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Towards grapevine (*Vitis vinifera* L.) mildews resistance: molecular defence mechanisms and New Breeding Technologies

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Abstract: One of the main challenges for viticulture is to sustainably maintain the production of high-quality grape varieties in the face of climate change. Current models predict an increasing disease pressure for grape, mainly because of warmer conditions in late spring-early summer. New strategies to address this challenge can stem from a deeper understanding of the grape biology and of the plant interaction with some major biotic stresses, such as mildew diseases. Grape thwarts the attack and invasion of pathogens using a composite molecular array, whose components and interactions are not fully known. This review aims to provide insights into the current understanding of plant defense mechanisms against fungal pathogens, and to discuss the set of cellular molecules that have been functionally identified in grape. It also highlights information related to the activation of grapes' immunity by using high-throughput genome-wide screenings and New Breeding Techniques as a powerful tool to achieve long-lasting and broad-spectrum resistance. Finally, the review provides food for thought to improve the sustainability of viticulture through the integration of genetic and biotechnological strategies for pathogen resistance.

Keywords: grapevine; fungi resistance; conventional breeding; New Breeding Technologies; QTLs; genomic approaches

1. Introduction

The cultivated grape (*Vitis vinifera* L.) has become the world's leading fruit crop. It is grown in almost 90 countries for wine, juice, raisins, and table grapes production. Human selection has led to a wide array of varieties endowed with useful traits related to yield, phenology, and berry chemical composition. However, these intensive breeding processes caused the loss of several other traits, such as resistance to biotic stresses, in the cultivated gene pool that were present in crop wild relatives (Khan et al., 2020). Furthermore, several grapevine pathogenic microorganisms are not indigenous to Eurasia, and therefore, vines have been not undergone selection pressure to evolve resistance. As a result, the *V. vinifera* varieties are susceptible to various pathogens responsible for severe crop losses. Nowadays, growers rely on fungicides applications and vineyard management practices to handle pathogenic fungi. However, the impact of chemicals on humans and agrobiodiversity has been widely demonstrated (Dry et al., 2019). In March 2019, nineteen Focus Group experts from different wine-growing regions of the European Union discussed and shared research needs to increase the resilience of grapevines to pests and diseases and to support the productivity of the sector in sustainable ways (EIP-AGRI, 2019). One strategy is to shift from a treatment-oriented to a disease-prevention approach by developing fungus-resistant varieties (Rousseau et al., 2013). They offer significant advantages due to their cost-effectiveness, safety, and low environmental impact.

Worldwide, the most economically important grape diseases are downy (DM), and powdery (PM) mildews caused by the ascomycete fungi *Plasmopora viticola* and *Erysiphe necator*, respectively. Until recently, no European grape *V. vinifera*, with a single exception (Hoffmann et al., 2008), has exhibited resistances to them, but wild North American vines (i.e., *V. labrusca*, *V. aestivalis*, *V. berlandieri*, *Muscadinia rotundifolia*) are significantly more resistant to pathogenic fungi (Mullins et al., 1992). For more than a century, grape breeders have attempted to introduce genetic resistance from North American *Vitis* spp. into European cultivars. Interspecific hybrids have not been successful for their low wine quality (Teissedre, 2018). Molecular breeding allowed the development of fungus-resistant grapes carrying both disease-resistance genes and a significant percentage (more than 85%) of *V. vinifera* genome in their pedigree (Sivčev et al., 2010). These achievements were possible also thanks to knowledge on the molecular basis of disease resistance. In the last three decades, impressive progress in deciphering plant immune mechanisms has been made, particularly in the model species *Arabidopsis thaliana* (Zhang et al., 2018). In grapevine, insights into the mechanisms regarding its immune machinery have only begun to be available in in recent years.

In this review, we first summarize the current understanding on the molecular mechanisms of plant resistance. We then discuss the set of specialized molecules in the immunity pathways active in grapevine against mildews and report the main breeding achievements in delivering fungi-resistant grape varieties. Finally, we discuss the need of translating current knowledge to strategies to improve grapevine varieties.

2. How plant defends against fungi: the immunity pathways

Protection of grape against fungi depends on both passive and active defense mechanisms. Broadly speaking, passive defense mechanisms are pre-existing and independent of the pathogen, while active defense mechanisms are activated only after pathogen recognition. To gain access to nutrients or to the replication machinery of the host cell, pathogens must first breach the passive defenses. These are the natural barriers of healthy plants, such as physical (e.g., wax, cuticle, cell wall, stomatal aperture, lenticels) or chemical (e.g., inhospitable pH, inhibitory compounds, phytoanticipins, lack of stimulatory compounds needed for pathogen development) (Ziv et al., 2018; Wang et al., 2020). On the counterpart, the active defense mechanisms encompass complex networks of genes and proteins. The current understanding of active plant defense is nicknamed the new "central dogma of plant pathology". It consists of three main response mechanisms, the ETI (Effector-Triggered Immunity), the ETS (Effector-Triggered Susceptibility), and the PTI (PAMP-Triggered Immunity) (Figure 1).

