

REVIEW PAPER

Past, present, and future of genetic strategies to control tolerance to the main fungal and oomycete pathogens of grapevine

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Abstract

The production of high-quality wines is strictly related to the correct management of the vineyard, which guarantees good yields and grapes with the right characteristics required for subsequent vinification. Winegrowers face a variety of challenges during the grapevine cultivation cycle: the most notorious are fungal and oomycete diseases such as downy mildew, powdery mildew, and gray mold. If not properly addressed, these diseases can irremediably compromise the harvest, with disastrous consequences for the production and wine economy. Conventional defense methods used in the past involved chemical pesticides. However, such approaches are in conflict with the growing attention to environmental sustainability and shifts from the uncontrolled use of chemicals to the use of integrated approaches for crop protection. Improvements in genetic knowledge and the availability of novel biotechnologies have created new scenarios for possibly producing grapes with a reduced, if not almost zero, impact. Here, the main approaches used to protect grapevines from fungal and oomycete diseases are reviewed, starting from conventional breeding, which allowed the establishment of new resistant varieties, followed by biotechnological methods, such as transgenesis, cisgenesis, intragenesis, and genome editing, and ending with more recent perspectives concerning the application of new products based on RNAi technology. Evidence of their effectiveness, as well as potential risks and limitations based on the current legislative situation, are critically discussed.

Keywords: Cisgenesis, downy mildew, dsRNA, genome editing, gray mold, powdery mildew, *Vitis*.

Introduction

Currently, grapevine cultivation has become enormously important at both economic and food production levels on a global scale. In 2019, according to the economic note of the world wine sector drawn up by the International Organization of Vine and Wine (OIV), the world area under vines, that is the total area planted with vineyards for all uses (wine, table grapes, and raisins), was estimated at 7.4 Mha. In the following year, the area covered by grapes for use in worldwide wine production was estimated at ~258 Mhl (OIV, 2020), with ~159 Mhl produced in Europe and 99 Mhl in the rest of the world. Italy is the world's leading wine producer, with a share of 18%, equal to 47.2 Mhl. Wine, understood as the main product of vine cultivation, is inextricably linked to the complex interactions of the vine with the surrounding environment. Moreover, the high quality and excellence of a wine often reflect the genetic characteristics typical of a single genotype, making it necessary to avoid the recombination of characters and therefore sexual reproduction. The vine, consequently, is identified as a static genetic entity, basically represented by one or a few genotypes perfectly adapted to a territory and multiplied vegetatively by means of cuttings or clones. The large global cultivation area and the need for genetic standardization within one or more territories make the vine particularly susceptible to attack by pathogens and abiotic stresses. In addition, the threat of pathogens in a purposely static genetic context can be intensified with the phenomenon of climate change, leaving room for emerging phytosanitary problems, as well as increasingly frequent and highly unfavorable atmospheric extremes affecting crop fitness. Next-generation viticulture will need to consider environmental trends toward high temperature. In addition to reviewing the traditional 'suitability' of different territories, the geographical redistribution of the vines, and their adaptation to cultivation techniques, it will be necessary to intensify the contribution that genetics and biotechnologies can provide to solve phytosanitary and qualitative problems linked to defense. At the end of the 19th century, mildews and phylloxera (*Daktulosphaira vitifoliae*) from North America put the entire European supply chain in jeopardy. Still today, some of the most disruptive grape diseases in the Eurasian region are downy mildew caused by the oomycete *Plasmopara viticola*, and powdery mildew and gray mold caused by the ascomycetes *Erysiphe necator* and *Botrytis cinerea*, respectively (Elad *et al.*, 2016; Buonassisi *et al.*, 2017; Pirrello *et al.*, 2019). Genetic systems have already been widely exploited to confer desired characteristics and to counter impending problems. The most emblematic example is the development and use of rootstocks. The root system of the European grapevine, in fact, was found to be highly vulnerable to infestation by phylloxera, while the wild species of American grapevine showed resistance to aphid attacks (Forneck *et al.*, 2001). Initially, attempts were made to combat the insect by hybridizing European and American species to obtain varieties resistant to phylloxera. However, the

obtained hybrids did not offer total resistance and did not adapt well to the calcareous, poor, and dry soils that characterize viticulture. Furthermore, the product obtained from these hybrids showed a lower organoleptic quality compared with European varieties. Therefore, the best solution was to select rootstocks derived from interspecific crosses between some non-*vinifera* species, mainly *Vitis berlandieri*, *V. riparia*, and *V. rupestris*, or between these and *V. vinifera*. This intense hybridization work led to the selection of many rootstocks that are still used today and guarantee resistance of the root system to phylloxera as well as greater adaptability to all soil and climatic conditions. The fight against phylloxera represented, in the context of vine cultivation, the trigger toward a growing importance in the selection of rootstocks, which had to confer resistance not only to the aphid but also to the main biotic agents, especially nematodes (Wilcox *et al.*, 2015) and related viruses (Walker *et al.*, 1994), and also needed to show good adaptability to stressful abiotic conditions, such as deficiencies in the absorption and transport of water and mineral elements (Marguerit *et al.*, 2012; Corso and Bonghi, 2014; Meggio *et al.*, 2014; Vannozzi *et al.*, 2017). The need for rootstocks, and more generally for resistant cultivars, also stemmed from a problem directly related to phytosanitary defense or to improvements in plant fitness, namely the use of insecticides, nematicides, soil fumigants, and chemical fertilizers, the indiscriminate use of which can lead to water contamination and environmental toxicity. For these reasons, chemical strategies for disease management are increasingly regulated by the EU to alleviate the potential negative effects of pesticides on workers' health and field biodiversity and to promote initiatives that fall within the broader framework of integrated pest management (IPM; Pertot *et al.*, 2017).

In this regard, grapevine breeding remains a valuable tool aimed not only at scavenging and exploiting natural resistance traits but also at coupling them with the market-dictated fruit quality and agronomical characteristics of elite varieties (Topfer *et al.*, 2011). In fact, the cultivation of disease resistance genotypes has made it possible to limit the number of pesticide treatments in viticulture in many areas of the world (Topfer and Hausmann, 2022). Nonetheless, the increasingly widespread development of these cultivars raises several important issues related to their qualitative potential and marketing, and, more importantly, to the management of the durability of resistance, with several cases of the erosion or breakdown of resistance already reported in Europe (Guimier *et al.*, 2019). In recent decades, the development of new biotechnological approaches such as cisgenesis, genome editing, and RNAi has expanded the range of tools that can be used to improve the tolerance to pathogens in difficult crops such as grapevine. However, it should be noted that, similar to conventional breeding, these new biotechnological techniques also have advantages and disadvantages that must be taken into consideration. In 2017, the EU Explanatory Note

(EU Commission and Directorate-General for Research and Innovation, 2017) introduced the definition of conventional breeding technologies, consisting of traditional breeding and marker- (MAB) and genomic-assisted breeding (GAB), to be considered separate from these new breeding biotechnologies (NBTs).

