AGC GAT G	660
2 (VRP1)	dT_{11}CC + GGA AGA CAA C	570
3 (VRP1)	dT_{11}GA + GGA AGA CAA C	850
4 (VRP2)	dT_{11}GA + AGA AGC GAT G	670
5 (VRP2)	dT_{11}GC + AGA AGC GAT G	720
6 (VRP2)	dT_{11}GC + AGA AGC GAT G	570
7 (VRP3)	dT_{11}GC + AGA AGC GAT G	425

Cloning and sequencing of DDRT-PCR products

Seven differentially observed DDRT-PCR products were excised from the gel matrix and allowed to diffuse into TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) over night at 4°C. Extraction was enforced by eluting in 0.5 M NH_4Acetate, 10 mM MgAcetate, 1 mM EDTA pH 8.0 and 0.1% SDS incubating 30 min. at 100°C. The isolated DNA fragment was precipitated with Ethanol and recovered in 10 µl of TE buffer. 5 µl of the eluate was reamplified using the original primers and PCR conditions. The PCR products were cloned using the "Sure Clone® Ligation Kit" (Pharmacia, Freiburg, Germany) according to the manufacturers instructions and transformed into competent cells (Hanahan, 1983) of *Escherichia coli* DH5α. Recombinant clones were identified on selective plates containing 100 µg ampicillin/ml. The screening for insertions using the alpha-complementing fragment of β-galactosidase in combination with the *lac*ZΔM15 deletion (Beckwith and Zipser, 1970) was done by including 40 µg IPTG/ml and 40 µg X-Gal /ml into selective LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl, 16 g agar per liter deionized water) (Bertani 1951, 2004). Plasmid DNA of recombinant clones was isolated using the "High pure Plasmid Isolation Kit" (Roche Diagnostics, Germany), characterized by restriction analysis and sequenced with the "ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit" (PE Applied Biosystems, Weiterstadt, Germany). Data processing was performed with DNASIS™ for Windows (Hitachi, San Bruno, CA, USA).

Southern and Northern hybridizations

Southern and Northern hybridizations were performed for the three classes of differentially displayed transcripts detected (VRP1, 2 and 3). Specific primer pairs for each group were designed: VRP1f (forward): GGAAGACAACTACACAC, VRP1r (reverse): GTGCAAGCATGAAGCCTC; VRP2f (forward): GTGTATCCCAATGCCACTGCA, VRP2r (reverse): GCCAGCGACTTCGTGCTCAG; VRP3f (forward): CAAGATGCGATGATGATCAC, VRP3r (reverse): GCCCAGAGATATGTGG. These primers were used for PCR-based synthesis of Digoxigenin-dUTP labelled probes employed in Southern hybridizations. Genomic DNA was cut with restriction endonucleases *Bam*HI, *Eco*RI, or *Hin*dIII (Fermentas, St.Leon-Rot, Germany) and the fragments resolved at 1.5 V/cm by gel electrophoresis on 1.2 % agarose (FMC Bioproducts

Biozym, Oldendorf, Germany) in TAE buffer (40 mM Tris-Acetate, 2 mM EDTA pH 7.9). Transfer of the fragments to positively charged nylon membranes (Roche Diagnostics, Germany) was done according to Southern (1975). The DNA was fixed to the membrane by baking for 2 hours at 80°C. Prehybridization, hybridization and detection were performed using the "DIG Easy Hyb" system and CDP-Star (Roche Diagnostics, Germany) according to the supplier's instructions.

Poly A^+ mRNA for Northern hybridizations was prepared by binding the molecules to biotin-labeled $d(T)_{20}$ oligonucleotides (ARK Scientific, Darmstadt, Germany) and purified with streptavidin-covered metallic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) with magnetic recovery. The mRNA was loaded onto denaturing agarose gels prepared in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) containing 2.47 % formaldehyde and resolved for 2 hours at 5V/cm. The RNA was transferred onto Nytran Plus membranes (Schleicher and Schüll, Kassel, Germany) in a similar way as described above (Ausubel et al., 1999) and crosslinked to the membrane with UV light of 254 nm wave length. Probes were labeled with alpha ^{32}P-dCTP (10 mCi/ml resp. 3.7x 10^8 Bq/ml) using the "Random primed labeling kit" (Roche Diagnostics, Germany) according to the instructions of the supplier. The filters were prehybridized in Church-buffer (Church and Gilbert, 1984) for 30 to 60 min. and hybridized in the same buffer containing the probe in 10 ml/100 cm^2 membrane over night at 65°C. The membrane was washed twice for 30 min. at 65°C in wash buffer 1 (1 mM EDTA, 5% SDS, 40 mM $NaHPO_4$, pH 7.2), for 10 min. at room temperature in wash buffer 2 (1 mM EDTA, 1% SDS, 40 mM $NaHPO_4$, pH 7.2), wrapped in foil and exposed to Kodak MS X-ray film at -70°C. The membranes were re-used for hybridizations after stripping by boiling in 0.5% SDS, cooling down and drying at room temperature for at least 30 min.. A β-tubulin probe derived from *Arabidopsis thaliana* (U. Conrath, University of Kailserslautern) served as a control for even RNA loading.

BAC library, screening and sequencing of clone B18G20

The VRP1 primer pair was used to screen a BAC (bacterial artificial chromosome)- library constructed from grapevine cv. "Regent" through PCR. This BAC library has an average insert size of 102.4 kb and a statistical 10.2 -fold coverage of the "Regent" genome, with

47616 clones. The library has been constructed in service through Genome Express (Meylan, France). It contains a contamination of cpDNA of 3.36% as shown by hybridizing of high density nylon filters with 9216 clones to a Digoxigenin-labeled PCR probe produced with primer pair *trn*M and *rbc*L from cpDNA (Demesure et al., 1995). Half of the library (50% *Hin*dIII and 50% *Mbo*I clones) was organized into three dimensional pools of 6 x 384 clones in microtiter plates, rows and colums through stacks of six 384 microtiter plates each and individual plate pools of 384 recombinant strains. A positive clone, carrying the largest `Regent´ DNA segment (app. 134 kb), was submitted to sequencing (MWG Biotech, Ebersberg, Germany).

Sequence analysis of clone B18G20

The DNA sequence of clone B18G20 was scanned for putative protein coding regions and splicing sites using GeneScan software. Only ORFs (open reading frames) encoding more than 100 amino acids were considered as putative genes. The predicted protein sequences were annotated by protein-protein Blast analysis (blastp) performed in the NCBI database. Blastx analysis in the same database was used in addition to identify possible genes not detected by blastp. The PileUp program was used for multiple sequence alignments. Motif searches of the putative resistance VRP1 genes were done with the MotifScan software available in the Swiss Protein Database. The COIL program (http://www.ch.embnet.org/software/COILS_form.html) was used to scan the VRP1 genes for the possible formation of coiled-coil structures. The GeneScan and PileUp software programs were from the HUSAR (Heidelberg Unix Sequence Analysis Resources, http://genome.dkfz-heidelberg.de) software package.

Genetic mapping of the resistance candidate genes

Specific primers were designed for the VRP1 resistance candidate genes to integrate them independently into the map constructed by Welter et al. (2007), using the cross population of “Regent” x “Lemberger”. Due to the high sequence similarity of the three genes in the coding region, the primers were designed within an intron. The primer sequences are: VRP1-1F: GGTAAAATATCGTCTTTTCCAT, VRP1-1R:

AGCCAATCGAAGATTATTTG, VRP1-2F: TGCATCGAATTAAGTATTGC; VRP1-2R: CTTACATTCGTGTCGTCAAA; VRP1-3F: CCATCGTAGCTTGCTCTTAT, VRP1-3F: GGAATGAAAATGGAGAGACA. The PCR products were separated on Serdogel SSCP gels (Serva, Heidelberg, Germany) and visualised by silver staining. JoinMap 3.0 software (Van Ooijen and Voorrips, 2001) was employed to map the markers.

Results

Timing of mRNA extraction and Differential Display

Comparative enzyme activity assays and cytological investigations of processes involved in pathogen defense and hypersensitive reaction had shown that the defense reaction of `Gloire de Montpellier´ resulted in induction of cytoplasmic peroxidase activity with the highest level reached at 10 to 15 hours after inoculation with *P. viticola* sporangia (Kortekamp, 2001). Hence this time point was chosen for total RNA extraction from infected and non-infected leaf tissue of the resistant variety `Gloire de Montpellier´ and the susceptible cultivar `Riesling´. The mRNA obtained was reverse transcribed and used in differential display analysis. PCR reactions combining anchor primers with random decamer primers displayed transcripts differentially expressed. Transcripts detectable exclusively in infected `Gloire de Montpellier´ leaves (and absent in infected or non-infected `Riesling´ resp. non-infected `Gloire de Montpellier´) were chosen for cloning and sequencing. Seven transcripts in a size range of 340 to 850 b met these criteria (Table 1). Some of them showed strong homology with each other and were grouped into classes. The three transcripts composing class 1 (VRP1 – *Vitis* resistance to *Plasmopara,* sizes 660, 570 resp. 850 b) exhibit pronounced similarities to resistance genes known from other plant species. The second class (VRP2), also containing three transcripts (670, 720 resp. 570 b), showed strong homology to a protein of unknown function from *Arabidopsis thaliana* and *rice* (*Oryza sativa*). The seventh transcript (VRP3, 425 b) has rather weak similarity to a MADS-box binding protein from *Silene latifolia* of uncertain significance. Scanning the putative peptides encoded in the transcripts with `ProSite´ software indicated the presence of conserved motifs known from resistance genes in the VRP1 peptides. All

three putative peptides contain an LRR (leucine rich repeat) domain, and motifs for protein kinase C phosphorylation, cAMP/cGMP-dependent protein kinase phosphorylation, casein kinase II phosphorylation, N-glycosylation and N-myristoylation.

To verify the plant origin of these transcripts specific primers were designed for each of the three classes and tested in PCR reactions with genomic DNA from grapevine cultivars (`Gloire de Montpellier´, `Riesling´, `Kerner´, `Regent´) and *P. viticola*. Specific amplificates of the expected sizes (452 bp for VRP1, 360 bp for VRP2 and 395 bp for VRP3) were obtained from all the *Vitis* cultivars tested, but never from pathogen DNA (data not shown). In addition, Southern hybridization experiments were performed with genomic DNA of the above mentioned grapevine cultivars and pathogen DNA (data no shown). Again, the probes constructed from VRP1, 2 and 3 reacted only with plant genomic DNA. This result supports the host origin of the cDNAs. Several polymorphic hybridizing bands were observed for VRP1 (up to five *Hin*dIII fragments) and VRP2 (up to four *Hin*dIII fragments), indicating the presence of several copies constituting small gene families, in agreement with their repeated appearance in the sequenced transcript variants.

Northern hybridizations using the same plant materials and infection conditions as originally implemented in the differential display analysis revealed varying levels of expression for VRP1, VRP2 and VRP3. VRP1 showed a higher signal of basal expression under non-infected conditions in the resistant variety `Gloire de Montpellier´ as compared to susceptible `Riesling´. In addition, the hybridization signal appeared induced under infection with *P. viticola* to a significantly higher level as in infected `Riesling´ (Fig. 1). VRP2 was expressed at very low level and its signal even appeared reduced during infection with the pathogen. A signal hybridizing to the VRP3-specific probe was barely detectable, indicating a very low level of expression (data not shown).