The initial stages of the plant-pathogen 'arms race' start with the plant immunity defense evolution, the ETI response mechanism. This mechanism consists of restoring the host species resistance status through specific resistance (R) genes. Commonly, the result of defense activation involving R-genes is the programmed cell death (PCD), known as the hypersensitive response (HR). It prevents the pathogen from obtaining nutrients and completing its life cycle (Mur et al., 2008). ETI is only effective against one or a few strains of a particular pathogen that possesses an Avr (Avirulence) protein called effector, recognized by an R-protein (Dry et al., 2009). Due to high recognition specificity, ETI signaling evolved to be robust against pathogen effectors. However, plant pathogens are highly adaptable and have much faster life cycles than their plant hosts. Therefore, resistance conferred by single R-genes can be easily defeated and as new pathogen strain appears, ETI is frequently broken (van Esse et al., 2020).

The effectors mentioned above are one of the principal components of another mechanism, the ETS. It is based on the specific pathogen ability to become "adapted" to plant species by evolving effectors. These interact with specific host proteins (effector targets), suppressing parts of their innate immunity. For instance, *E. necator* and *P. viticola* are the only mildew species, that have become adapted to *V. vinifera*. One of the well-characterized examples of effector targets are the S-proteins (Susceptibility

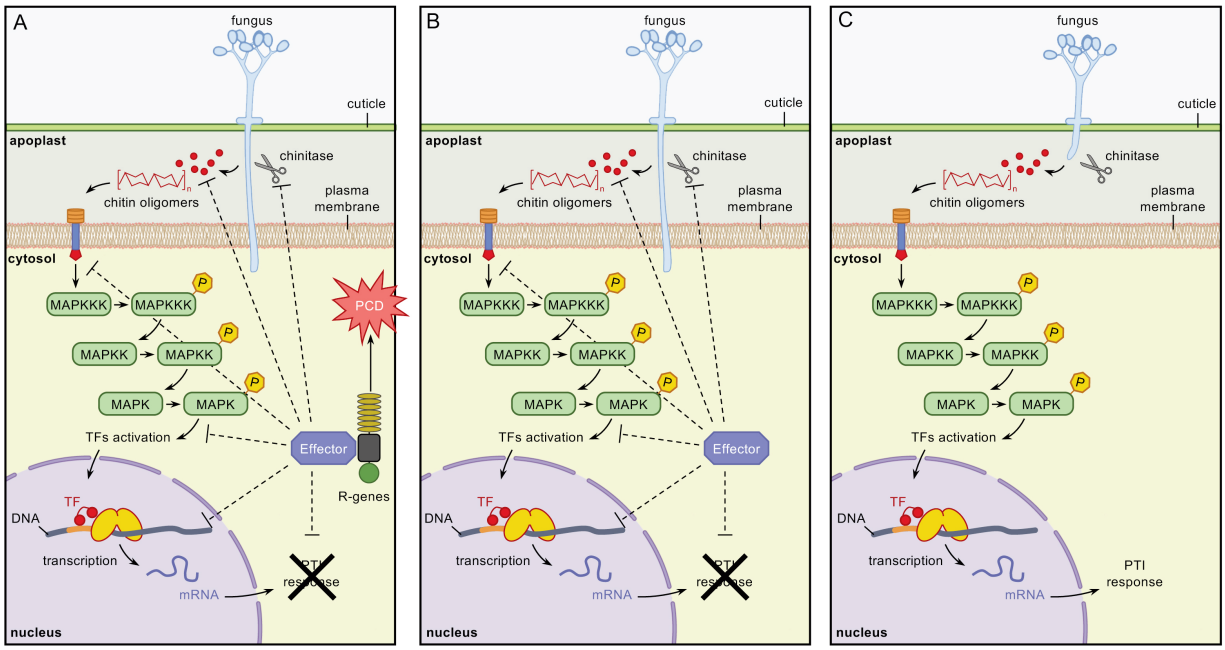

Figure 1. Representation of the three main response mechanisms of plants triggered by pathogenic fungi: the ETI (Effector-Triggered Immunity) (A), the ETS (Effector-Triggered Susceptibility) (B) and the PTI (PAMP-Triggered Immunity) (C). MAPK: mitogen activated protein kinase; PCD: programmed cell death; TF: transcription factor; R-genes: resistance-genes.

proteins), encoded by the S-genes. They are pathogen-specific molecules that facilitate the development of a disease by inviting and helping a pathogen gain entrance into a plant, negatively regulating the R-genes, or providing nutrients to support pathogen proliferation. Thus, the deactivation of S-genes can make a plant resistant to pathogens. Very recently, Moniruzzaman et al. (2020) published a list of more than 100 S-genes identified in plants, of which only 18 have been characterized. For most of them, the exact function is not very clear. The authors reported that S-genes can interfere with the expression of genes involved in plant resistance at different levels, from pathogen recognition (e.g., receptors in the epidermal cells) to molecule production.