This study aims to review the contribution of conventional and new breeding techniques to the genetic improvement of grapevine, starting with MAB, moving on to the use of transgenesis, cisgenesis, and post-transcriptional silencing mechanisms, and ending with genome editing. Parallel to the description of the main results obtained from the research in this area, an attempt was also made to provide the regulatory framework at a national and European level.

Conventional genetic improvement of grapevine for fungal and oomycete diseases

Cross-breeding exploits the genetic segregation that occurs through controlled sexual reproduction to obtain progeny with broad genetic diversity. It was first used in grapevine in the 18th century, when unsuccessful attempts to cultivate *V. vinifera* subsp. *vinifera* in America finally resulted in the development of ‘American hybrids’ adapted to local climatic conditions with better characteristics for winemaking than the local wild species (Topfer *et al.*, 2011). In Europe, cross-breeding for resistance started at the beginning of the 20th century to cope with emerging pests coming from the New World, such as downy mildew, powdery mildew, and gray mold. The first interspecific cross attempts were conducted by private French breeders leading to so-called ‘first-generation hybrids’ or ‘direct producers’ that were ungrafted. Given their process of co-evolution with pathogens, American wild species were considered resistant sources (Toffolatti *et al.*, 2018); however, unfortunately, they were not suitable for wine production in terms of quality (Eibach *et al.*, 2010) (Box 1). In fact, although this technique proved effective for rootstocks, the unsatisfactory viticultural properties of the French hybrids forced the breeding programs in France to stop and led to the advent of laws that still rigorously regulate hybrids in the EU wine market today (Meloni and Swinnen, 2013). Nonetheless, subsequent breeding activities in other countries resulted in cultivars being successfully grown and vinified throughout Europe (Topfer *et al.*, 2011).

Given the need to make the most of genetic resources for resistance from wild species, gene introgression has been employed. This process consists of an interspecific hybridization between a wild individual carrying the resistance trait (donor plant) and an individual from a commercial variety (recipient plant). The recipient plant is then used for subsequent backcrosses coupled with the recurrent selection of the resistance trait. Unfortunately, as described by Dalla Costa *et al.* (2017), this strategy is labor- and time-consuming for a woody crop

Box 1. The genetic diversity of the *Vitis* genus

The members of the *Vitaceae* family are classified into five tribes (Wen *et al.*, 2018), consisting of 16 genera and 950 species (Lu *et al.*, 2018). The grape genus *Vitis* L., with 75 species, belongs to the *Viteae* tribe. The *Vitis* genus includes the *Muscadinia* [(Planch.) Rehder] ($2n=40$ chromosomes) and *Vitis* ($2n=38$) subgenera, to which the *V. vinifera* wild subsp. *sylvestris* and cultivated subsp. *sativa* belong. Species belonging to the *Vitis* subgenus are interfertile. The genetic proximity between *sylvestris* and *sativa*, and the occurrence of intermediate genotypes corroborate the widely accepted theory that cultivated subspecies originated from the domestication of wild individuals (Grassi and Arroyo-Garcia, 2020). The autogamous nature of *V. vinifera* determines low levels of genetic polymorphism compared with hybrids and wild species, which, however, results in interspecific and even intervarietal genomic variations (Cardone *et al.*, 2016). This high level of genetic plasticity is a crucial resource for the exploitation of resistance traits to the main fungal pathogens (Vezzulli *et al.*, 2022). Interestingly, the recent awareness that spontaneous gene introgression in wild species initially contributed to the establishment of resistance traits to biotic stresses (Morales-Cruz *et al.*, 2021) led to new studies on the ancestry of *Vitis* members (Foria *et al.*, 2022). Moreover, self-fertilization represents a primal selection tool that, on the one hand, favors the establishment of lethal recessive alleles but, on the other hand, naturally selects surviving homozygous individuals as ‘good performers’. For these reasons, several *vinifera* and non-*vinifera* core collections have been created in the last few years (Cipriani *et al.*, 2008; Le Cunff *et al.*, 2008; Emanuelli *et al.*, 2013; Migliaro *et al.*, 2019).

with a long juvenile phase such as grapevine, without the guarantee of a satisfactory result in agronomical terms.

MAB can partially overcome the aforementioned drawbacks and accelerate the process of selection of resistant cultivars obtained from interspecific crosses by tracing the introgression of the loci of interest (Di Gaspero and Cattonaro, 2010). In the 2000s, a large collection of molecular markers associated with quantitative trait loci (QTLs) was developed to allow the construction of genetic maps (Hvarleva *et al.*, 2009). The first to be used were the simple sequence repeats (SSRs) which, despite their high mutation rate, are still the best selective markers for their stability and co-dominance (Adam-Blondon *et al.*, 2004). Over the years, many other highly polymorphic markers have been exploited (Vezzulli *et al.*, 2019a), such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP; Fischer *et al.*, 2004), and sequence characterized amplified regions (SCARs; Welter *et al.*, 2007). Given their ease of use, reproducibility, and abundance in the

grapevine genome (Velasco *et al.*, 2007), single nucleotide polymorphisms (SNPs) have been used as integration tools for SSRs (Emanuelli *et al.*, 2013) and to create dense genetic maps (Vezzulli *et al.*, 2008).

As a consequence of the development and application of new molecular markers in recent years, there has been a significant increase in the number of identified *R* loci as dominant sources of resistance. In this regard, North American species have contributed most to the collection of *R* loci thus far (Table 1). In recent years, the investigation of QTLs for resistance has expanded from *Muscadinia* through new American and Asian *Vitis* species up to *V. vinifera*. However, despite the large updated list of *R* loci (Table 1) against downy mildew, powdery mildew, and gray mold, the exploitation of resistance traits in unexplored wild species is rapidly proceeding, but the search for new resistance genes is also needed in cultivated resistant varieties (e.g. *Rpv27* identified in Norton by Sapkota *et al.*, 2019). Moreover, the recent loosening of the regulation on the cultivation of hybrids, which now allows them to be classified with a Protected Geographical Indication [PGI; EU Council, 2009], has given a new impetus to the development of resistant species from interspecific cross-breeding. These new cultivars are considered *V. vinifera* varieties and are defined as pilzwiderstandsfähig (PIWI), meaning 'fungal disease resistant'. The same definition is used by an international working group aimed at the promotion of fungus-resistant grape varieties via different breeding programs in Europe and North America (PIWI International; <https://piwi-international.de>). Currently, resistant varieties are widely cultivated in many regions of the world (Fig. 1; Supplementary Table S1). Among the top 30 resistant wine grape varieties, Concord is the most widespread and covers 0.24% of the worldwide area cultivated with vines; it is mostly cultivated in Brazil, the USA, and Japan. Similarly, Bianca covers 0.22% and is concentrated in Russia, Hungary, and Moldova. Seyval Blanc, Regent, Chambourcin, Villard Noir, and Baco Noir follow with 0.06–0.02% of the worldwide share and covering countries on all five continents (Anderson and Nelgen, 2020) (Fig. 1; Supplementary Table S1; Table 2). Not much is known about the genetic traits that determine resistance in these cultivars. While Norton and Golden Muscat have only one *R* locus against *P. viticola*, Seyval blanc, Regent, and Solaris carry five *Rpv* and *Ren* loci. Four loci for resistance to *E. necator* and *P. viticola* are carried by Cabernet Cortis, with three by Phoenix and Johanniter, and two by Bianca and Chambourcin (Table 2); no data are available for the other cultivars.