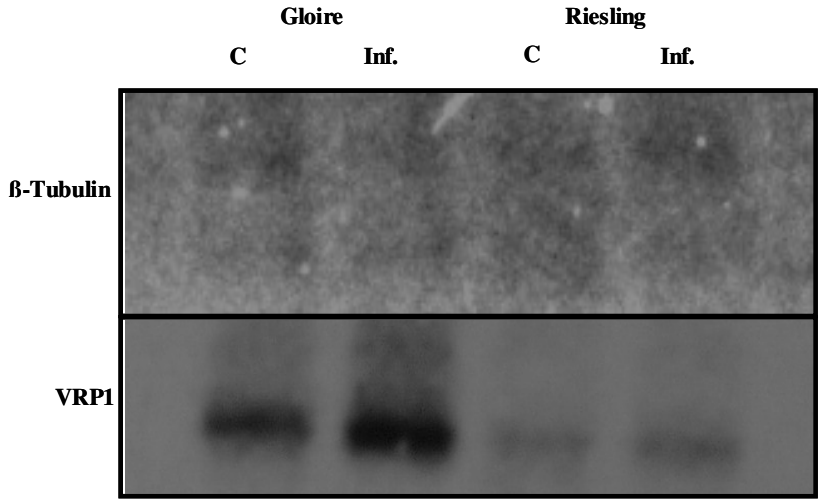


Figure 1. Northern-Blots showing the expression of the reference gene ß-Tubulin and the target gene VRP1. The expression was evaluated with mRNA extracted from leaves of `Gloire de Montpellier´ (resistant) and `Riesling´ (susceptible) 12 h after infection with Plasmopara viticola (Inf.) in comparison to non-infected plants (C = control)

Genomic organization and structure of BAC clone B18G20

Considering the high amino acid sequence similarity to previously characterized resistance genes and the inducibility detected by challenging `Gloire de Montpellier´ with *P. viticola*, the VRP1 transcript class was chosen for further investigation. Orthologous VRP1 resistance candidate genes were thus isolated, characterized and genetically mapped in the cv. `Regent´. This cultivar was chosen due its resistance to *P. viticola,* the availability of a genetic mapping population (Welter et al., 2007) and a comprehensive BAC (bacterial artificial chromosome) library.

The original VR1 primer pair was used to screen the BAC library by PCR. Six positive clones were detected. Clone B18G20 carrying the largest `Regent´ genomic DNA fragment (app. 134 kb) was submitted to sequencing (MWG Biotech, Ebersberg, Germany). The assembly of the shotgun sequences resulted in six large contigs, which were then linked to each other by direct sequencing of PCR products obtained from contig-specific gap-outreaching primers, resulting in a total length of 134,392 bp (Fig. 2).

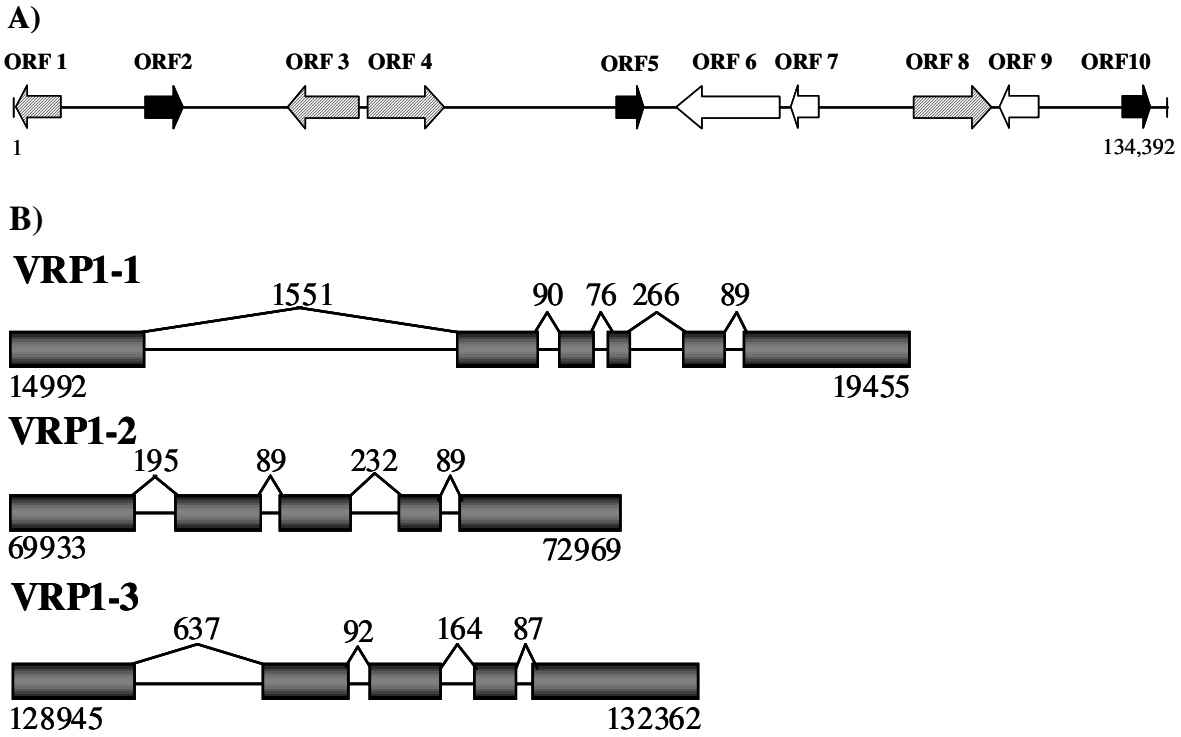


Figure 2. A) Genetic organisation of the 134,392 bp grapevine genomic segment of BAC B18G20. The positions and orientations of 10 ORFs identified with GeneScan software are indicated. The three CC-NBS-LRR resistance candidate genes *VRP1* are represented by black arrows. Lined arrows represent ORFs that code for polypeptides showing homology to transposable element proteins. B) Structure of the predicted *VRP1* genes. Exons are represented by black boxes and introns by lines. Intron sizes are indivated above their location. The numbers left and right represent the position of the genes in the BAC B18G20 sequence. The number at the left side represents the first base of the start codon (ATG) and the number at the right side the last base of the stop codon (TGA).

The sequence of BAC clone B18G20 was scanned for ORFs (open reading frames) using GeneScan software with the parameter matrix from *Arabidopsis*. The BlastP algorithm was applied to search for similarities to known proteins. Only ORFs homologous to known proteins were considered. Under this condition 10 ORFs were identified. Their position and orientation is shown in Figure 2. The putative function of the predicted polypeptides encoded is listed in Table 2. Half of the ORFs encode for proteins known from transposable elements (ORF 1, 3, 4, 8 and 9).

Table 2. Predicted positions, putative functions and lengths of the open reading frames (ORFs) detected in the `Regent´ BAC clone B18G20.

ORF[a]	Predicted position[b]	Strand	Putative protein function[c]	Organisms	%[d]	E-value	Length[e]
1	5187-?	-	Retrotransposon protein	*Beta vulgaris*	38	4e-11	474
2	**14992-19455**	**+**	**CC-NBS-LRR**	***Nicotiana benthamiana***	**40**	**2e-170**	**798**
3	40006-31724	-	Retrotransposon protein, Ty3-gypsy subclass	*Oryza sativa*	42	2e-10	723
4	40877-49840	+	Contains an integrase core domain and a zinc finger	*Medicago truncatula*	60	3e-50	757
5	**69933-72969**	**+**	**CC-NBS-LRR**	***Nicotiana benthamiana***	**40**	2e-158	**811**
6	89103-77033	-	Tandem Ubiquitin	*Arabidopsis thaliana*	60	1e-34	401
7	93866-90460	-	Heat shock protein binding	*Arabidopsis thaliana*	58	7e-08	364
8	104557-113681	+	Reverse transcriptase	*Medicago truncatula*	58	1e-27	258
9	119343-114773	-	Transposable-like protein with hAT dimerisation domain	*Medicago truncatula*	31	4e-14	253
10	**128945-132362**	**+**	**CC-NBS-LRR**	***Nicotiana benthamiana***	**39**	**5e-156**	**813**
*	12117-11685	-	Contains an integrase core domain and a zinc finger	*Beta vulgaris*	45	3e-13	-
*	56062-55497	-	Integrase core domain	*Oryza sativa*	38	2e-31	-
*	84807-84634	-	Integrase core domain	*Medicago truncatula*	39	5e-04	-

[a] Open reading frames detected by GeneScan software
[b] from start codon until the stop codon [?: stop codon not found (the sequence finished with an exon)]
[c] Determined by protein BLAST in NCBI database
[d] Percentage identity
[e] Length of the predicted peptide sequence
* No ORFs were predicted in these regions but significant homology was found by blastx analysis in the NCBI database

The polypeptides encoded by ORFs 2, 5 and 10 exhibit structural features similar to previously characterized plant resistance genes. They share a high similarity to each other and are practically identical to the original VRP1 transcripts detected in the differential display analysis. These resistance candidate genes were hence designated VRP1-1, VRP1-2 and VRP1-3. A polypeptide sharing high homology to two ubiquitin domains arranged in tandem as found in the phosphatidylinositol 4-kinase type II protein from *Arabidopsis thaliana* (Müller-Roeber and Pical, 2002) could be encoded by ORF 6. However this putative gene seems to have undergone major rearrangements through retrotranspositional insertion. The two ubiquitin-like domains are separated by retrotransposonal sequences (positions 84807 – 84634, table 2) and a putative 4-kinase protein domain is missing. ORF 7 encodes a heat shock protein and the polypeptide encoded by ORF 4 has homology to a zinc finger protein. Additional small regions showing similarity to retroelements on DNA sequence level not represented in Fig. 2 were identified by blastx analysis (Table 2, bottom).

Structure of the resistance candidate genes VRP1

The three VRP1 resistance candidate genes present on BAC clone B18G20 share the same orientation (Figure 2). The lengths of VRP1-1, VRP1-2 and VRP1-3 genes from the presumed start to the stop codons are 4464, 3037 and 3418 bp, respectively. The different lengths of the ORFs are mainly due to a variable length of intron 1. According to GeneScan splicing predictions VRP1-1 possesses the longest intron 1 (1551), followed by VRP1-3 (637) and VRP1-2 (195) (Fig. 2). VRP1-2 and VRP1-3 are spliced into five exons, resulting in a predicted polypeptide of 811 and 813 amino acids, respectively. In VRP1-1 the third exon seems to have additional splice sites, leading to a deletion and a smaller protein of 798 amino acids. The different splicing results from point mutations. Although the splice sites were not confirmed by sequencing cDNA, blastx alignments performed in the NCBI database support these presumptions, showing five stretches of high similarity to the *NRG1* gene from *N. tabacum*, exactly corresponding to the five predicted exons.