The third mechanism is the PTI. It is based upon recognizing conserved components of pathogenic surfaces termed PAMPs (Pathogen-Associated Molecular Patterns), alternatively known as elicitors. PAMPs are recognized in the plasmalemma of plant cells by specialized molecules, named PRRs (Pattern-Recognition Receptors). They transmit the signal of infection into the cytoplasm and activate PTI responses, such as cytoskeleton rearrangements, callose deposition and induction of antimicrobial compounds (Zipfel, 2009). The PTI response usually ends up with resistance against pathogen attack, as in the case of *V. vinifera* with the non-adapted mildews, such as *E. cichoracearum* and *P. infestans*. PTI is effective against most pathogens due to their highly conserved nature. Due to the low recognition specificity, PTI cannot be very effective against well-adapted pathogens. It can still provide good immunity with low fitness costs against potential pathogens that are not well adapted.

3. Molecules that participate in plant-fungi interaction in grapevine

Plant immunity relies on a set of specialized molecules. Some of them have been studied in grapevine and have been used in breeding to obtain resistant varieties. In the following section, we will summarize all the molecules that have been proved to play a role in enhancing resistance in grapevine. We organized this information into the three main host-perception pathways leading to immunity responses (ETI, ETS, and PTI).

3.1. Molecules eliciting the Effector-Triggered Immunity (ETI)

In the ETI, plant response mainly occurs by recognizing pathogen effectors via plant disease resistance proteins encoded by R-genes. The major class of R-genes is the nucleotide oligomerization domain (NOD)-like receptors (NLRs), which encode nucleotide-binding site (NB) and leucine-rich repeat (LRR) gene families (Michelmore et al., 2013). Historically, NLRs are divided into two classes, namely, TIR–NB–LRR (TNL) and CC–NB–LRR (CNL) (Meyers et al., 1999; Andolfo et al., 2019). Nowadays, 43 loci encoding both TNL and CNL proteins capable of conferring increased resistance to downy (31 loci), and powdery (12 loci) mildews have been reported from grapevine. They are listed and described in Table 1 with the correlated references. Most of them have been identified from the wild grapevine species. Among powdery mildew R genes, Run1 (Resistance to *U. necator* 1), *Run2* (Resistance to *U. necator* 2), and *Ren5* (Resistance to *E. necator* 5) were identified in North American species *Muscadinia rotundifolia*; *Ren1* from Central Asian *V. vinifera* subsp. *Sylvestris*; *Ren6*, and *Ren7* from Chinese *V. piasezkii*; and *Ren2* and *Ren4* from Chinese *V. cinerea* and *V. romanetii*, respectively. The remaining minor powdery mildew R loci are *Ren3*, *Ren8*, *Ren9*, and *Ren10*, reported from North American *Vitis* species of unknown origin. These genes are distributed on 8 out of 19 grape chromosomes. Among them, *Run1*, *Run2*, *Ren1*, and *Ren4*-6 have proven to confer the highest levels of resistance to powdery mildew both in *in-vitro* and in the field. Interestingly, some of them (e.g. *Run1* and *Ren4)* confer PCD resistance and have been highly effective in all genetic backgrounds evaluated (Pauquet et al., 2001). Concerning downy mildew, 31 R loci (*Rpv1* to *Rpv31*) from wild and cultivated grapevine species have been reported to confer some level of increased resistance to *P. viticola*. These loci are located on almost all chromosomes excepted chromosomes 1, 13, 17, and 19. Most of them have been identified from the wild species *V. amurensis* (*Rpv8, Rpv10, Rpv12, Rpv22-26*), *V. riparia* (*Rpv5, Rpv6, Rpv9, Rpv13*), and *V. rupestris* (*Rpv19, Rpv28, Rpv3*). The remaining loci originated from *V. aestivalis* (*Rpv27*), *V. piasezkii* (*Rpv15* and *Rpv16*), *M. rotundifolia* (*Rpv1* and *Rpv2*), *V. cinerea* (*Rpv14*). *Rpv1* and *Run1* are the only pathogen resistance locui that have been cloned from any grapevine species. They have been introduced into susceptible *V. vinifera* grapevine cultivars, including 'Shiraz', 'Tempranillo', 'Portan', 'Maccabeu', and 'Carignan' by *Agrobacterium*-mediated transformation. Transgenic grapevines expressing Run1 were found to induce PCD in 67–78% of the penetrated epidermal cells, compared with a mean value of 88% in the powdery mildew-resistant line and 11–15% in untransformed lines. At the same time, downy mildew penetration tests of infected leaf discs at seven days post-inoculation (dpi) revealed profuse *P. viticola* hyphal growth throughout the mesophyll cell layer of susceptible *Rpv1* transgenic lines and non-transformed cultivars (Feechan et al., 2013). In contrast, in those lines, hyphal growth and subsequent sporangiophore development were severely restricted by PCD induction (Feechan et al., 2013). In addition to the wild grapevine species, other *Vitis* species showed efficient resistance to mildews. Very recent studies provided evidence that even the cultivated grape *V. vinifera* could be a source of resistance. In the reference Pinot Noir genome, Goyal et al. (2020) identified 386 *NBS-LRR* genes; 63 of them were responsive to powdery mildew stress, and other new classes of resistance gene families (such as *EDS1, NDR1, PAD4, NPR, RAR1*, and *PR*) involved in ETI pathway. Furthermore, Sargolzaei et al. (2020) identified three novel NLR encoding R genes on chromosomes 14 (*Rpv29*), 3 (*Rpv30*), and 16 (*Rpv31*) associated with a low level of *P. viticola* sporulation using a genome-wide association (GWA) approach in a population of Georgian *V. vinifera* accessions. A different set of data has been published by Andolfo et al. (2019) who studied a phylogenetically distinct class of plant R genes, called *RNLs* because they carry a special N-terminal-resistance domain RPW8 (Resistance to Powdery mildew 8). The authors investigated RNL genes and transcripts in five *V. vinifera* cultivars ('Aglianico', 'Falanghina', 'Sultanina', 'Tannat', and 'Nebbiolo') plus the reference genome of 'Pinot' noir. Based on both RNASeq public dataset and expression analysis, their results highlighted that such gene family is present in grapevine varieties and experienced inter- and intra-specific expansions.