Regarding the wild and cultivated sources of resistance, one of the main issues of their investigation is that not all individuals belonging to a species show the same resistant phenotype, raising the need to test every single germplasm with local strains of the pathogen (Dry *et al.*, 2019). Moreover, as proven by Peressotti *et al.* (2010), resistance conferred by major QTLs cannot be considered a long-term solution since it is highly race specific and can be overcome by more

virulent strains of the pathogen; on the other hand, partial resistance is the result of an additive effect of many genes and is more durable but still prone to erosion (Stuthman *et al.*, 2007). The ability and time to overcome resistance by a pathogen are determined by several factors, such as its morphological and reproductive characteristics and the permanence of the crop in the field (Dry *et al.*, 2019). Stam and McDonald, (2018) calculated the combination of four *R* genes as a minimum to guarantee total impermeability of the resistance barrier in herbaceous crops, but it is not yet possible to establish whether these data are also valid for perennial crops such as grapevine and how much they should be combined with other control strategies (Feechan *et al.*, 2015; Zini *et al.*, 2019). In this regard, gene stacking (pyramiding) is proposed to improve breeding efficiency because it takes advantage of known resistance QTLs to downy mildew and powdery mildew (Miedaner, 2016; Mundt, 2018). Grapevine breeding programs aimed at stacking *R* loci are widespread throughout the world. In Europe, the collaboration between INRAE, Julius Kühn-Institut (JKI), Staatliches Weinbauinstitut (WBI), and Agroscope in the INRA-ResDur program (Merdinoglu *et al.*, 2018) made it possible to release the four ResDur1 cultivars 'Voltis', 'Artaban', 'Vidoc', and 'Floreale', carrying *Run1*, *Ren3*, *Rpv1*, and *Rpv3*. This collaboration is still working on ResDur2 (with *Run1*, *Ren3.2*, *Rpv1*, and *Rpv10*) to reach ResDur3 cultivars with six *R* loci. Moreover, the Fondazione Edmund Mach (FEM) recently registered four new varieties ('Termantis', 'Nermantis', 'Valnosia', and 'Charvir' in the Italian grapevine variety catalog (Pecile and Zavaglia, 2013), and its breeding program produced progeny with individuals carrying from two to seven *R* loci to downy mildew and powdery mildew (Vezzulli *et al.*, 2019c). Furthermore, Foria *et al.* (2019), at the University of Udine, were able to combine two *Rpv* and two *Ren/Run* loci into 'elite' parenting offspring, releasing 'Cabernet Volos', 'Fleurtaï', 'Merlot Kanthus', and 'Soreli'. Overseas, Riaz *et al.* (2019) are working on combining up to three *Ren/Run* loci to develop advanced lines with a *V. vinifera* background. In Chile, Agurto *et al.* (2017) obtained *Ren1Run1Rpv1* table grape cultivars, and Saifert *et al.* (2018) were able to stack *Rpv1* and *Rpv3.1* in elite genetic material through a collaboration between Ecuadorian and Brazilian universities.

The ability to move from a genetic map to a genetic sequence was certainly provided by the many genome sequencing works on *Vitis* cultivars (Jaillon *et al.*, 2007; Velasco *et al.*, 2007; Carrier *et al.*, 2012; Gambino *et al.*, 2017; Roach *et al.*, 2018; Minio *et al.*, 2019; Magris *et al.*, 2021; Foria *et al.*, 2022) and American species (Girollet *et al.*, 2019; Cochetel *et al.*, 2021). Since the first SNP detection in grapevine (Owens, 2003), the harnessing of SNPs in breeding has been inevitably coupled with systematically increasing the genetic information obtained through next-generation sequencing (NGS), third-generation sequencing (TGS; Varshney *et al.*, 2009; Kumar *et al.*, 2012), and cost-efficient amplicon sequencing (AmpSeq; Yang *et al.*,

Table 1. Resistance (R) loci to *B. cinerea*, *E. necator*, and *P. viticola* in grapevine (Topfer and Hausmann, 2022)