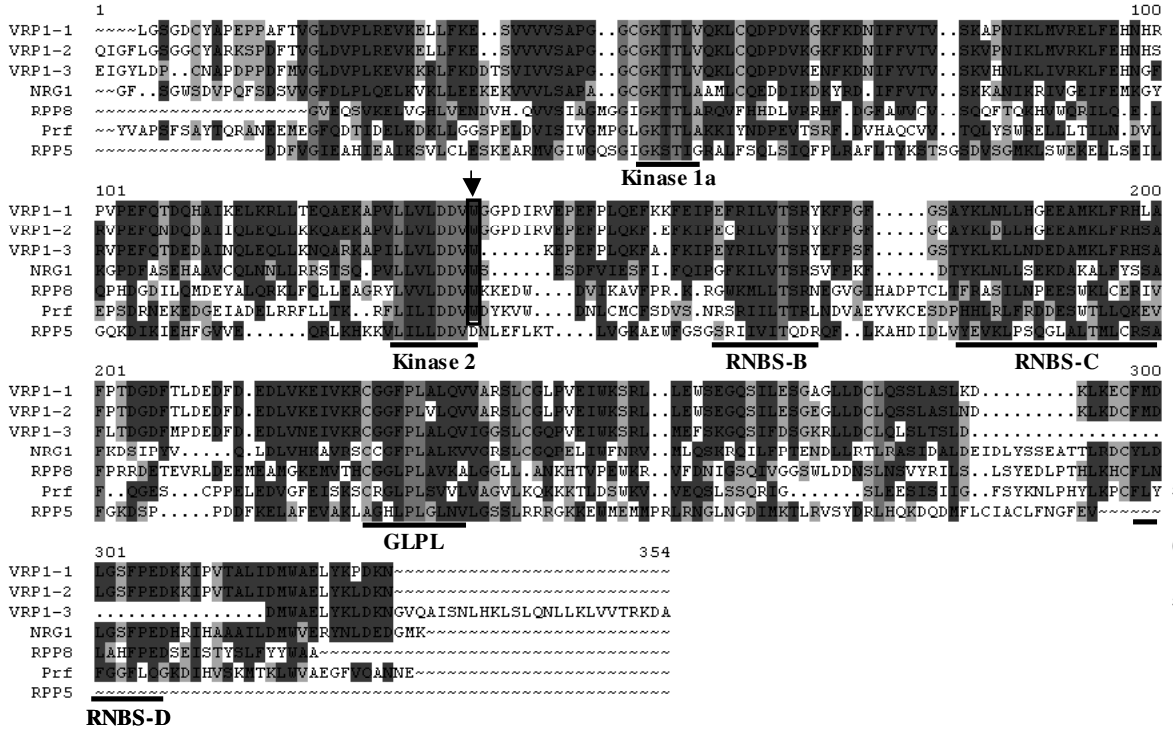
The deduced polypeptides of the three resistance candidate genes are closely resembling each other. Pairwise comparison of the deduced polypeptides showed that the VRP1-2 and

VRP1-3 are most similar, with 87% amino acid identity, whereas VRP1-1 shares 70% resp. 72% of its amino acids with VRP1-2 and VRP1-3. The most significant divergence of VRP1-1 in comparison to the other two genes is a loss of 24 amino acids, resulting from the presumably different splicing. All three polypeptides closely resemble well characterized resistance genes from other plants, of which the tobacco mosaic virus CC-NBS-LRR resistance gene *NRG*1 is the most similar. VRP1-1 shows 40% amino acid identities with NRG1 at an E-value of $2e^{-170}$.

The *VRP*1 genes clearly belong to the CC-NBS-LRR class of resistance candidate genes. The presence of the NBS and LRR domains were confirmed by checking the VRP1 polypeptide sequence with the Swiss Protein Database using the Motif Scan software. In the same analysis an RPW8 domain (Xiao et al., 2001) was found at the C-termini of the genes. A comparison involving the NBS domain of the predicted VRP1 polypeptides and other previously characterized resistance-involved proteins (NRG1, RPP8, Prf and RPP5) is illustrated in Figure 3. These comparisons highlight the presence of the NBS conserved motifs Kinase 1A, Kinase 2, RNBS-B, RNBS-C, GLPL and RNBS-D in the VRP1 polypeptides, also present in the *NRG*1 resistance gene.

The structural LRR domain of the VRP1 polypeptides is composed of seven well recognizable repeats. Each unit is composed of 24 amino acids. The individual repeats show high divergence inbetween each other at the variable amino acid positions. In Figure 4 the five most conserved LRR repeats are compared. Similar to the NBS domain (Fig. 3), the LRR repeats of VRP1-2 and VRP1-3 are practically identical. Only four amino acid residues diverge between them. The most significant change occurred in the fifth LRR repeat ("e"), which is located within the β-strand/β-turn domain. In contrast, 24 amino acids substitution were found in VRP1-1, as compared to the two others. The formation of coiled-coil structure at the C-termini of the VRP1 peptides is supported by analysis performed with COILS software. Furthermore, amino acid residues that have been found to be unique for the CC-NBS-LRR class of resistance genes (Pan et al., 2000) were detected in the NBS region of the VRP1 peptides. The last tryptophan of the kinase 2 motif (labeled with an arrow) is found only encoded in the CC-NBS-LRR genes (*VRP1-1, 2* and *3*, *RPP8* and *Prf*) and it is not present in *RPP5*, a TIR-NBS-LRR resistance gene (Fig. 3).

Figure 3. Multialignment showing the homology of predicted VRP1 proteins with the product of previously characterized CC-NBS-LRR resistance genes *NRG1*, *RPP8* and *Prf* and the TIR-NBS-LRR resistance gene *RPP5*. The region compared corresponds to 300 amino acids of the NBS domain. Amino acids were aligned using the PileUp program. Consensus residues above 50% are indicated by shading. The conserved domains kinase 1a, kinase 2, RNBS-B, RNBS-C and GLPL are labeled. The last tryptophane of the kinase 2 motif unique for CC-NBS-LRR class of resistance gene is highlighted by an arrow.



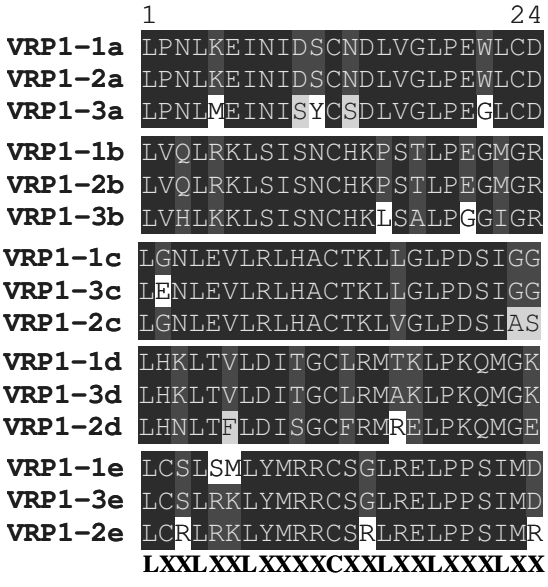


Figure 4. Amino acid comparison of five strongly conserved LRRs of the *VRP1* resistance candidate gene ORFs identified on BAC clone B18G20. The letter in annex to the gene indicates the individual LRR. Conserved residues have a black background. The majority of the LRRs show the conserved core sequence LxxLxxLxxxxCxxLxxLxxxLxx. Alignment of the LRRs was done using the PileUp program available in the Husar bioinformatic facilities.

Trials to align the sequence comprising the ORFs (including their introns) from *VRP1* genes with these contigs identified orthologous genes in PN40024. These orthologous genes from *VRP1-1*, *VRP1-2* and *VRP1-3* were found on contigs 18783, 18782 resp. 18773. Both the organization of the ORFs and their predicted polypeptide sequences are highly similar. Only two amino acid changes at positions 147 (Ser to Tyr) in the RPW8 domain and at 644 (Met to Leu) in the LRR region were detected comparing *VRP1-1* and the orthologous gene. Nine amino acid substitutions were detected between *VRP1-2* and the respective orthologue (two in the RPW8 domain at positions 25 and 127, five scattered allover the NBS domain, one in the LRR region (position 733) and one at position 497 inbetween the NBS and LRR parts of the presumptive protein). VRP1-3 and the ORF of contig 18773 diverge in a total of 34 amino acid changes and a deletion of four amino acids. The majority of these changes occurred at the N-termini within the RPW8 domain. Five, resp. four amino acid replacements occurred in the NBS and LRR regions.

To further understand the relationship between these genes, the sequence ranges 2000 bp upstream and 2000 bp downstream were analysed. The three *VRP1* genes from `Regent´ were at first compared to each other. *VRP1-2* and *-3* share a segment of 135 bp with high similarity (exhibiting only 7 nt changes) directly preceding the start codon. The same sequence is found 68 bp upstream of the start codon of *VRP1-1* due to an insertion. Further upstream of this region, *VRP1-1* differs considerably from the other two genes. *VRP1-2* and *-3* resemble each other closely over 1260 bp of the upstream sequence. Downstream of the stop codon, the three *VRP1* genes share 71 similar nucleotides. *VRP1-2* and *-3* are very similar overall, although some smaller insertions and deletions are evident.

Regions flanking the *VRP1* genes from `Regent´ were also compared to the sequence context of the genes identified in the sequence of PN40024. *VRP1-1* and its orthologue detected on contig 18783 are very similar to each other including the range of 2000 bp up- and downstream of the putative coding sequence. *VRP1-3* and ORFs from 18782 and 18773 share a high similarity, with contig 18773 ORFs being almost identical. *VRP1-2* shows a high similarity with the three others covering 1235 bp upstream of the start codon. The downstream 2 kb sequences of these four putative genes all resemble each other. The only differences are small insertion/deletions. (In the case of contig 18782 only 80 bp could be compared, as the corresponding gene lies at the end of contig.) Results of these comparisons are summarized in Figure 5.

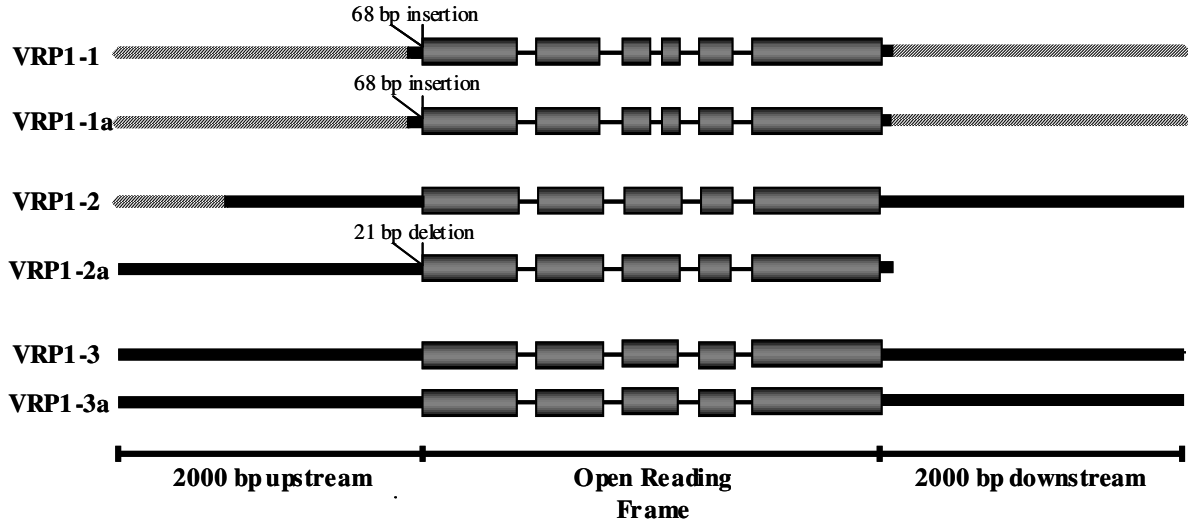


Figure 5. Comparison of the regions 2000 bp up and downstream of the open reading frame between the VRP1 genes and the respective orthologus genes (labeled by "a") identified in PN40024 contigs. Continues and dashed lines represent sequence similarity resp. no significant sequence homology. The open reading frames (exons and intron) are not drawn to scale.