Table 1. R-genes involved in ETI response to grapevine powdery and downy mildew modified from Dry et al. (2019).

	R-locus	Source of resistance	Origin of source	Chromosome	Resistance type	R-protein type	Reference
Powdery mildew genes	Run1	*M. rotundifolia*	North America	12	Major	TNL	Pauquet et al., 2001
	Run2	*M. rotundifolia*	North America	18	Major	NLR	Riaz et al., 2011
	Ren1	*V. vinifera subsp. sylvestris*	Central Asia	13	Major	CNL	Hoffman et al., 2008
	Ren2	*V. cinerea*	North America	14	Partial	NLR	Dalbo et al., 2001
	Ren3	unknown	North America	15	Partial	CNL	Zendler et al., 2017
	Ren4	*V. romanetii*	China	18	Major	NLR	Ramming et al., 2011
	Ren5	*M. rotundifolia*	North America	14	Major	NLR	Blanc et al., 2012
	Ren6	*V. piasezkii*	China	9	Major	NLR	Pap et al., 2016
	Ren7	*V. piasezkii*	China	19	Partial	NLR	Pap et al., 2016
	Ren8	unknown	North America	18	Minor	NLR	Zyprian et al., 2016
	Ren9	unknown	North America	15	Partial	NLR	Zendler et al., 2017
	Ren10	unknown	North America	2	Minor	NLR	Teh et al., 2017
Downy mildew genes	Rpv1	*M. rotundifolia*	North America	2	Partial	TNL	Merdinoglu et al., 2003
	Rpv2	*M. rotundifolia*	North America	3	Major	NLR	Merdinoglu et al., 2018
	Rpv4	unknown	North America	5	Minor	NLR	Welter et al., 2007
	Rpv7	unknown	North America	7	Minor	NLR	Bellin et al., 2009
	Rpv11	unknown	North America	9	Minor	NLR	Fischer et al., 2004
	Rpv17	unknown	North America	12	Minor	NLR	Divilov et al., 2018
	Rpv18	unknown	North America	12	Minor	NLR	Divilov et al., 2018
	Rpv20	unknown	North America	14	Minor	NLR	Divilov et al., 2018
	Rpv21	unknown	North America	14	Minor	NLR	Divilov et al., 2018
	Rpv27	*V. aestivalis*	North America	18	Partial	NLR	Sapkota et al., 2019
	Rpv8	*V. amurensis*	China	7	Major	NLR	Blasi et al., 2011
	Rpv10	*V. amurensis*	China	8	Partial	ERF	Scwander et al.,2012
	Rpv12	*V. amurensis*	North America	9	Major	CNL	Venuti et al., 2013
	Rpv22	*V. amurensis*	China	14	Partial	NLR	Song et al., 2018
	Rpv23	*V. amurensis*	China	15	Minor	NLR	Song et al., 2018
	Rpv24	*V. amurensis*	China	15	Minor	NLR	Song et al., 2018
	Rpv25	*V. amurensis*	China	15	Partial	NLR	Lin et al., 2019
	Rpv26	*V. amurensis*	China	16	Partial	NLR	Lin et al., 2019
	Rpv14	*V. cinerea*	China	10	Minor	NLR	Ochssner et al., 2016
	Rpv15	*V. piasezkii*	North America	11	Major	NLR	Pap et al., 2016
	Rpv16	*V. piasezkii*	North America	12	Minor	NLR	Divilov et al., 2018
	Rpv5	*V. riparia*	North America	5	Minor	NLR	Marguerit et al., 2009
	Rpv6	*V. riparia*	North America	6	Minor	NLR	Marguerit et al., 2009
	Rpv9	*V. riparia*	North America	7	Minor	NLR	Moreira et al., 2010
	Rpv13	*V. riparia*	China	9	Minor	NLR	Moreira et al., 2010
	Rpv19	*V. rupestris*	North America	14	Minor	NLR	Divilov et al., 2018
	Rpv28	*V. rupestris* × *V. riparia*	North America	18	Partial	NLR	Bhattarai et al., 2020
	Rpv3	*V. rupestris x V. lincecumii*	North America	4	Partial	TNL	Welter et al., 2007
	Rpv29	*V. vinifera*	Southern Caucasus	18	n.d.	NLR	Sargolzaei et al., 2020
	Rpv30	*V. vinifera*	Southern Caucasus	18	n.d.	NLR	Sargolzaei et al., 2020
	Rpv31	*V. vinifera*	Southern Caucasus	18	n.d.	NLR	Sargolzaei et al., 2020