Pathogen	Locus	Chr	Source	Genotype	Associated marker	Origin	Type	Reference
<i>B. cinerea</i>	Unnamed	2	<i>V. aestivalis</i>	Norton	VMC6F1, VMC3B10	NA	Mj	(Sapkota et al., 2019)
	QTL							
	<i>Ren1</i>	13	<i>V. vinifera</i>	Kishmish vatkana	UDV020, VMC9H4-2, VMCNg4e10.1	CA	Mj/Pa	(Hoffmann et al., 2008)
<i>E. necator</i>	<i>Ren2</i>	14	<i>V. cinerea</i>	Illinois 547-1	CS25	NA	Pa	(Dalbó et al., 2001)
	<i>Ren3</i>	15	unknown	Regent	UDV015b, Wlv67	NA	Pa	(Weiter et al., 2007)
					ScORAT-760			(Akkurt et al., 2007)
	<i>Ren4</i>	18	<i>V. rotundifolia</i>	C166-043	VChr15CenGen02	CH	Mj/Pa	(van Heerden et al., 2014)
				C87-41	GF15-28/Wlv67			(Zypryan et al., 2016)
	<i>Ren5</i>	14	<i>M. rotundifolia</i>	Regale	GF15-42			(Zendler et al., 2017)
	<i>Ren6</i>	9	<i>V. piasezkii</i>	DVIT2027	VMC7f2			(Riaz et al., 2012)
	<i>Ren7</i>	19	<i>V. piasezkii</i>	DVIT2027	SNPs			(Mahanil et al., 2012)
	<i>Ren8</i>	18	Unknown	Regent	VMC9c1	NA	Mj/To	(Blanc et al., 2012)
	<i>Ren9</i>	15	Unknown	Regent	PN9-057, PN9-068	CH	Mj/To	(Pap et al., 2016)
<i>P. viticola</i>	<i>Ren10</i>	2	Unknown	Seyval blanc	Wlp17.1, VMC9a2.1	CH	Pa	(Pap et al., 2016)
					UDV117, SPS_P_SNP632GF	NA	Mn/Pa	(Zypryan et al., 2016)
	<i>Ren11</i>	15	<i>V. aestivalis</i>	Tamiami	CenGen6	NA	Pa	(Zendler et al., 2017)
	<i>Run1</i>	12	<i>M. rotundifolia</i>	VRH3082-1-42	S2_17854965	NA	Mn	(Teh et al., 2017)
	<i>Run2.1</i>	18	<i>M. rotundifolia</i>	Magnolia	Haploblock validation	NA	Pa	(Kam et al., 2021)
	<i>Run2.2</i>	18	<i>M. rotundifolia</i>	Trayshed	rh_chr15_15294725 rh_	NA	Pa	
	<i>Rpv1</i>	12	<i>M. rotundifolia</i>	28-8-78	chr15_13822901 rh_chr15_13698923	NA	Mj/To	(Barker et al., 2005)
	<i>Rpv2</i>	18	<i>M. rotundifolia</i>	8624	VMC4f3.1, VMC8g9	NA	Mj/To	(Feechan et al., 2013)
					49MRP1.P2, GB53.54	NA	Mj/Pa	(Riaz et al., 2011)
	<i>Rpv3</i>	18	<i>V. rupestris</i>	Regent	VMC7f2, VMCNg1e3	NA	Pa	
				Bianca	VVin16, VMC7f2, VMC7f2	NA	Pa	(Riaz et al., 2011)
	<i>Rpv3.1</i>	18	<i>V. rupestris</i>	Regent	VMC7f2	NA	Pa	(Merdinoglu et al., 2003)
	(=Rpv3 ²⁹⁹⁻²⁷⁹)			Seibel 4614	WVlb32	NA	Mj/To	(Wiedemann-Merdinoglu et al., 2006)
	<i>Rpv3.2</i>	18	<i>V. rupestris</i>	Villard blanc	UDV112, UDV305, VMC7f2,	NA	Pa	(Weiter et al., 2007)
	(=Rpv3 ^{full-297})			Munson	VMC7f2	NA	Pa	(Bellin et al., 2009)
	<i>Rpv3.3</i>	18	<i>V. labrusca</i>	Noah	UDV305, UDV737	NA	Pa	(van Heerden et al., 2014)
	(=Rpv3 ^{full-271})			Merzling	UDV305, UDV737	NA	Pa	(Di Gaspero et al., 2012)
	<i>Rpv3³²¹⁻³¹²</i>	18	<i>V. riparia</i>	Noah	GF18-06, GF18-08	NA	Pa	(Zypryan et al., 2016)
			Noah	UDV305, UDV737	NA	Pa	(Di Gaspero et al., 2012)	
<i>Rpv3³⁶¹⁻²⁸⁹</i>	18	<i>V. rupestris</i>	Ganzin	GF18-06, GF18-08	NA	Pa	(Zypryan et al., 2016)	
			Ganzin	UDV305, UDV737	NA	Pa	(Di Gaspero et al., 2012)	
<i>Rpv3²⁹⁹⁻³¹⁴</i>	18	<i>V. rupestris</i>	Ganzin	VIN16, UDV737	NA	Pa	(Mezulli et al., 2019b)	
			Ganzin	UDV305	NA	Pa	(Di Gaspero et al., 2012)	
			Ganzin	UDV737	NA	Pa		
			Ganzin	UDV305	NA	Pa	(Di Gaspero et al., 2012)	
			Ganzin	UDV737	NA	Pa		
			Ganzin	UDV305	NA	Pa	(Di Gaspero et al., 2012)	
			Ganzin	UDV737	NA	Pa		

Table 1. Continued

Pathogen	Locus	Chr	Source	Genotype	Associated marker	Origin	Type	Reference
	<i>Rpv3^{nuil-287}</i>	18	<i>V. rupestris</i> <i>V. labrusca</i>	Bayard (Couderc 28-112)	UDV305 UDV737	NA	Pa	(Di Gaspero et al., 2012)
	<i>Rpv4</i>	4	Unknown	Regent	VMC7h3, VMCNg2e1	NA	Mn	(Weiter et al., 2007)
	<i>Rpv5</i>	9	<i>V. riparia</i>	G. de Montpellier	WVlo52b	NA	Mn	(Marguerit et al., 2009)
	<i>Rpv6</i>	12	<i>V. riparia</i>	G. de Montpellier	VMC8g9	NA	Mn	(Marguerit et al., 2009)
	<i>Rpv7</i>	7	Unknown	Bianca	UDV097	NA	Mn	(Bellin et al., 2009)
	<i>Rpv8</i>	14	<i>V. amurensis</i>	Ruprecht	Chr1 4V015	CH	Mj	(Blasi et al., 2011)
	<i>Rpv9</i>	7	<i>V. riparia</i>	W63	CCoAOMT	NA	Mn	(Moreira et al., 2011)
	<i>Rpv10</i>	9	<i>V. amurensis</i>	Solaris	GF09-46	CH	Pa	(Schwander et al., 2012)
	<i>Rpv11</i>	5	Unknown	Regent	VWMD27	NA	Mn	(Fischer et al., 2004)
				Chardonnay	CS1E104J11F			(Bellin et al., 2009)
	<i>Rpv12</i>	14	<i>V. amurensis</i>	Solaris 99-1-48	VCHR05C UDV014	CH	Mj	(Venuti et al., 2013)
	<i>Rpv13</i>	12	<i>V. riparia</i>	20/3	UDV304, rgwvin180, UDV370	NA	Mn	(Moreira et al., 2011)
	<i>Rpv14</i>	5	<i>V. cinerea</i>	W63	VMC1g3.2	NA	Mn	(Ochssner et al., 2016)
	<i>Rpv15</i>	18	<i>V. piasezkii</i>	Bömer	GF05-13	CH	Mj	-
	<i>Rpv16</i>	9	<i>V. piasezkii</i>	DVIT2027		CH	Mn	-
	<i>Rpv17</i>	8	Unknown	DVIT2027		CH	Mn	
	<i>Rpv18</i>	11	Unknown	Horizon		NA	Mn	(Divilov et al., 2018)
	<i>Rpv19</i>	14	Unknown	Horizon		NA	Mn	(Divilov et al., 2018)
	<i>Rpv20</i>	6	<i>V. rupestris</i>	B38		NA	Mn	(Divilov et al., 2018)
	<i>Rpv21</i>	7	Unknown	Horizon		NA	Mn	(Divilov et al., 2018)
	<i>Rpv22</i>	15	<i>V. amurensis</i>	Horizon		NA	Mn	(Divilov et al., 2018)
	<i>Rpv23</i>	2	<i>V. amurensis</i>	Shuanghong		CH	Pa	(Fu et al., 2020)
	<i>Rpv24</i>	18	<i>V. amurensis</i>	Shuanghong		CH	Mn	(Fu et al., 2020)
	<i>Rpv25</i>	15	<i>V. amurensis</i>	Shuanghong		CH	Mn	(Fu et al., 2020)
	<i>Rpv26</i>	15	<i>V. amurensis</i>	Shuangyou	Marker561375, Marker549779	CH	Pa	(Lin et al., 2019)
	<i>Rpv27</i>	18	<i>V. amurensis</i>	Shuangyou	Marker525926, Marker526446	CH	Pa	(Lin et al., 2019)
			<i>V. aestivalis</i>	Norton	VVCS1H077H16R1-1	NA	Pa	(Sapkota et al., 2019)
	<i>Rpv28</i>	10	<i>V. rupestris</i>	B38	UDV737			
	<i>Rpv29</i>	14	<i>V. vinifera</i>	Mgaloblishvili	VVIH01, UDV-073	NA	Mj	(Bhattarai et al., 2021)
	<i>Rpv30</i>	3	<i>V. vinifera</i>	Mgaloblishvili	chr14_21613512_C_T	CA	Mj	(Sargolzaei et al., 2020)
	<i>Rpv31</i>	16	<i>V. vinifera</i>	Mgaloblishvili	cn_C_T_chr3_16229046	CA	Mn	(Sargolzaei et al., 2020)
					il_T_C_chr16_21398409	CA	Mn	(Sargolzaei et al., 2020)

The locus name, the chromosome (Chr), the resistance source (Source), the genotype, the associated marker, the geographical origin (NA: North America, CA: Central Asia, CH: China) and the type of resistance (To: total, Mj: major, Pa: partial, Mn: minor) are given.