Genetic mapping of VRP1 genes

Specific primer pairs were designed to map the three *VRP1* genes individually. They were located in the genetic map constructed by Welter et al. (2007), employing a segregating population derived from the cross of `Regent´ x `Lemberger´. The genes were positioned in the inferior extremity of LG 10, 24 cM distant from the microsatellite marker UDV59. No recombination events were found between them using a progeny of 144 individuals. Genomic regions associated with resistance factors against *P. viticola* were previously detected by QTL analysis in the cv. "Regent" (Fischer et al., 2004; Welter et al., 2007). However, so far no stable QTL was detected on LG 10.

Discussion

There is a very little knowledge about the molecular mechanisms involved in the interaction between the host grapevine and its pathogen *Plasmopara viticola*. For this reason we initiated this study investigating the molecular response of the resistant *V. riparia* selection `Gloire de Montpellier´ to *P. viticola* infection, applying the differential display approach (Liang and Pardee, 1992). This technique identified transcripts that appeared especially in the case of the incompatible interaction. Comparison of their DNA sequences revealed that basically three different cDNA types had been recovered, although different primer combinations had been used. Two sets of three sequences each, class VRP1 and class VRP2, exhibited very strong similarities to each other, although they had shown different lengths at the level of DDRT-PCR amplification products.

The results of Southern hybridizations are in agreement with the possibility that these bands originate from a small gene family and hence represent the activities of several closely related genes. Hybridizations with *VRP1*- and *VRP2*-specific probes revealed several hybridizing fragments with polymorphic patterns when resistant and susceptible grapevine cultivars were compared. This argues against an alternative possibility that the bands may have arisen through partial mispriming due to the shortness of the primers employed, as sometimes observed in DDRT-PCR (Zhao et al., 1995). Incomplete or alternative splicing could be another mechanism explaining the length-variable copies observed from the same gene, but is counter-argued by the results of genomic hybridizations. Quite in contrast, the DDRT-PCR product *VRP3* was obtained as a unique sequence and also exhibited a less complex hybridization pattern in genomic Southern analysis, hybridizing to only one major fragment in all cases tested.

Expression analysis of *VRP1* suggested that it is induced during pathogen attack in the resistant variety `Gloire de Montpellier´. Also its basal level appears higher than the basic level of the susceptible cv. `Riesling´ as estimated from Northern hybridization experiments. In `Riesling´ only a very faint increase of *VRP1* band intensity could be observed during infection as compared to the non-infected control. In this case the difference between resistance and susceptibility does not rely on the presence or absence of specific genetic factors, but rather is reflected by a variation in the kinetics and intensity of gene regulation during defense. How this differential gene regulation is achieved, awaits further analysis. The fact that the *VRP1* sequence had a high similarity to well

characterized resistance genes from other plant species revealed it as an interesting resistance candidate gene and encouraged its further analysis.

Using the *VRP1* partial sequence revealed through DDRT-PCR we tried first to isolate the complete gene(s) by PCR walking. This strategy failed, probably due to the high similarity of the genes in the gene family, resulting in the generation of redundant amplified sequences. Then we decided to isolate the orthologous *VRP1* resistance candidate genes from a BAC-library constructed of the *P. viticola* resistant cv. `Regent´. One of the positive clones (B18G20) identified by using the original VRP1 primers was completely sequenced over its length of 134.392 bp. Three ORFs identified in clone B18G20 encode for polypeptides carrying conserved domains commonly present in plant resistance genes that confer resistance to biotrophic pathogens. These three polypeptides are similar to each other and are practically identical to the original *VRP1* sequences obtained from `Gloire de Montpellier´, confirming their identity. The identification of three putative resistance candidate genes is in agreement with the results obtained by Southern hybridization, which suggested the organization of *VRP1* in a small gene family. We can not exclude the possibility of additional genes as members of this gene family. Due to the high similarity to the original *VRP1* sequences and considering them as paralogs of a putative resistance gene family, they were designated *VRP*1-1, *VRP*1-2 and *VRP*1-3, according to their relative position on the BAC sequence. Motif searches and Blast analysis allowed to classify these genes into the CC-NBS-LRR resistance gene class. The tobaco *NRG*1 resistance gene shows the highest amino acid homology to the *VRP*1 genes. This recently characterized gene also encodes a CC-NBS-LRR protein and is involved in the resistance response against tobacco mosaic virus (Peart et al., 2005). Interestingly, NRG1 has not only high amino acid identity to the VRP1 polypeptides, but also its general gene structure is very similar, since its mRNA is spliced into five exons with similar length just like *VRP1*, resulting in an ORF encoding 851 amino acids.

Different investigations have shown that variation at the sequence level, especially in the LRR domain, can lead to an alternative recognition of pathogen strains (see reviews Hulbert et al., 2001; Xiao, 2006). As demonstred by Bryan et al. (2000), the change of a single amino acid within the LRR domain of the rice blast resistance gene resulted in susceptibility to the disease. Comparisons of the LRRs of *VRP*1 genes have shown some amino acids substitutions, especially when comparing *VRP*1-1 with the two others.

Considering the function of these genes, these substitutions could lead to an alternative interaction with signal molecules of the pathogen(s). However, to support this hypothesis more experimental studies are necessary. A weak homology was found between the *VRP*1 genes and the *RPW*8 resistance gene at the N-terminus. *RPW*8 was isolated from *Arabidopsis* and confers broad-spectrum resistance to powdery mildew (Xiao et al., 2001). An alternative hypothesis would be a non-specific activation of *VRP*1 genes during pathogen attack. As suggested by Seo et al. (2006), the activation of a resistance gene by a pathogen may lead to non-specific activation of other resistance genes.

Although the experiments at transcriptional level show induction of the expression of *VRP*1 genes when challenging the plants with the biotrophic pathogen *P. viticola*, the *VRP*1 genes mapped in a genomic region (LG-10) where no stable QTL conferring resistance to pathogens could be detected so far. Considering that *VRP*1 genes would play a major role in the detection of the pathogen by the host followed by the downstream activation of resistance responses, a significant change in the phenotype may be expected. However, the lack of QTLs in this genomic region can not be used as conclusive argument to exclude the involvement of this gene family in the resistance mechanisms to the pathogen. It suggests that the *VRP*1 gene products may require additional gene functions to mediate resistance against *P. viticola*, just like its nearest homologue *NRG*1, a cofactor of N-mediated resistance to tobacco mosaic virus in tobacco that requires the TIR-NBS-LRR N gene product to function (Peart et al., 2005).

A non-specific activation of *VRP*1 genes during pathogen attack could be an alternative explanation. The activation of non-specific resistance genes could enhance the defense response, involving the induction of different defense pathways (Seo et al. 2006). Genes encoding protein(s) responsible for the regulation of the *VRP*1 genes could be co-located with QTL regions with a significant phenotypic effect on resistance. Further investigations are necessary to verify the exact functional role of this gene family.

The cv. `Regent´ shows a complex pedigree consisting of wild North American *Vitis* species (donor of resistance) crossed with traditional *V. vinifera* cultivars (donor of quality features). Therefore, the high homology between the *VRP*1 genes from `Regent´ and the orthologous genes from the Pinot noir clone sequenced (PN40024) could suggest that the genes isolated from `Regent´ could in fact be inherited from a *V. vinifera* cultivar.

Considering that all *V. vinifera* cultivars used during the breeding process of `Regent´ are susceptible to *P. viticola*, we would not expect that these genes alone have a considerable effect in the expression of resistance against the pathogen. Otherwise, small differences in regulatory regions of resistance genes are sometimes enough to deactivate a resistance gene. The isolation of orthologous *VRP*1 genes from wild *Vitis* species or hybrids (e.g `Gloire de Montpellier´) will help to elucidate this question.

This investigation represents the first publication of a full sequence of resistance gene analogs from grapevine. The importance of this class of genes in grapevine has been recognized in other investigations. Barker et al. (2005) demonstrated that the *Run*1 locus that confers complete resistance to *Erysiphe necator* is composed of a NBS-LRR gene family. Additionally, RGA markers co-located with genomic regions associated with resistance to different pathogens were detected (Di Gaspero et al., 2007). However, the way in which these genes act in grapevine is currently unknown. The availability of the full genome sequence of grape in the near future should offer highly efficient support for these investigations. To better understand the molecular interactions between grape and *P. viticola*, both the host and the pathogen must be systematically analyzed.

Very little is known about the diversity of the pathogen. A classification of the pathogen into potentially given races and the identification of the corresponding specific resistance genes can lead to the development of control strategies of the pathogen, either by traditional breeding, supported by marked assisted selection, or by transfer of resistance genes into traditional susceptible grapevine cultivars via genetic transformation. This is a very exciting challenge for the future. It could lead to a better elucidation of the molecular mechanisms involved in the expression of resistance in grape, not only for *P. viticola*, but also for other important pathogens.

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8. General discussion and conclusions

8.1 The advantageous use of microsatellite markers for grapevine genetic map construction

The construction of genetic maps based on microsatellite markers has many advantages. These markers are co-dominant, highly polymorphic and very specific usually amplifying only a single locus of the genome. The arrangement of several microsatellite-flanking primers in single PCR-reaction mixtures (multiplex) may be combined with automation and allows high-throughput microsatellite genotyping. The transferability of these markers within and amongst grape species is very high, enabling the construction of both intra (Adam-Blondon et al., 2004) and inter-specific (Lowe and Walker, 2006) grapevine genetic maps.

Grapevine is highly heterozygous and characterized by high inbreeding depression. The development of homozygous lines as commonly used in annual crop plants as basic material for genetic mapping studies, is very difficult if not impossible in grapevine. Consequently, no F_2 or doubled-haploid populations can be generated for mapping purposes. To overcome this problem, encountered also in other fruit crops such as apple, a mapping strategy, termed double-pseudo testcross (Grattapaglia and Sederoff, 1994) was employed. In this strategy, genetic maps can be constructed using F_1 populations directly, considering exclusively heterozygous markers present in one parental type or double heterozygous markers of both parents The recombination frequencies of independent meioses (formation of maternal and paternal gametes) are used to construct two independent parental maps. By the use of co-dominant markers and dominant double hets the parental maps can be integrated into a single map. In the present investigation the use of co-dominant microsatellite markers easily permitted the integration of the parental maps from `Regent´ and `Lemberger´ into a single map (Welter et al., 2007). The integration of several independent maps into a single integrated map is possible by using transferable markers, as demonstrated by Doligez et al. (2006). At the Institute two additional segregating populations were used in genetic mapping studies employing microsatellite markers. The maps obtained from all three populations will be integrated into a single map.