3.2. Molecules participating in Effector-Triggered Susceptibility (ETS)

Successful pathogens evolved ETS mechanisms to evade recognition or to suppress PTI interfering with signaling or defense. This interference is made by a heterogeneous class of effectors able to block PTI at different levels. Here, we focus our attention on the plant side, and, therefore, the fungal effectors will not be discussed. The S-genes characterize the ETS mechanism activated by the plant transcriptional machine. Among them, the *MILDEW RESISTANCE LOCUS O* (*MLO*) genes family has been investigated in grapevine. Its members are characterized by the presence of seven transmembrane and C-terminal calmodulin-binding domain proteins of unknown biochemical activity localized at the plasma membrane (Acevedo-Garcia et al., 2014). The precise mechanism through which the reduction of MLO genes expression ends up in resistance to mildews is not completely clear. Some authors hypothesized that the resistance is linked to secretory vesicle traffic (Miklis et al., 2007; Feechan et al., 2011) and to the formation of cell wall appositions called papillae (Consonni et al., 2006). MLO-based resistance is recessive and non-race specific. In grapevine, 17 MLO genes have been identified in the genome, and three of them *VvMLO3*(EU812234.1), *VvMLO4*(EU591723.1), and *VvMLO17*(EU812238.1), were found to be significantly induced in grape leaves within 8 h of *E. necator* inoculation, which coincided with the beginning of fungal penetration (Feechan et al., 2008).

3.3. Molecules eliciting the PAMP-Triggered Immunity (PTI)

Plant PRRs can perceive elicitors and induce a specific set of defense-related genes and associated signal transduction pathways. PTI first component is the penetration resistance against non-adapted mildews, which involves three penetration (*PEN*) genes acting in two different but synergic pathways. In the first, *PEN1* (a member of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor family) mediates membrane fusion events and modulates the trafficking of secretory vesicles to the plasma membrane that contain molecules required for penetration resistance against mildews. In the second, *PEN2* (encoding for a peroxisome-associated glycosyl hydrolase) positively regulates the biosynthesis of antimicrobial molecules delivered to the site of mildew infection via the ATP-binding cassette (*ABC*) transporter *PEN3* (Qiu et al., 2015). In grapevine, only *VvPEN1* (VIT08s0032g01150) has been cloned from *V. vinifera* 'Cabernet Sauvignon' (Feechan et al., 2013). Its functional complementation in the *Arabidopsis* pen1 mutant demonstrated that it shares the same trafficking pathway with *VvMLO3*/*VvMLO4* and undergoes endocytic recycling from the mildew to the powdery mildew attack site (Feechan et al., 2013). Studies on *PEN2*/*PEN3* are not available in grapevine (Qiu et al., 2015).