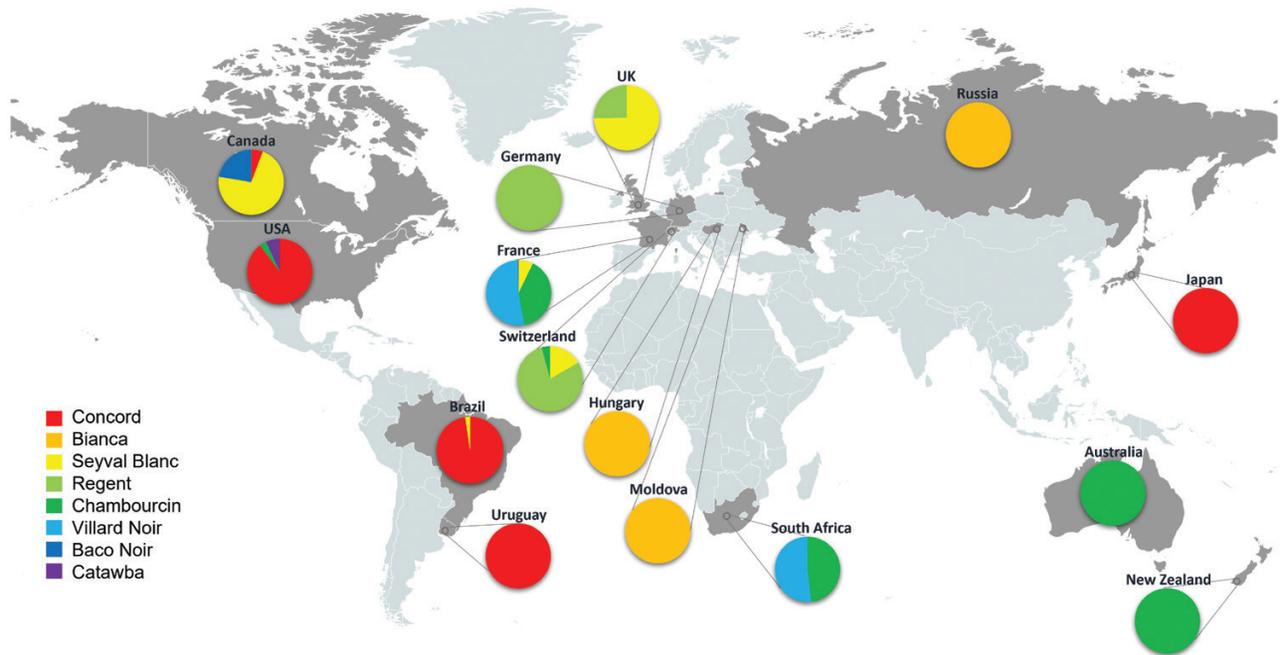


Fig. 1. Distribution of the top resistant cultivated grapevine varieties. The map shows the distribution of the eight most resistant cultivated wine grape varieties throughout the world. Data were retrieved from [Anderson and Nelgen \(2020\)](#). Detailed information is provided in [Supplementary Table S1](#).

2016; Pirrello *et al.*, 2021). The advent of genome-wide association studies (GWAS) based on grouping shared genetic variants led to the development of 9K and 18K SNP chips (Myles *et al.*, 2010; Le Paslier *et al.*, 2013), which allowed extensive studies on population genomics, germplasm genetic diversity, and linkage mapping (Myles *et al.*, 2011; Mercati *et al.*, 2016; Riaz *et al.*, 2018). Later, the more genetic diversity-focused restriction site-associated DNA sequencing (RAD-seq) and genotyping by sequencing (GBS; Peterson *et al.*, 2014; Campbell *et al.*, 2015) techniques were established to separate the analysis from the bias of using a pre-determined set of point markers, resulting in high-resolution maps with a large density of detected variant sites (Barba *et al.*, 2014; Hyma *et al.*, 2015; Marrano *et al.*, 2017).

Cisgenesis, intragenesis, and transgenesis approaches for fungal and oomycete pathogen resistance in grapevine

Despite the availability of several resistance genes isolated within *Vitis* spp. (Table 3), the lack of efficient promoters and selection markers made the application of cisgenesis (Box 2) in this genus very difficult (Limera *et al.*, 2017). This would explain why cisgenesis attempts aimed at improving fungal and oomycete tolerance in *V. vinifera* are rare. Based on our literature, only Dhekney *et al.* (2011) claimed the successful application of cisgenesis approaches in grapevine through the transfer of the *VvTL-1* gene (encoding a thaumatin-like

protein with broad-spectrum antifungal activity) from Chardonnay to Thompson. Some cisgenic lines regenerated from somatic embryos showed a delay of 7–10 d in the development of powdery mildew symptoms in greenhouse conditions. However, although the authors recognize it as the first report of a cisgenic approach for fungal and oomycete disease resistance, the resulting engineered plants were known to contain viral promoters as well as reporter/marker genes. In reality, based on the strictest definitions, no study has successfully developed fungal-resistant cisgenic or intragenic grapevines, and the aforementioned study could be placed halfway between cisgenesis and transgenesis. Similarly, other studies have used a hybrid approach based on both stable and transient transformation with genes derived from the same species or sexually compatible taxa, as well as viral promoters [i.e. cauliflower mosaic virus (CaMV) 35S], bacterial selection markers (i.e. *nptII* and *hptII*), and reporter genes [i.e. green fluorescent protein (GFP)].

For example, full or increased tolerance to powdery mildew was observed by transferring genes isolated from *V. quinquangularis*, *V. vinifera*, and *V. pseudoreticulata* into *V. vinifera*. These genes belong either to the stilbene synthase family (*VpSTSgDNA2* and *VqSTS6*; Dai *et al.*, 2015; Cheng *et al.*, 2016) or to the pathogenesis-related (PR) protein pathway (*VpPR4-1*, *VvNPR1.1*, and *VpEIFP1*; Le Henanff *et al.*, 2011; Dai *et al.*, 2016; Wang *et al.*, 2017).