Due to the specificity and conservation of the microsatellite markers, the resulting maps are comparable to other published grapevine maps based on the same microsatellite markers. The specificity of the microsatellite markers allows also anchoring the present genetic map in the genome sequence of PN40024 (Jaillon et al., 2007). This is especially important for the identification of candidate genes in QTL regions. The mapping population employed in map construction segregates for resistance against grapevine powdery and downy mildew and quantitative traits (QTLs) conferring resistance to both pathogens have been localized in the genetic map (Fischer et al., 2004; Welter et al., 2007).

Various microsatellite markers were localized within the confidence interval comprised by these QTLs (they are linked with the resistance traits) and can be directly tested for their usefulness in marker-assisted selection (MAS). Microsatellite markers linked to the QTL conferring resistance to powdery mildew (LG-15) are being used for the map-based isolation of the gene(s) responsible for the resistance.

8.2 Detection of QTLs conferring resistance to grape powdery and downy mildew

Quantitative traits conferring resistance to powdery and downy mildew were localized in the `Regent´ x `Lemberger´ genetic map (Welter et al., 2007). Only one single major QTL conferring resistance to powdery mildew was detected on LG-15. For downy mildew, two QTLs, one with a major effect located on LG-18 and one with a minor effect located on LG-04, were found. All the resistance QTLs detected were inherited from the resistant cv. `Regent´. `Regent´ combines high wine quality and resistance to powdery and downy mildew. Its resistance traits were introgressed by complex crossbreeding between susceptible traditional *Vitis vinifera* cultivars (contributing high wine quality) and resistant wild *Vitis* species or interspecific hybrid genotypes as donors of resistance. Consequently, the resistance genes have been probably inherited from different wild species and therefore may result in durable resistance.

Durable resistance is a central aspect of the breeding program at the Institute. One approach taken to increase the sustainability of the resistance is the combination of various resistance genes to a specific pathogen into a single cultivar (pyramiding of resistance genes). It is more difficult for a pathogen to overcome the resistance conferred by a range

of genes than only one mechanism specified by a single resistance gene. The accumulation of resistance genes in new breeding lines is very hard to follow only by phenotypic evaluations. The use of molecular markers tightly linked to resistance genes is an effective tool to monitor the pyramiding of gene (MAS – marker-assisted selection). The practical application of resistance gene pyramidization in grapevine using molecular tools was investigated. This was accomplished by analyzing F_1 progeny derived from the cross between *VHR 3082-1-42* and `Regent´. Both parents are resistant to powdery and downy mildew but the resistance background is divergent. Microsatellite markers co-located with the major resistance QTL to powdery and downy mildew detected in `Regent´ were included in this analysis (see annex 1, Eibach et al. 2007). Although no knowledge is yet given about how tightly these markers are linked with the underlying resistance genes, the use of them in MAS appeared very promising to the transfer of the resistance factors from `Regent´ to new breeding lines. In general the individuals of the progeny containing the microsatellite allele linked with resistance were phenotypically more resistant. This correlation was especially well pronounced for downy mildew. Exceptions to the rule may be a result of recombination events that occurred between the microsatellite markers and the resistance genes. The identification and characterization of the resistance genes underlying the QTLs will allow the design of molecular markers within these genes avoiding the segregation between molecular marker and resistance genes, consequently improving the efficiency of MAS for these genes.

8.3 Positional candidate genes

Although QTLs conferring resistance to powdery and downy mildew were detected it is not possible to sufficiently resolve these QTL to identify the causative genes and generate tightly linked markers that could be used in MAS. The identification of genes (CG) that co-segregated with QTLs (positional candidate gene) may facilitate the isolation of the causative genes. This approach had been successfully applied either in other plant species (e.g., Calenge et al. 2005; Xu et al. 2005) as well as in grapevine (Donald et al. 2002) to isolate resistance genes. Therefore, we integrated structural and functional genes related to diseases resistance in the `Regent´ x `Lemberger´ genetic map (Welter et al., 2008a and b). The functional genes were selected from an EST- (expressed sequence tags) library from

`Regent´ (Salmaso et al., 2004), the structural ones were resistance gene analogs (RGAs) provided through the work of Di Gaspero and Cipriani (2002 and 2003). At that time no information about the genome sequence of grapevine was available.

Our results were especially promising for the QTL conferring resistance to downy mildew detected on LG18 (Fischer et al., 2004 and Welter et al., 2007). Two functional (IIIb08 and Ia01) and three structural (RGA – Resistance Gene Analogs) genes were localized within the genetic region comprised by the QTL. Sequencing analysis confirmed the presence of these genes in wild grapevine species and complementary these genes could also be localized in the genome sequence of PN40024. The RGA-genes mapped in this region putatively encode proteins containing the conserved domains NBS (Nucleotide Binding Site) and LRR (Leucine Reach Repeat). About two thirds of the R genes (conferring resistance to biotrophic and hemibiotrophic pathogens) so far isolated from other plants are members of the NBS-LRR resistance gene class (Xiao, 2006). IIIb08 codes for protein containing a LRR domain (at its N-Terminus) plus a serine/threonine kinase domain (at the C-Terminus). Such proteins belong to another class or R genes known in plants. One example is the *Xa21* gene from rice that encodes a transmembrane receptor carrying a large extracellular LRR domain and an intracellular protein kinase domain (Song et al., 1995). This gene confers broad-spectrum resistance to rice leaf blight caused by different strains of *Xanthomonas oryzae* pv. *oryzae* (Wang et al., 1996). Functional analysis provided strong evidence that R genes are involved in specific direct or indirect pathogen recognition, triggering a strong defense response (Jones and Dangl, 2006).

A parallel and complementary investigation performed by Di Gaspero et al. (2007) allowed the genetic mapping of further RGAs within this region. Looking at the grapevine genome sequence of PN40024 that recently became available, a cluster of these gene classes can be found in this position of the genome. All these results together strongly suggest the involvement of such genes in the resistance response to the biotrophic pathogen downy mildew in grapevine. As these gene clusters are also present in susceptible genotypes (e.g. PN40024), there is an important question to be answered: Are there allelic forms of these genes present only in resistant wild species that are responsible for the resistance or were new genes generated during the co-evolution between grapevine and downy mildew or both? This question will be addressed in the near future by the characterization of gene diversity complemented by more phenotypical and functional analyses. The use of the

genome sequence of PN40024 as a framework should allow the rapid isolation of homologous (or allelic forms) of these candidate genes from resistant genotypes. In general the pathogen recognition by R-genes is very specific, and it may be possible that the interaction of several of these genes (or their allelic forms) are required to confer resistance to the different variants of the pathogen. This would explain the high number of NBS-LRR genes present in this genomic region.

8.3 Powdery mildew-inducible genes

The application of the microarray technology for analysis of differential gene expression and the identification of genes involved in the defense response to powdery mildew was very useful. Although the microarray slides available for this investigation contained only about half of the putative grapevine genes and the sequences were obtained predominantly from *V. vinifera* cultivars, a set of differentially expressed genes could be identified. Quantitative Real Time PCR analysis confirmed the responsiveness of part of this set of genes detected in the microarray analysis. These genes belong to different functional gene classes and their induction indicates parallel activation of complementary pathways and genes to combat the pathogen. This is supported by the finding of activated transcription factors (e.g. WRKY, MYB and ERF) that have been shown to modulate the expression of specifically transactivated sets of defense-related genes, such as of PR-proteins or enzymes involved in the secondary metabolism (Sugimoto et al., 2000; Chakravarthy et al., 2003; Qiu et al., 2007).

The induction of PR-proteins and key genes composing the phenylpropanoid pathway was confirmed by qRT-PCR. The phenylpropanoid pathway is an important branch of the secondary metabolism, leading to the biosynthesis of many molecules that may play a range of roles in the defense response to pathogens, such as acting as signalling molecules (e.g. acid salicylic); mediating antimicrobial activity (e.g. resveratrol) and enhancing structural barriers (e.g. lignin) (Dixon et al., 2002). The induction of the PR (pathogenesis related protein)-10 and PR-5 seems also to be an important component of the grape defense response. The great majority of the genes analysed were clearly stronger induced in the resistant cv. `Regent´ than in the susceptible cv. `Chardonnay´.

The responsiveness of these genes to infection with powdery mildew strongly suggests their involvement in the defense response. The activity of these genes could play an important role during the defense response. Therefore, it was of interest to define the genomic location of these genes. The position of the most interesting genes (e.g. transcriptions factors) was defined by genetic mapping. The complete set of genes evaluated by qRT-PCR was also localized in the genome sequence of PN40024. Genetic mapping and genome sequence analysis mapped the genes to the same chromosome in every case. None of these genes appeared directly associated with the major QTL conferring resistance to powdery mildew, detected on LG-15 (Fischer et al., 2004 and Welter et al., 2007). This finding suggests that these genes are ranked downstream of other gene(s) probably involved in the initiation of defense response. Such higher order regulators may be contained in the R-gene(s) found within the QTL region (possibly explaining the major effect of the QTL). These may be responsible for pathogen recognition and consequently triggering a strong defense response, which includes the genes evaluated in this study. There are many reasons that can explain the missing identification of such genes by microarray analysis, such as constitutive expression or their absence on the microarray slides.

This experiment rendered new insights in the grapevine defense response against powdery mildew infections, such as identification of transcription factors with induced expression, the involvement of PR-proteins and the activation of the secondary metabolism. However, these results are only the beginning. Technical advances in sequencing allow the rapid analysis of new EST libraries constructed from wild species. In combination with the availability of the whole genome sequence of grapevine, this should allow the development of new microarray chips representing the whole grapevine gene set complemented with selected genes from wild species (resistant genotypes). The use of such microarray chips will contribute greatly in the elucidation of resistance mechanisms involved in the defense response of grapevine, as demonstrated in other plant/pathogen interactions (e.g. Maleck et al., 2000).

8.4 The isolation and characterization of candidate genes for resistance to diseases

In a previous investigation employing differential display analysis transcripts differentially expressed in the resistant genotype `Gloire de Montpellier´ have been identified after infection with downy mildew (*Plasmopara viticola*, 12 hour post infection). Three of these transcripts shared high nucleotide similarity to each other and resembled characterized resistance R-genes of the NBS-LRR class isolated from other plant species. The responsiveness of these genes to downy mildew was confirmed by Northern blot analysis (Kortekamp, 2001). This strongly suggested some involvement of these genes in the defense response to downy mildew. Their homology to R-genes indicates that these genes could play a functional role through pathogen recognition by triggering the defense response. In this case, we could expect that these transcripts are co-localised with resistance QTLs detected to downy mildew. Attempts to map these transcripts failed. Therefore the complete sequences of orthologous genes were isolated from the cv. `Regent´.