After fungi penetration, elicitors are recognized by host PRRs at the apoplastic level. Elicitoromic studies in fungus-grapevine interactions showed that these structures, such as chitin, chitosan, and ergosterol, are cultivar-non-specific (Wan et al., 2008; Granado et al., 1995; Brulè et al., 2019). The PTI response network after elicitor perception can be highly variable among species. PTI employs a massive transcriptional reprogramming generated by a complex cascade of signaling events, including fluxes of ions such as Ca2+, the production of reactive oxygen species (ROS) and nitric oxide, and the activation of mitogen-activated protein kinases (MAPKs) (Knogge et al., 2009). These events are activated at the plasma membrane level by receptors able to bind the elicitors and transduce the signal into the cell. The first chitin-binding PRR was identified in rice as the LysM-RLP *CEBiP* (chitin elicitor-binding protein). This is a plasma membrane receptor-like protein (RLP) characterized by an extracellular domain containing two predicted lysin motifs (LysMs) at the N-terminus and a short membrane-spanning domain at the C-terminus. In grapevine, among 15 CEBiP orthologs, named VvLYKs, three are putative direct orthologues of the *Arabidopsis AtCERK1*/*LYK1* and the rice *OsCERK1a*, namely *VvLYK1-1*, *VvLYK1*-2, and *VvLYK1*-3 (Brulé et al., 2019). Functional complementation of the *Arabidopsis atcerk1* mutant demonstrated the constitutive expression of *VvLYK1*-1, the inducible expression of *VvLYK1*-2, and the absence of *VvLYK1*-3. These data provided evidence that *VvLYK1*-1

can completely restore the chitin-triggered immune responses and it plays an important role in basal resistance against *E. necator* (Brulé et al., 2019).

Downstream the grape receptor activation, hexamers of chitin and chitosan elicit the phosphorylation of MAPKs (mitogen-activated protein kinase) cascade comprising three interconnected kinases modules: MAPK, MAPKK, and MAPKKK. MAPKKK proteins function at the beginning of the cascade, receiving signals from upstream sensors to initiate the pathway and activate the MAPKK proteins by phosphorylating the activation loop's serine/threonine residues. *V. vinifera* genome contains 14 *MAPKs*, five *MAPKKs*, and 62 *MAPKKKs*. Among them, only *MAPKKK* involvement in mildews response has been investigated. Wang et al. (2014) observed that *E. necator* caused a substantial increase of transcripts of *VviMAPKKK31, 32, 34, 38, 46,* and *50*. In particular, *VviMAPKKK50* showed the highest transcript abundance, between 6 and 27-fold the control. MAPK cascade activates the phytoalexin production and the positive regulation of defense genes expression by transcription factors, such as WRKYs and DOFs (Brulé et al., 2019). Recently, Yu et al. (2019) reported that the overexpression of *VvDOF3* in *A. thaliana* enhances resistance to *Golovinomyces cichoracearum*, the expression of the SA-responsive defense-related gene PR1, and the concentration of SA in transgenic lines. Together, these data suggest that *VvDOF3* functions as a transcription factor in grape and enhances powdery mildew resistance through the SA signaling pathway. Rather than DOFs, the most studied class of TFs in this context are the WRKYs. Through the analysis of various genomic and proteomic grapevine databases, Wang et al. (2014) identified 59 putative grapevine WRKY transcription factors (VvWRKYs). Several of them were upregulated in 'Cabernet Sauvignon' leaves infected with powdery mildew, included five WRKYs (*VvWRKY47, 24, 16, 08,* and *51*) previously reported to be upregulated after 1-hour inoculation with the same fungi (Fung et al., 2008). Marchive et al. (2013) reported that even *VvWRKY1* and *VvWRKY2* are involved in regulating fungal disease resistance. In plants, ubiquitin E3 and Really Interesting New Gene (RING) proteins have been recognized to play a role in immunity pathways. Among the E3 ubiquitin ligases, the *Arabidopsis thaliana* Toxicos en Levadura (ATL) proteins have been deeply studied in the last years. The ATL grapevine family has 96 members likely to be involved in several physiological processes through protein ubiquitination (Ariani et al., 2016). The analysis of co-expression networks among grapevine ATL genes across a set of transcriptomic data related to biotic stressors revealed strong correlations between VviATL148 and VviATL156 proteins and suggested their role as putative key regulators of fungal infection responses in grapevine (Wong et al., 2018).

4. Development of fungi-resistant grape varieties: achievements and challenges

During the 20th century, the breeding programs active across Europe led to several fungus-resistant grape varieties. The German 'Regent' ('Silvaner' × 'Müller-Thurgau') × 'Chambourcin', cross 1967) and 'Solaris' ('Merzling' × ('Severnyi' × 'Muscat Ottonel'), cross 1975), and the Hungarian 'Bianca' ('Eger 2' ('Villard Blanc') × 'Bouvier', cross 1963) are probably the most successful cultivars derived from such programs, where the achievement of field disease resistance matched the wine quality (Guedes de Pinho and Bertrand, 1995; Basler and Pfenninger, 2003; Eibach and Töpfer, 2003; Ruehl et al., 2015). These varieties were obtained employing classic breeding strategies, based on phenotypic selection of superior genotypes within progenies obtained from crosses. Such methodology has been proved to be extremely labour-intensive and time-consuming to such an extent that from the first cross to the cultivar release up to 35 years could be necessary (Töpfer et al., 2011). This is due to several grapevine-specific limitations, such lengthy juvenile phase (3-5 years), a large plant size, a high inbreeding depression, and a limited propagation rate (Eibach and Topfer, 2015; Di Gaspero and Foria, 2015). A breakthrough in the classic grapevine breeding was the advent of molecular markers. They have been pivotal tools for developing genetic maps, providing the framework required for the discovery and localization of genes and quantitative trait loci (QTL). In grapevine, molecular markers have