Stilbenes represent phenolic compounds that accumulate at the infection site of the main fungal and oomycete pathogens and show antimycotic activity by inhibiting conidial germination (Hasan and Bae, 2017), while PR proteins are a class of

Table 2. List of the 30 most cultivated wine grape resistant varieties throughout the world

Rank among hybrids	Rank among varieties	Name	Country of origin	Parent 1	Parent 2	R loci	Cultivated area (ha)	World share (%)
1	64	Concord	USA	Catawba	<i>V. labrusca</i>		10 544	0.24
2	69	Bianca	Hungary	Villard Blanc	Bouvier	<i>Rpv3, Rpv7</i>	9766	0.22
3	151	Seyval Blanc	France	Seibel 5656	Rayon D'Or	<i>Ren3, Ren9, Ren10, Rpv3.2, Rpv3.3</i>	2699	0.06
4	173	Regent	Germany (PIWI)	Diana	Chambourcin	<i>Ren3, Ren9, Rpv3.1, Rpv4, Rpv11</i>	1974	0.04
5	254	Chambourcin	France (PIWI)	Joanne Seyve 11369	Plantet	<i>Rpv3.1, Rpv3.2</i>	968	0.02
6	282	Villard Noir	France	Chancellor	Subereux		777	0.02
7	288	Baco Noir	France	Folle Blanche	Riparia Grand Glabre + <i>V. riparia</i>		735	0.02
8	316	Catawba	USA	<i>V. labrusca</i>	Semillon		626	0.01
9	358	Couderc 13	France	<i>V. aestivalis</i>	Couderc 162- 5		474	0.01
10	401	Norton	USA	<i>V. aestivalis</i> var. Lincecumii Buckley	<i>V. vinifera</i> subsp. <i>vinifera</i>	<i>Rpv27</i>	328	0.01
11	441	Aurore	France	Seibel 788	Seibel 29		255	0.01
12	449	Vignoles	France				241	0.01
13	450	Traminette	USA	Joannes Seyve 23416	Gewürztraminer		239	0.01
14	456	Maréchal Foch	France	Millardet et Grasset 101 O.P.	Goldriesling		229	0.01
15	465	Cayuga White	USA	Seyval blanc	Schuyler		217	<0.01
16	469	Frontenac	USA	Riparia 89	Landot Noir		212	<0.01
17	505	Marquette	USA	Minnesota 1094	Ravat Noir		166	<0.01
18	554	Solaris	Germany (PIWI)	Merzling	Geisenheim 6493	<i>Ren3, Ren9, Rpv3.3, Rpv10, Rpv11</i>	118	<0.01
19	564	Johanniter	Germany (PIWI)	Riesling Weiss	Freiburg 589-54	<i>Ren3, Ren9, Rpv3.1</i>	111	<0.01
20	576	De Chaunac	France	Seibel 5163	Seibel 793		102	<0.01
21	583	La Crescent	USA	St. Pepin	Elmer Swenson 6-8-25		94	<0.01
22	594	Chardonel	USA	Seyval blanc	Chardonnay		90	<0.01
23	604	Léon Millot	France	Millardet et Grasset 101 O.P.	Goldriesling		85	<0.01
24	687	Rondo	Germany (PIWI)	Zarya Severa	Saint Laurent	<i>Rpv10</i>	51	<0.01
25	689	Golden Muscat	USA (PIWI)	Muscat Hamburg	Diamond		50	<0.01
26	706	Phoenix	Germany	Bacchus Weiss	Villard blanc	<i>Ren3, Ren9, Rpv3.1</i>	46	<0.01
27	730	Cabernet Cortis	Germany (PIWI)	Cabernet Sauvignon	Solaris	<i>Ren3, Ren9, Rpv3.3, Rpv10</i>	38	<0.01
28	734	Chancellor	France	Seibel 5163	Seibel 880		38	<0.01
29	770	Rathay	Austria (PIWI)	Blauburger	Klosterneuburg 1189-9-77		32	<0.01
30	809	La Crosse	USA	Minnesota 78 x Seibel 1000	Seyval		26	<0.01

Classification and distribution data were retrieved from [Anderson and Nelgen \(2020\)](#). Origin, parentage, and R loci data were retrieved from [Topfer and Hausmann \(2022\)](#) and [Maul \(2022\)](#).

soluble proteins that are generally expressed following abiotic and biotic stresses ([Ali *et al.*, 2018](#)). PR proteins are also involved in increased resistance to *P. viticola*, as observed by engineering *V. vinifera* with genes isolated from *V. pseudoreticulata* (*VpPR10.1*; [Su *et al.*, 2018](#)). Improved resistance to *P. viticola* was also observed in *V. vinifera* transformed with *VaHAESA*, a gene isolated from *V. amurensis* that is highly similar to pattern recognition receptor (PRR)-like kinase 5 (RLK5; [Liu *et al.*,](#)

[2018](#)). Finally, the only hybrid approach (i.e. combining cis-genesis and transgenesis elements) aimed at reducing the susceptibility of grapevine to *Botrytis cinerea* included transforming 41B rootstock and cv. Sugraone with the stilbene synthase-coding gene *VST1* under the control of the alfalfa PR 10 and 35S promoters, respectively. A strong reduction in symptoms was linked in both cases to the accumulation of resveratrol ([Coutos-Thévenot *et al.*, 2001](#); [Dabauza *et al.*, 2015](#)).

Table 3. List of the most representative examples of *Vitis* genetic transformation to improve resistance against *E. necator*, *P. viticola*, and *B. cinerea*