As expected, a gene family of putative R-genes was detected on the sequence of a BAC-clone of cv. `Regent´. Three CC-NBS-LRR genes (VRP1-1, -2 and -3) showing high amino acid similarity to each other were identified. By the design of specific primers inside intron regions these genes could be independently integrated in the `Regent´ x `Lemberger´ genetic map. These genes mapped to LG-10, where no resistance QTL to downy mildew was detected. For that reason, we do not expect that these genes play a major role in the resistance response to downy mildew in the cv. `Regent´, from which the genes were isolated. Unfortunately, no QTL data are currently available from `Gloire de Montpellier´.

The responsiveness to downy mildew infection in `Gloire de Montpellier´, however, suggests the involvement of VRP1 genes in the defense response in some way. There are many possible explanations. The first would be a divergent regulation of these genes between `Regent´ and ´Gloire de Montpellier´. The isolation of orthologous VRP1 genes from `Gloire de Montpellier´ followed by functional analysis will help to elucidate this open question. Additionally, the VRP1 peptides show highest similarity to the NRG1 gene, which participates together with the N gene (TIR-NBS-LRR) to mediate resistance to tobacco mosaic virus in tobacco (Peart et al., 2005). In the same way, the VRP1 genes could interact with other R-genes to confer resistance to diseases, explaining their

induction. An alternative explanation is a non-specific activation of VRP1 genes during pathogen attack. As demonstrated by Seo et al. (2006) R-genes can be induced in a non-specific manner. In this investigation an R gene (TIR-NBS-LRR) was identified by inoculating common beans (*Phaseolus vulgaris*) with a Gemini virus reporter. However, functional analysis revealed that this gene did not confer resistance to the reporter Gemini virus, but it did activate a resistance-related response (systemic necrosis) to seven strains of cucumber mosaic virus (CMV) from pepper or tomato, but not to a CMV strain from common bean. This suggests that the VRP1 genes could also play a major role in another grape/pathogen interaction. Such possible non-specific activation of resistance genes is thought to enhance the defense response, involving the induction of different defense pathways. Functional analysis of the VRP1 genes will elucidate these assumptions. Further investigations are necessary to verify the exact functional role of this gene family.

9. Summary

Powdery (*Erysiphe* syn. *Uncinula necator*) and downy mildew (*Plasmopara viticola*) are the two most important diseases in grapevine. Both pathogens are native from North America and were introduced in Europe app. in the middle of the 19th century. The European grapevine (*Vitis vinifera*) is very susceptible against these two pathogens. As all internationally acknowledged grapevine cultivars used for wine production are members of the *V. vinifera* species, enormous amounts of fungicides - widely exceeding the amounts used in other crops- are applied in vineyards every year to protect grapevine from the injuries caused by the mildew diseases. Resistance sources can only be found in wild species (or cross derivates) natives from American and Asian grape gene pools. Introgression of these resistance genes into the European grapevine by traditional breeding is difficult and very time consuming due to the low wine quality from the wild species. Many generations of backcrossing to grapevine cultivars are required to re-establish wine quality. The use of molecular tools should render the breeding process more precise and efficient, reducing the time required to breed new resistant elite grapevine cultivars.

Considering all these aspects, the present thesis had as general objective the genetic and molecular analysis of mildew disease resistance in grapevine. These investigations should provide new basic knowledge of the interaction between grapevine and mildew diseases and make available molecular tools to be employed in breeding programs. Based on this conception, different investigations were performed: i) Construction of a genetic map and localisation of quantitative traits conferring resistance to powdery and downy mildew; ii) Integration of functional and structural resistance candidate genes into the genetic map; iii) Transcriptional analysis of grapevine after infection with powdery mildew and iv) Isolation and *in silico* characterization of a resistance gene analog family.

Based on the double-pseudo test cross strategy, a grapevine genetic map was constructed employing 144 F_1 progeny derived from the cross between the mildew-resistant grapevine cv. `Regent´ and the mildew-susceptible cv. `Lemberger´. In total, 122 microsatellite markers were used to genotype the mapping population. The information obtained with these microsatellite markers was combined to previously generated information with other classes of molecular markers (mainly AFLP and RAPD) to construct a new integrated map

of `Regent´ x `Lemberger´. Co-dominant microsatellite markers permitted the integration of the two parental maps into a single genetic map of `Regent´ x `Lemberger´. The new integrated map contains 398 markers aligned along 19 linkage groups, covers a total length of 1,631 cM and shows an average distance between markers of 4.67 cM. This genetic map was then used to localize QTLs (quantitative trait loci) conferring resistance to powdery and downy mildew pathogens transmitted from `Regent´. For downy mildew two stable QTLs were detected, one with major effect located on linkage group (LG) 18 and one with minor effect located on LG 4. Only one major QTL conferring resistance to powdery mildew was detected on LG-15 (Welter et al., 2007). Microsatellite markers linked to QTLs were detected and their potential for marker-assisted selection was afterwards tested (Eibach et al 2007).

Additionally to microsatellite markers, functional and structural CGs were genetically mapped using the `Regent´ x `Lemberger´ population. This aimed at the identification of CGs associated with the QTLs conferring resistance to mildew diseases. Genes linked with either the major resistance QTL to powdery as well as to downy mildew were found. These results seem especially promising for downy mildew resistance. Two functional (IIIb08 and Ia01) and three structural (RGA – Resistance Gene Analogs) genes were localized within the genetic region comprised by the QTL. The putative RGA-genes mapped in this region encode proteins containing the conserved domains NBS (Nucleotide Binding Site) and LRR (Leucine Reach Repeat) (Welter et al., 2008a and b). About two thirds of the R genes (confer resistance to biotrophic and hemibiotrophic pathogens) so far isolated from other plant species are members of the NBS-LRR resistance gene class. IIIb08 codes for a protein containing a LRR domain (N-Terminus) plus a serine/threonine kinase domain (C-Terminus). Such proteins belong to another class of R genes isolated from plants. These CGs give first insights about the putative functional role of this QTL and may be used for the isolation of the full gene sequence and the development of practical molecular markers to be tested in MAS.

Grapevine transcriptional analysis after infection with powdery mildew allowed the identification of differentially expressed genes. Initially, differential microarray hybridizations permitted the identification of a battery of mildew-responsive genes 10 hpi. Subsequently, a subset of these genes were selected and analyzed with more detail by quantitative real time PCR (qRT-PCR) by repeating the infection conditions. From the 27

genes investigated, 24 showed at least two-fold induction in `Regent´ infected with powdery mildew, when compared to the control. The great majority of the genes evaluated were significantly stronger induced in the resistant cv. `Regent´. The induced genes are members of different components of the defense response, such as transcription factors, PR-proteins and enzymes involved in the secondary metabolism. Four classes of transcription factors [WRKY, Myb, Ethylene-responsive factor (ERF) and CZF1/ZFAR1] were investigated and their expression is induced. These interact with divergent cis-acting elements commonly found in the promoter regions of pathogenesis-related genes, modulating the expression of a complementary set of genes. PR-10 and PR-5 were the most strongly activated genes in the incompatible interaction, showing 50- and 15-fold induction, respectively. Also the phenylpropanoid pathway was activated. The expression of phenylalanin ammonia-lyase (PAL) was 12-fold induced. None of the genes tested here are associated with the resistance QTL to powdery mildew detected on LG-15.

Finally, the full gene sequence of three CC-NBS-LRR resistance gene analogs were isolated, *in silico* characterized and genetically mapped. Differential display and northern blot analysis had shown that these genes were induced in the resistant `Gloire de Montpellier´ after downy mildew infection. These genes were isolated from the cv. `Regent´ using a BAC-library. A positive BAC-clone (app. 134 kb) was submitted to sequencing. The assembly of the shotgun sequences resulted in six contigs, which were then linked to each other by direct sequencing of PCR products obtained from contig-specific gap-outreaching primers, resulting in a single contig of 134,392 bp length. Bioinformatic analysis allowed the discovery of three resistance gene analogs, referred to as VRP1-1, -2 and -3. They all exhibit the typical structure of CC-NBS-LRR resistance genes and putatively encode proteins of 798, 811 and 813 amino acids, respectively. They share high similarity to each other and were mapped on LG-10, where so far no resistance QTL to downy mildew was detected (Kortekamp et al., submitted).

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11. Zusammenfassung

Echter (*Erysiphe necator*) und Falscher Mehltau (*Plamopara viticola*) sind weltweit die wirtschaftlich bedeutendsten Krankheiten in Weinreben. In der vorliegenden Arbeit wurden verschiedene Untersuchungen durchgeführt, um neue Grundlagenerkenntnisse über die Interaktion zwischen Weinreben und Mehltau-Pathogenen zu erarbeiten und darüber hinaus nutzbare molekulare Werkzeuge für die Züchtung zur Verfügung zu stellen. Zunächst wurden 144 Nachkommen der Kreuzung `Regent´ x `Lemberger´ benutzt, um eine bestehende genetische Karte zu verbessern. Für diese Analyse wurden hauptsächlich Microsatelliten- Marker benutzt. `Regent´ ist widerstandfähig und `Lemberger´ ist anfällig gegenüber beiden Mehltau-Krankheitserregern. Quantitative Resistenzfaktoren gegenüber diesen beiden Krankheiten wurden auf der genetischen Karte lokalisiert. Mit Resistenzfaktoren gekoppelte Microsatelliten-Marker wurden identifiziert und stellen ein potenzielles Werkzeug für Marker-gestützte Selektion dar. Obwohl Resistenzfaktoren identifiziert werden konnten, reicht die Auflösung dieser Analyse nicht aus, um die verantwortlichen Resistenzgene zu lokalisieren. Um Kandidaten-Gene zu ermitteln, wurden anschließend potenzielle Resistenzgene in der genetischen Karte lokalisert. Interessanterweise konnten mit Resistenzfaktoren gekoppelte Kandidatengene gegen beide Krankheiten identifiziert werden. Die meisten besitzen Ähnlichkeit zu bekannten Resistenzgenen aus anderen Pflanzen und stellen sehr interessante Kandidaten Gene dar, die weiter untersucht werden müssen. Ein weiterer Ansatz war die Transkriptomanalyse von Weinreben nach Infektion mit dem Echten Mehltau. Hierfür wurde zunächst die DNA-Chip Technologie zur Identifizierung differenziell exprimierter Gene angewendet. Anschließend wurde die Expression einer Reihe hochregulierter Gene durch qReal Time PCR überprüft. Die Aktivierung der Genexpression konnte bestätigt werden. Diese Gene gehören zu unterschiedlichen funktionellen Genklassen und spielen vermutlich unterschiedliche Rollen bei der Abwehrreaktion. Dazu gehören Transkriptions-Faktoren, PR- (pathogenesis realated) Proteine und Gene, die am pflanzlichen Sekundärmetabolismus beteilig sind. Schließlich wurde eine potenzielle Resistenzgenfamilie aus der Sorte `Regent´ isoliert und charakterisiert. Diese Gene kodieren für CC-NBS-LRR (coiled-coil - nucleotide binding site - leucine rich repeat) Proteine, welche bei anderen Pflanzen in der Erkennung des Pathogens beteiligt sind und daraufhin eine starke Abwehrreaktion auslösen.