been used to dissect the genetic bases of resistance to powdery and downy mildew, revealing an oligogenic architecture for both traits (Vezzulli et al., 2019). Moreover, to accelerate and enhance cultivar development, these tools have been integrated into classic breeding schemes in a process called marker-assisted selection (MAS) where individuals are chosen based on QTL-linked markers that have major effects (e.g. >10%) on the phenotypic variation of the target trait (Collard and Mackill, 2008). MAS combined with multiple backcrossing with *V. vinifera* varieties has been used to efficiently recover the recurrent parent (*V. vinifera*) to a significant percentage (more than 85%) while preserving the wild trait of interest (Töpfer et al., 2011). Through the application of these strategies, several fungus-resistant grape varieties, possessing desirable agronomic and oenological attributes, have been recently developed in North America (named FRG, fungi-resistant grapes) and Europe (called PIWI, from German: Pilzwiderstandsfähige, "disease-resistant"). They are accepted as *V. vinifera* varieties in European catalogues (Sivčev et al., 2010) for both conventional and organic farming (Pedneault and Provost, 2016). It is expected that these new varieties will lessen the average treatment frequency from 12, currently observed for the traditional varieties, to 2 (Delrot et al., 2020). MAS has also great potential for efficient gene pyramiding; namely, combining multiple resistance QTLs acting against a disease in a single variety to achieve a better chance of resistance durability. In 2007, Eibach and collaborators gave an example of pyramiding resistance loci, two for resistance against *E. necator* and two for resistance against *P. viticola*. More recently, RUN1 and REN1 were pyramided in the cultivar Crimson Seedless, leading to an enhanced resistance to powdery mildew (Agurto et al., 2017).

MAS hold great promise for grape breeding for fungi resistance. However, examples of successful, practical outcomes are still rare (Vezzulli et al., 2019). Does this mean that MAS is not able to deliver its expected benefits in grape breeding programmes for resistance? Not exactly. Empirical applications of this procedure have shown that the success of MAS relies upon several factors, including the genetic complexity of the target trait (many QTLs involved), the interaction between genes (epistasis), and the difficulty of finding the same QTL across multiple environments (due to QTL × environment interactions) (Francia et al., 2005). Most loci responsible for the genetic control of the resistance traits account for 10%, or less of the phenotypic variation (the so-called minor QTLs) and their reliable detection has always been challenging. Thus, grape varieties lacking minor QTLs for fungi resistance can fail to provide the expected phenotypic response (Merdinoglu et al., 2018). Given that, how is it possible to facilitate the fine-association mapping of these QTLs with minor, but significant effects? The adoption of the new high-throughput sequencing within grape breeding programs can certainly help in increasing the power of QTL mapping. For example, the dropping sequencing costs enable the discovery of tens of thousands of markers in grape genome (Myles et al., 2010; Le Paslier et al., 2013). Such huge number of markers lay solid foundations for empowering the exploitation of the most powerful breeding methods for dissecting complex traits, namely Genome-wide association study (GWAS) and genomic selection (GS) (D'Amelia et al. 2018). However, before the full potential of such strategies can be routinely used in grapevine, some challenges associated with the genetic structure of grape germplasm and grape genome biology must be addressed (Delrot et al., 2019).

The enormous breeding effort of developing grape resistant varieties has led researchers to look for new strategies through the application of new breeding technologies (NBTs). They comprise several techniques that can modify the genetic makeup of a plant variety in a targeted way to introduce new traits or modify existing ones. The most promising is genome editing (GE). It is an unprecedented technological breakthrough whereby punctual targeted mutations can be introduced into a plant genome through the action of sequence-specific nucleases (SSNs, such as Cas9) that generate a DNA double-strand break (DSB) at a specific genomic target (Chen and Gao, 2014; D'Amelia et al., 2018). The DSB triggers a DNA repair process that leads to the addition or deletion of a few DNA letters. The resulting edited sequences can lead to a gene's deactivation, which is useful if a specific gene makes, for example, a crop susceptible to disease infection. Despite many commonalities, edited crops and genetically modified organisms (GMOs) differ for a noteworthy aspect: the use of foreign DNA. Indeed, GMOs are commonly