Target	Gene source	<i>V. vinifera</i> host	Protein (gene)	Promoter	Selection and reporter genes	Transformed tissue	Reference
<i>E. necator</i>	<i>V. vinifera</i>	Thompson Seedless	Thaumatin-like protein 1 (<i>Vvfl-1</i>)	CaMV 35S	npIIL (GFP)	Leaf	(Dhekne <i>et al.</i> , 2011)
	<i>V. vinifera</i>	Chardonnay	Non-expressor of pathogenesis related 1 (<i>VvNPR1.1</i>)	CaMV 35S	npIIL (GFP)	Anther	(Le Henanff <i>et al.</i> , 2011)
<i>P. viticola</i>	<i>V. pseudoreticulata</i>	Red Globe	Pathogenesis-related protein 4-1 (<i>VpPR4-1</i>)	CaMV 35S	npIIL	Anther	(Dai <i>et al.</i> , 2016)
	<i>V. pseudoreticulata</i>	Red Globe	F-box/Kelch-repeat protein (<i>VpEIFP1</i>)	CaMV 35S	npIIL	Anther	(Wang <i>et al.</i> , 2017)
<i>B. cinerea</i>	<i>V. pseudoreticulata</i>	Chardonnay	Stilbene synthase (<i>VpSTSgDNA2</i>)	CaMV 35S	hpIIL	Anther, ovary, flower	(Dai <i>et al.</i> , 2015)
	<i>V. quinquangularis</i>	Thompson Seedless	Stilbene synthase 6 (<i>VqSTS6</i>)	CaMV 35S	npIIL	Anther	(Cheng <i>et al.</i> , 2016)
<i>O. sativa</i>	<i>O. sativa</i>	Pusa seedless	Rice chitinase (<i>Chi11</i>)	maize ubiquitin promoter	hpIIL, bar	Leaf	(Nirala <i>et al.</i> , 2010)
	<i>O. sativa</i>	Neo Muscat	Rice chitinase (<i>RCC2</i>)	CaMV 35S	npIIL	Ovule	(Yamamoto <i>et al.</i> , 2000)
<i>Trichoderma spp.</i>	<i>A. thaliana</i>	Thompson Seedless	Resistance to powdery mildew 8 locus (<i>RPW8.2</i>)	CaMV 35, native promoter	npIIL	Anther	(Hu <i>et al.</i> , 2018)
	<i>Trichoderma spp.</i>	Thompson Seedless	Endochitinases (<i>ech42</i>) + <i>N</i> -acetyl- β -D-hexosaminidase (<i>nag70</i>)	CaMV 35S	npIIL	Bud	(Rubio <i>et al.</i> , 2015)
<i>P. viticola</i>	<i>V. pseudoreticulata</i>	Thompson Seedless	Pathogenesis-related protein 10.1 (<i>VpPR10.1</i>)	CaMV 35S	npIIL (GFP)	Anther	(Su <i>et al.</i> , 2018)
	<i>V. amurensis</i>	Thompson Seedless	Thaumatin-like protein (<i>VaTLP</i>)	CaMV 35S	npIIL	Anther	(He <i>et al.</i> , 2017)
<i>B. cinerea</i>	<i>F. graminearum</i>	Crimson Seedless	Chitinase and β -1,3-glucanase	maize ubiquitin promoter	hpIIL, bar	Leaf	(Nookaraju and Agrawal, 2012)
	<i>V. vinifera</i>	Thompson Seedless	Thaumatin-like protein (<i>Vvfl-1</i>)	CaMV 35S	npIIL (GFP)	Leaf	(Dhekne <i>et al.</i> , 2011)
<i>V. vinifera</i>	<i>V. vinifera</i>	Sugraone	Stilbene synthase 1 (<i>Vvst1</i>)	CaMV 35S	npIIL (GFP)	n.s.	(Dabauza <i>et al.</i> , 2015)
	<i>V. vinifera</i>	41B (Chasselas x Berlandieri)	Stilbene synthase 1 (<i>Vvst1</i>)	alfalfa <i>PR10.1</i> promoter	npIIL	Immature embryo	(Coutos-Thévenot <i>et al.</i> , 2001)

Box 2. Transgenesis, intragenesis, and cisgenesis in plants

By definition, transgenesis is the genetic alteration of a plant with one (or more) gene(s) derived from any unrelated and sexually incompatible plant taxa or even from non-plant organisms (Schouten *et al.*, 2006). In contrast, cisgenesis and intragenesis represent two possible alternatives that rely on the use of genetic material from the same species or from closely related species capable of sexual hybridization being transformed (Holme *et al.*, 2013).

While cisgenesis involves genetic modification using a complete gene unit, including its regulatory elements (i.e. the entire original transcriptional unit), intragenesis refers to the *in vitro* recombination and transfer of elements (i.e. promoters, introns, exons, and terminators) isolated from different genes and assembled into a chimeric construct. In both cases, the result is a product free of non-plant DNA sequences and, consequently, any selection markers or residual vector sequences at the level of transformed plants are not expected (Rommens *et al.*, 2007; Holme *et al.*, 2013). In contrast to intragenesis, cisgenesis events also occur spontaneously in nature or by means of conventional breeding. However, cisgenesis has great potential to overcome the two major bottlenecks typical of traditional breeding: much longer times and the risk of linkage drag (i.e. the possibility to transfer genes with deleterious effects that are associated with the gene of interest).

Regarding transgenic approaches, the chitinase family has aroused enormous interest due to its involvement in the response to *E. necator*. Two rice chitinase genes (*Chi11* and *RCC2*) were effective in reducing the effects of this pathogen in transgenic lines of *V. vinifera* (Yamamoto *et al.*, 2000; Nirala *et al.*, 2010). Acquired resistance to the above-mentioned fungus was also obtained by engineering grapevine with both an *ech42-nag70* double gene construct (i.e. a 42 kDa extracellular endochitinase combined with an *N*-acetyl- β -D-hexosaminidase) isolated from *Trichoderma* spp. and the *RESISTANCE TO POWDERY MILDEW 8* (*RPW8.2*) gene isolated from *Arabidopsis thaliana* (Rubio *et al.*, 2015; Hu *et al.*, 2018). Reduced rates of lesion expansion caused by *B. cinerea* were observed by transforming *V. vinifera* with a gene isolated from *Pyrus* spp. and coding for a polygalacturonase-inhibiting protein (pGIP; Agüero *et al.*, 2005). Finally, a chitinase gene isolated from scab-infected Sumai-3 wheat and introduced into cv. Crimson Seedless produced enhanced tolerance to *P. viticola* infection (Nookaraju and Agrawal, 2012). The development and application of transgenesis and cisgenesis strategies are linked to several technical and biological advantages

Box 3. Genome editing and CRISPR/Cas9 in plants

The advent of the CRISPR/Cas9 system has been revolutionizing genome editing approaches, allowing the modification of cellular DNA with a much higher level of versatility and precision and with a wide extension of applications with respect to previous technologies (Ren *et al.*, 2021). The system is based on the recognition of a pre-determined DNA target site through the action of a complementary RNA sequence, namely the guide RNA (gRNA), and the Cas endonuclease, followed by the DNA double-strand break at the target site, which determines the onset of insertions, deletions, or nucleotide substitutions (Scintilla *et al.*, 2022). This technique is less expensive, simpler, and faster than other enzyme-based techniques, such as zinc finger nuclease (ZFN) or transcription activator-like effector nuclease (TALEN; Ghogare *et al.*, 2021). Therefore, especially in the case of the genetic improvement of elite varieties of woody plants, such as grapevine, CRISPR/Cas9 technology ensures genotype preservation while resulting in targeted and specific genetic modifications. Although there is enormous scientific interest in applying this technology to grape varieties, there are few examples of successes. On the one hand, the decrease in the application of this technique could be due to the disillusionment caused by the short-sightedness of national and international organizations that still associate genome editing with transgenesis and with the generation of GMOs. On the other hand, especially in grapevines, there are technical impediments to using these processes, including the difficulty and timing of regeneration to create stable edited lines and the difficulty of creating plants free of exogenous DNA through segregation of the induced mutation by the CRISPR/Cas transgenic construct.

and disadvantages. Moreover, these strategies often give rise to biosafety issues and public concerns. All these aspects are summarized later in Box 5.

Application of CRISPR/Cas9 to improve tolerance to the main fungal pathogens in grapevine

A first example of the application of the CRISPR/Cas9 [clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated protein] system (Box 3) in grapevine involved the *Agrobacterium*-mediated transformation of Chardonnay cell suspensions with a single plasmid containing a specific single guide RNA (sgRNA) able to target the *L-idonate*

dehydrogenase (IdnDH) gene to alter the biosynthetic pathway of tartaric acid (Ren *et al.*, 2016). Later, the gene encoding *phytoene desaturase (VvPDS)* was effectively knocked out in embryogenic calli of Neo Muscat (Nakajima *et al.*, 2017), Chardonnay, and 41B rootstock by means of CRISPR/Cas9 binary vectors (Ren *et al.*, 2019).