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13. Curriculum vitae

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15. ANNEX 1

Vitis **46** (2), 120–124 (2007)

The use of molecular markers for pyramiding resistance genes in grapevine breeding

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Summary

The practical application of pyramiding resistance genes by the use of molecular markers was investigated in a F1 progeny derived from the cross of *VHR 3082-1-42* x 'Regent'. *VHR 3082-1-42* is a cross between *Muscadinia rotundifolia* x *Vitis vinifera*, backcrossed another four times with V. vinifera (Pauquet *et al.* 2001). It carries the *Run1*-gene which causes resistance to powdery mildew and the *Rpv1*-gene which is related to resistance against downy mildew. Both genes were introduced from *Muscadinia rotundifolia* (Bouquet *et al.* 2000; Wiedemann-Merdinoglu *et al.* 2006). 'Regent' is a new cultivar with quantitative resistance against downy and powdery mildew (Eibach and Töpfer 2003) released in Germany in 1996 for commercial use. 119 individuals of the F1 progeny were screened with a molecular marker for the *Run1*-gene (Donald *et al.* 2002), with two SSR-markers for the *Rpv1*-gene (Wiedemann-Merdinoglu *et al.* 2006) and with several markers from 'Regent' that showed good correlation to powdery and downy mildew resistance (Zyprian *et al.* 2002, Salakhutdinov *et al.* 2003, Akkurt 2004, Fischer *et al.* 2004, Akkurt *et al.* 2007). Phenotypic evaluation for downy mildew resistance was done by artificial inoculation of leaf discs, and for powdery mildew by natural infection in a greenhouse. Comparison of the phenotypic data with the results of the molecular marker analyses showed a clear correlation between the degree of resistance and the presence of the resistance related alleles. According to the phenotypic data, 20 genotypes of the offspring were free of powdery and downy mildew infections. Based on a marker-assisted evaluation, out of these 20 genotypes a subset of four carried all the resistance related alleles for powdery and downy mildew indicating that resistance genes from both parents were effectively combined.

K e y w o r d s : downy mildew, powdery mildew, *Vitis*, resistance, marker assisted selection, breeding.

Introduction

The use of molecular markers is becoming increasingly important for breeding purposes in a lot of agricultural crops like wheat (Adhikari *et al.* 2004, Gupta *et al.* 2005, Yang *et al.* 2005, Sardesai *et al.* 2005), rice (Sharma *et al.* 2004, Ashikari and Matsuoka 2006) maize (Widstrom *et al.* 2003) but also grapes (Striem *et al.* 1996, This *et al.* 2000, Doligez *et al.* 2002, Mejia and Hinrichsen 2003). Of particular relevance is this method for polygenic traits such as resistance against the mildews in grapevine. From the breeding point of view, it is highly desirable to combine as many resistance genes as possible in a new cultivar in order to make resistance as sustainable as possible. Based only on phenotypic evaluation data, it is hardly feasible to track the accumulation of resistance genes in a new breeding line. The use of molecular markers provides a new tool for breeders and may help to overcome this problem (Dalbó 1998, Dalbó *et al.* 2001, Luo *et al.* 2001, Pauquet *et al.* 2001, Fischer *et al.* 2004).

Material and Methods

Investigations were carried out on a F1 progeny of 119 individuals derived from a cross between *VRH3082-1-42 x* 'Regent'. Population was grown with one plant per genotype in a greenhouse with natural soil and at the time of investigation the plants were four years old. The female parent *VRH3082-1-42* can be traced back to an initial cross between *Muscadinia rotundifolia G52* x Malaga seedling No. 1 and four further backcrosses with *Vitis vinifera* cultivars (Pauquet *et al.* 2001). It carries the *Run1*-gene which is responsible for resistance against powdery mildew and is linked to the CAPS-marker GLP1-12 (Donald *et al.* 2002). It also carries the *Rpv1*-gene which confers good resistance against downy mildew (Wiedemann-Merdinoglu *et al.* 2006). 'Regent' is a new cultivar with complex resistance against powdery and downy mildew, released in Germany for commercial use in 1996 (Eibach and Töpfer 2003).

Phenotypic screening for downy mildew was performed with artificial inoculation tests. One leaf per plant was collected from a well developed shoot in the middle of the cane. For harmonizing leaf age the 7th leaf from shoot top was harvested respectively. Four leaf discs per leaf were generated. They were placed in a plastic box on wet filter paper. 20 µl of a spore suspension with a concentration of 50.000 spores/ml was used for artificial inoculation. Screening of downy mildew infection was done one week after infection. Ratings followed the OIV descriptor list for grapevine cultivars and *Vitis* species (Anonymous 1983) while not the degree of resistance but the degree of infection was rated (1 = no infection, 9 = heavy infection). Experiments were done twice. Natural appearance of powdery mildew infection on leaves was rated in a greenhouse in 2005 and 2006 (1 = no infection, 9 = heavy infection).

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For both mildews the highest degree of infection for the individual genotypes was used for further calculation.

DNA was isolated from young, healthy leaves following the protocol of THOMAS *et al.* (1993) or using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). According to the protocol of DONALD *et al.* (2002), the CAPS marker GLP1-12 was used to evaluate the progeny for the presence or absence of the *Run1*-gene. Screening for the *Rpv1*-gene was done using two linked SSR-markers, VMC_8g9 and VMC_1g3.2, according to WIEDEMANN-MERDINOGLU *et al.* (2006). FISCHER *et al.* (2004) developed a genetic map using the progeny of a cross between 'Regent' x 'Lemberger'. A major QTL for downy mildew was located on linkage group 18 and another one for powdery mildew on linkage group 15. Three SSR-markers located within the QTL for downy mildew and three SSR-markers located within the QTL for powdery mildew were used for genetic analysis. In addition the SCAR-marker ScOR A7-760 linked to the powdery mildew QTL (AKKURT *et al.* 2007) was included in the investigations.

PCR for SSR loci were performed in standard reactions of 10 µl. Primer pairs were labelled with ABI fluorescent dyes and analyzed by capillary electrophoresis on an ABI 3100 Genetic Analyser. PCR for the SCAR marker was carried out in standard assays in a total volume of 25 µl. Products were separated on a 1.5 % agarose gel.

Results and Discussion

No powdery mildew infection was observed on 48 % of the genotypes of the F1 progeny (Fig. 1 A). By dividing them into two subgroups with and without the *Run1*-gene, all the genotypes carrying the *Run1*-gene proved to be free of powdery mildew infection (Fig. 1 B). However those genotypes not carrying the *Run1*-gene were distributed over all classes of infection, with 9 % showing either no infection or severe infection (Fig. 1 B). The frequency distribution of the classes of infection for those genotypes carrying neither the *Run1*-gene nor the individual resistance related alleles of 'Regent' is demonstrated in Fig. 2 A. Considering the absence of all resistance related alleles of 'Regent', no individual was rated as free of infection. Compared to this group, the presence of the individual resistance related alleles of 'Regent' led to a significant shift of the frequency distribution towards the lower infection classes (Fig. 2 B). 19 % of the individuals with all four resistance related markers of 'Regent' proved to be free of infection. All individuals with the combination of the presence of the *Run1*-gene and the absence of individual resistance related alleles of Regent were free of powdery mildew infection (Fig. 2 C). Phenotypically this group didn't differ from the group of individuals which carried the *Run1*-gene as well as the individual resistance related markers of 'Regent' (Fig 2 D). Genotypically it can be stated that, within the latter group, powdery mildew resistance genes derived from both parents are accumulated.

Fig. 3 demonstrates the frequency distribution of the progeny for downy mildew. Thirty-six percent of the population showed no downy mildew infection while 6 % showed severe infection (Fig. 3 A). The genotypes carrying the *Rpv1*-markers exhibit a frequency distribution shifted towards the lower infection classes compared to the individuals without the *Rpv1*-markers (Fig. 3 B).

The frequency distribution for the degree of infection for those genotypes carrying neither the *Rpv1*-markers nor the individual downy mildew resistant related markers of 'Regent' is shown in Fig. 4 A. Seventy-four percent of the individuals were rated 7 or worse when all the resistance related markers from 'Regent' were absent. The comparison with the group of genotypes having the individual 'Regent'-markers shows a significant shift of the frequency distribution towards the lower infection classes (Fig. 4 B). When all 'Regent'-markers were present, 96 % of the individuals were rated within the infection classes 1 and 3. The effect of the presence of the *Rpv1*-gene is demonstrated in Fig. 4 C. Compared to Fig. 4 A, there is a considerable shift towards the lower infection classes. 87 % of the vines were rated within the infection classes 3 and 5 but none was within infection class 1. The comparison of the groups shown in Fig. 4 B and Fig. 4 C demonstrates that the effect of the 'Regent'-related resistance markers led to a higher degree of resistance in the offspring than the *Rpv1*-gene related markers. The combination of the presence of the *Rpv1*-markers and the individual 'Regent'-markers is shown in Fig. 4 D. When all the 'Regent'-markers were present, these individuals showed no infection of downy mildew. This demonstrates that the combination of both

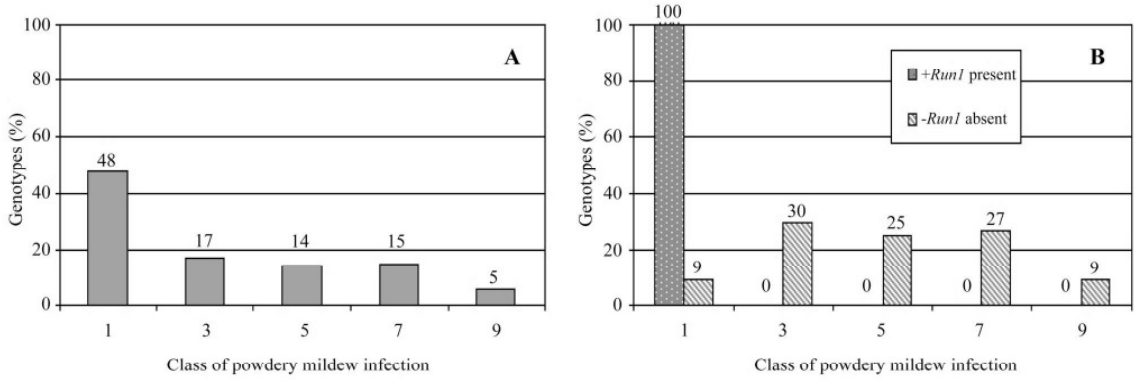

Fig. 1: Frequency distribution for powdery mildew infection (1 = no infection, 9 = heavy infection); **A**: entire progeny, **B**: progeny divided into genotypes with and without the *Run1*-gene.