made through the introduction of exogenous DNA into a given organism, a process called transgenesis. The genome-editing process, instead, does not require the use of foreign DNA and produce mutation of a few nucleotides, which is a frequent occurrence in the wild and a driver of evolution. This similarity between natural evolution and GE is an angle which can separate edited crops from GMOs (Doxzen and Henderson, 2020). In countries that follow product-based regulation (e.g., USA, Argentina, Australia, Brazil), it has been established that if no foreign DNA is present in a genome-edited variety, they are not subjected to additional regulatory oversight and risk assessment as in the case of GMOs (Eriksson et al., 2019). In these countries these new individuals (including grapevine) will be probably classified as clones. By contrast, in EU, where a process-based regulation exists, the Court of Justice has recently (25 July 2018, https://curia.europa.eu/jcms/upload/docs/application/pdf/2018-07/cp180111en.pdf) ruled that organisms modified by the NBTs have to be considered as GMOs and are, therefore, banned for cultivation, according to the European Directive 2001/18/EC. The European scientific community expressed a strong displeasure to the ruling of the Court of Justice, to safeguard genome editing from GMO legislation. Scientists claim that regulating edited organisms as GMOs will have noticeable negative consequences for agriculture, society and economy in Europe and hope that they will fall under the regulatory regime applied to classically bred varieties (European Plant Science Organization, 2018; Vlaamsche Institute Biologie, 2018). Among NBTs, the clustered regularly interspaced palindromic repeats (CRISPR)-associated system (Cas9) is the most precise technology (Cong et al., 2013; Jinek et al., 2012). It has not yet released any commercial variety but most likely will produce results in countries other than Europe. Nowadays, CRISPR/Cas9 has been successfully used to knock out genes in grapevine embryogenic callus (Ren et al., 2016; Nakajima et al., 2017) and very few studies reported the production of edited plants resistant to fungi (Table 2). Giacomelli et al. (2019) produced plants resistant to downy and powdery mildew through CRISPR/Cas9 knockout of multiple S genes. Wan et al. (2020) produced four VvMLO3-edited *V. vinifera* cv. Thompson Seedless lines with enhanced resistance to *E. necator*. These pioneering studies demonstrate that CRISPR/Cas9-targeted mutagenesis can be used to develop disease-resistant cultivars and facilitate the functional characterization of genes of interest in grapevine.

Table 2. *V. vinifera* cultivars used in genetic engineering for fungal resistance. The table reports, for each cultivar, the candidate gene, its function, the embryogenic tissues used for plant genetic transformation and the reference.

***V. vinifera* cultivar**	**Gene**	**Function**	**Tissue**	**Reference**
		Higher resistance to powdery mildew		
Neo Muscat	RCC2	Chitinase from rice	Ovaries-derived EC[a]	Yamamoto et al., 2000
Chardonnay	mag-02	Magainin	Anther-derived EC	Vidal et al., 2006
Merlot, Shiraz and Thompson Seedless	Vvtl-1	Thaumatin-like protein gene 1	Anther-derived EC	Gray et al., 2008
Pusa Seedless	RCC2	Chitinase from rice	EC	Nirala et al., 2010
Chardonnay	VvNPR1.1, VvNPR1.2	Non-expressor of Pathogenesis Related 1	Anther-derived EC	Le Henanff et al., 2011
Thompson Seedless	VqSTS6	Stilbene synthase from *V. quinquangularis*	Anther-derived EC	Cheng et al., 2016
		Higher resistance to downy mildew		
Crimson Seedless	chitinase and β-1,3-glucanase	Chitinase and β-1,3-glucanase	Leaf-derived EC	Nookaraju and Agrawal, 2012
Chardonnay	VpSTSgDNA2	Stilbene synthase from *V. pseudoreticulata*	Anther-derived EC	Dai et al., 2015
Brachetto	VvMLO6, 7, 11 and 13	S-genes	EC	Pessina et al., 2016
Thompson Seedless	VaTLP	Thaumatin-like protein	Anther-derived EC	Hert al., 2016
Thompson Seedless	VpPR4-1	Pathogenesis-related gene	Anther-derived EC	Hert al., 2016
Thompson Seedless	VpPR10.1	Pathogenesis-related gene	Anther-derived EC	Su et al., 2018

5. Conclusions

To cope with powdery and downy mildew invasions in viticulture, speeding-up breeding programs is a necessary endeavor. Grapevine has several limitations associated with its biology and the genetic architecture of resistant traits, which collectively hampers an efficient improvement. In this review, we outlined the progress made in characterization of the molecules involved in the grape defense mechanisms showing how this knowledge is not exhaustive and only partly exploited in grape breeding. However, the new opportunities offered by genomics and NBTs, such as genome editing with engineered nucleases, could push the boundaries of current breeding methodologies to translate the knowledge gained into practical applications.

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