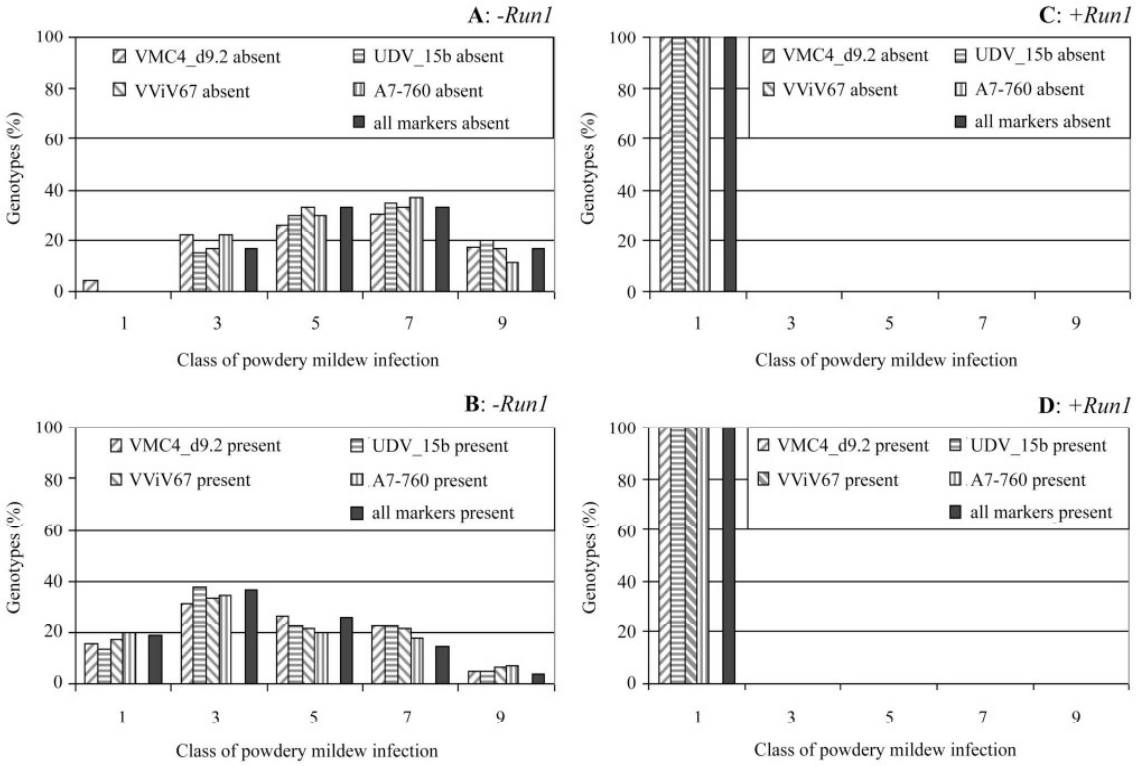

Fig. 2: Frequency distribution of powdery mildew infection (1 = no infection, 9 = heavy infection) for different groups of seedlings genotypes: **A**: genotypes not carrying the *Run1*-gene and sorted by the absence of individual powdery mildew resistance related alleles of 'Regent'; **B**: genotypes not carrying the *Run1*-gene and sorted by the presence of individual powdery mildew resistance related alleles of 'Regent'; **C**: genotypes carrying the *Run1*-gene and sorted by the absence of powdery mildew resistance related alleles of 'Regent'; **D**: genotypes carrying the *Run1*-gene and sorted by the presence of individual powdery mildew resistance related alleles of 'Regent'.

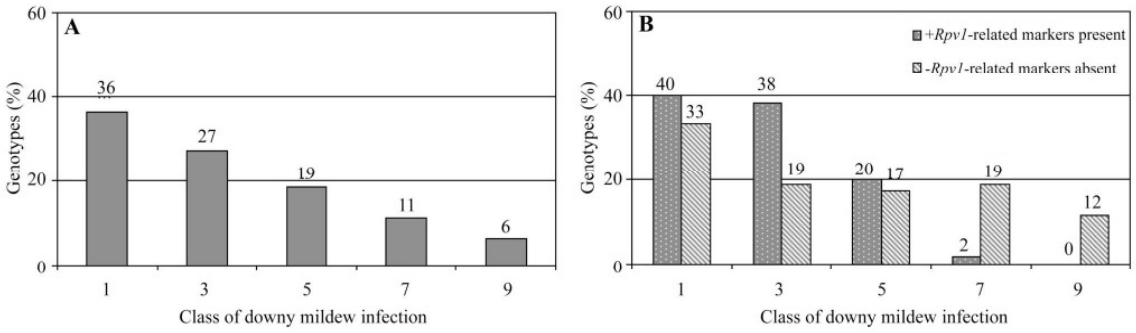

Fig. 3: Frequency distribution for downy mildew infection; **A**: total progeny, **B**: progeny divided into genotypes with and without the *Rpv1*-related markers VMC_8g9 and VMC_1g3.2.

resistance sources had an additive effect on the degree of resistance. Comparing the effect of the different resistance sources for both mildew diseases it can be stated that the effect of the resistance genes originated from 'Regent' is higher for downy mildew while for powdery mildew the effect of the *Run1*-gene is much higher, it even covers phenotypically the effect of the resistance genes originating from 'Regent'. Taking into account both mildew diseases, phenotypic screening led to 20 individuals which were totally free of any infections. By using marker assisted selection, a subset of four genotypes could be identified which carry each the *Run1*-gene and the *Rpv1*-related markers along with the entire set of powdery and downy mildew resistance related alleles originating from 'Regent'. Thus all markers associated with mildew resistance genes of both parents are present in the four individuals. By using only these vines for further breeding work it can be expected that the proportion of resistant individuals in the progeny will be higher and hence the efficiency of resistance breeding will increase.

Since marker assisted selection is still labour- and cost-intensive, a combined scheme of phenotypic and genetic

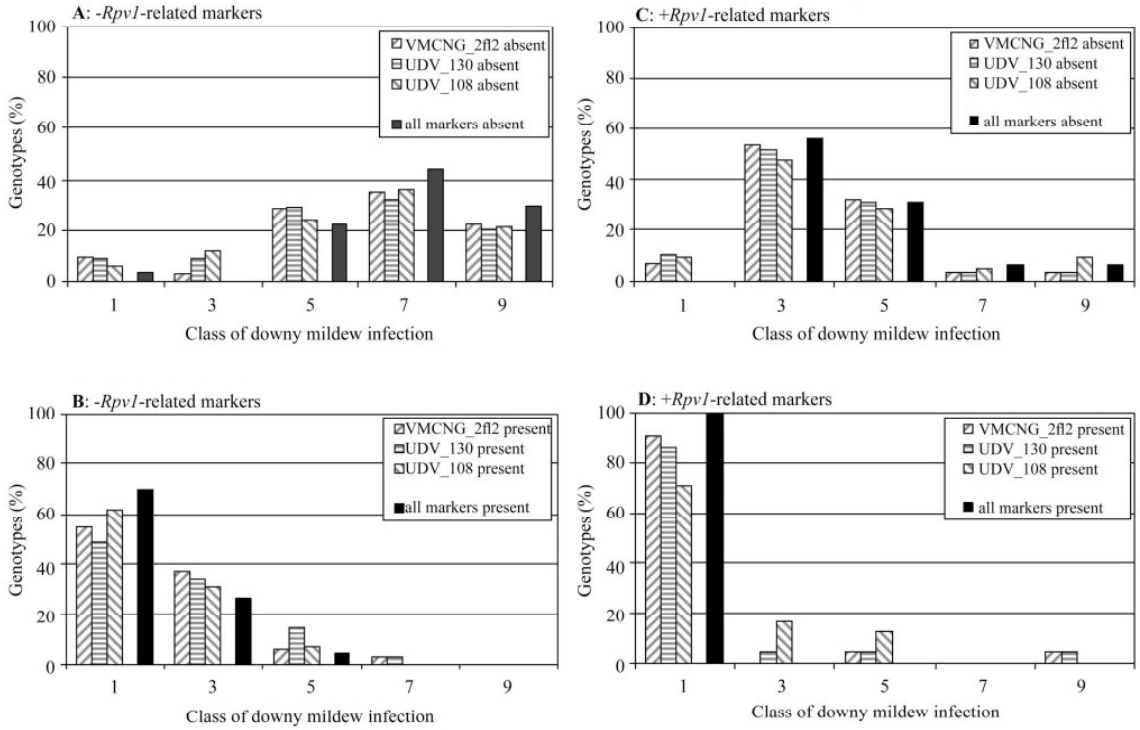

Fig. 4: Frequency distribution of downy mildew infection (1 = no infection, 9 = heavy infection) for different groups of seedlings genotypes; **A**: genotypes not carrying the *Rpv1*-related markers and sorted by the absence of individual downy mildew resistance related alleles of 'Regent'; **B**: genotypes not carrying the *Rpv1*-related markers and sorted by the presence of individual downy mildew resistance related alleles of 'Regent'; **C**: genotypes carrying the *Rpv1*-related markers and sorted by the absence of downy mildew resistance related alleles of 'Regent'; **D**: genotypes carrying the *Rpv1*-related markers and sorted by the presence of individual downy mildew resistance related alleles of 'Regent'.

selection is proposed. Fig. 5 clarifies the procedure of such a combined selection procedure applied to the investigated population as an example. In the first step the selection of downy mildew resistance can be executed rather easily by artificial inoculation of the seedlings with spores of downy mildew. In a second screening step those seedlings showing no infections are subjected to powdery mildew inoculation. Only the remaining seedlings without infections of downy and powdery mildew are processed for marker assisted selection. In the example demonstrated in Fig. 5, there are 20 seedlings, which is around 15 % of the initial seedling population. After the following marker assisted selection steps, four seedlings were identified posessing, as already mentioned, the entire set of powdery and downy mildew related alleles of both parents.

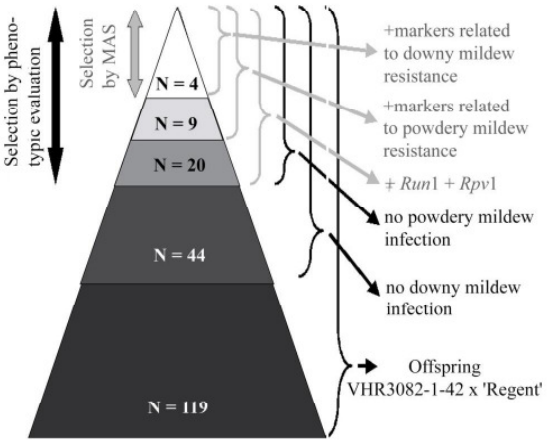

Fig. 5: Scheme for pyramiding mildew resistance genes by a combination of phenotypic evaluation and marker assisted selection (MAS). MAS reduced the number of elite individuals from 20 to 4.

Conclusions

The investigations carried out in an offspring of the cross *VRH3082-1-42* x 'Regent' show that the *Run1*-marker GLP1-12 is very tightly linked to powdery mildew resistance while the *Rpv1*-gene related markers are linked to downy mildew resistance. The evaluation of genotypes carrying neither the *Run1*-marker nor the *Rpv1*-gene related markers shows that the markers VMC_4d9.2a, UDV_015b,VVIv67 and ScOR A7-760 are related to powdery mildew resistance (19 % no infection class 1, 37 % slight infection class 3). The markers UDV_130, VMCNG2_f12 and UDV_108 are related to downy mildew resistance (71 % no infection, 25 % slight infection class 3). This relation is stronger compared to the markers for powdery

mildew. The application of marker assisted selection for the mildew resistance related markers leads to a pyramiding of resistance genes. For breeding purposes a combination of phenotypic evaluation and marker assisted selection is suggested.

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