The first attempt to edit the grapevine genome to increase resistance against *B. cinerea* was performed on the Thompson Seedless variety, targeting the transcription factor (TF) gene *VvWRKY52*. Induced mutations in this gene led to a significant reduction in the development of fungal colonies, especially in biallelic mutant lines (Wang *et al.*, 2018). In further studies, CRISPR/Cas9 technology was used to obtain different mutant lines of *V. vinifera* characterized by a lack of expression of *Downy Mildew Resistance 6 (DMR6)* and *Mildew Locus O (MLO)*. *DMR6* and *MLO* represent susceptibility (*S*) genes toward *P. viticola* and *E. necator*, and their knockout led to a significant increase in resistance to these fungal and oomycete pathogens (Giacomelli *et al.*, 2019, 2022, Preprint). The efficiency of CRISPR/Cas9-mediated mutagenesis in grapevine depends on factors common to other genetic transformation methods (technical methods, plant genotype, target gene, *in vitro* regeneration, and selective conditions) and on factors specific to this particular type of approach, such as the choice of the upstream Cas9 promoter and the sgRNA sequence (Ma *et al.*, 2015). With reference to this last point, CRISPR/Cas9-mediated genome editing in grape was first reported in 2016 (Ren *et al.*, 2016), and since then, the Arabidopsis AtU6 or AtU3 promoters have been used to regulate the expression of sgRNAs (Ren *et al.*, 2016; Nakajima *et al.*, 2017; Osakabe *et al.*, 2018; Wang *et al.*, 2018; Giacomelli *et al.*, 2019). Recently, Ren *et al.* (2021) identified four VvU3 and VvU6 promoters and two ubiquitin (UBQ) promoters in grapevine, and demonstrated that the use of the identified VvU3/U6 and UBQ2 promoters could significantly increase the editing efficiency in grape by improving the expression of sgRNA and Cas9, respectively. CRISPR components can be delivered within the cell in different ways: (i) in the form of nucleic acids, with genes coding for Cas9 and gRNA delivered using vectors; (ii) with genes coding for gRNA cloned within vectors and a recombinant Cas9 protein; and (iii) in the form of a ribonucleoprotein (RNP) complex, constituted *in vitro* with Cas9 protein and an *in vitro/in vivo* transcribed gRNA (Glass *et al.*, 2018). The major bottleneck in grapevine editing is that all the applications described thus far still exploit *Agrobacterium*-mediated genetic transformation using nucleic acids (i.e. DNA/mRNA coding for the entire system). Consequently, these obtained varieties would not overcome the limits highlighted for transgenic varieties (Capriotti *et al.*, 2020) (for a more detailed description of the advantages/disadvantages of genome editing, see Box 5). Clearly, a delivery strategy based on the use of the RNP complex represents

a valid methodology to edit the genome without the introgression of exogenous DNA. At the same time, however, an approach of this type cannot be accompanied by an *Agrobacterium*-mediated transformation, hence the need to develop or implement new methods to associate the use of RNP, which does not involve integration of non-host DNA into the edited species, into an efficient, inexpensive, and fast transformation system. Among the best candidate delivery systems are particle gun or polyethylene glycol (PEG)-mediated transfection. A first example of the direct delivery of CRISPR/Cas9 RNPs in grapevine protoplasts to obtain efficient DNA-free targeted mutations was achieved by Malnoy *et al.* (2016) in Chardonnay. In this study, protoplasts obtained from embryogenic calli were edited, and a site-specific mutation was determined at the level of the *MLO7* gene locus, which is one of the susceptibility factors to *E. necator*. A detailed protocol adapting the CRISPR/Cas9 system to grapevine plants using the direct delivery of CRISPR/Cas9 RNPs to achieve efficient DNA-free targeted mutations in grapevine protoplasts was described 2 years later by Osakabe *et al.* (2018). Clearly, one of the major impediments to the use of protoplasts/RNPs in genome editing approaches lies precisely in the difficulty encountered in the regeneration of plants starting from single cells. Numerous efforts are being made in this field to alleviate this bottleneck to applying genome editing successes to plants. Recently, a successful DNA-free methodology was used to obtain fully edited grapevine plants regenerated from protoplasts obtained from a *V. vinifera* cv. Crimson seedless L. embryogenic callus. The transfected protoplasts were edited on the downy mildew susceptibility gene *VvDMR6-2* (Scintilla *et al.*, 2022). Excluding the protoplast-based approach, in all other cases, the RNP complex needs to cross a substantial barrier, the cell wall, whose structure and composition are complex and tissue specific and can vary over time, making it a complicated structure to be crossed. Nanoparticles were shown to deliver different molecules (DNA, RNA, and protein) to mammalian cells. Interestingly, increased interest in their use in agriculture occurred with relative success in delivering different molecules to normal plant cells with cell walls. The scientific community is currently paying a lot of attention to a particular class of nanoparticles, the cell wall-penetrating peptide (CPP), which seems to be less cytotoxic to plants than other nanoparticles, such as gold or silicone nanoparticles. CPPs consist of short peptide sequences (5–30 amino acid residues) that facilitate cargo penetration through the cell membrane. Recent studies have demonstrated their efficacy in penetrating intact plant cells, and they can help deliver large cargo molecules such as proteins, DNA, and RNA (Numata *et al.*, 2018). The development of a methodology for CPP-mediated delivery of a CRISPR/Cas9 system to a regenerable grapevine plant material will be a game changer because it will align with the pursuit of

generating edited grapevine material that is free of genetically modified organisms (GMOs).

RNAi mechanisms and potential applications in grapevine defense

To date, conventional RNAi approaches (Box 4) have relied on the use of weakened recombinant plant viruses (the so-called virus-induced gene silencing, VIGS), *Agrobacterium*-mediated transiently expressed transgenes, and stably transformed transgenic plants that express dsRNAs to silence specific genes that

Box 4. RNA interference mechanisms in plants

RNAi is one of the most important natural regulatory and defense mechanisms that is implicated in the control of plant growth, development, and response to biotic or abiotic stresses. RNAi controls the expression of endogenous protein-coding genes in a sequence-specific manner and regulates the plant response to undesired nucleic acids, transposons, and transgene activity (Dubrovina *et al.*, 2016). The suggested RNA silencing mechanism coordinately involves key components, such as Dicer-Like (DCL) proteins, Argonaute (AGO) proteins, and RNA-dependent RNA polymerase (RDR) proteins, which are responsible for the genesis of 21–24 nucleotide (nt) sRNAs and their biological function (Vaucheret, 2006). Although a dsRNA molecule serves as a precursor for subsequent sRNA production, sRNAs can be classified as siRNAs, miRNAs, or trans-acting siRNAs (ta-siRNAs) based on their origin. A number of DCL, AGO, and RDR genes have been discovered in various plant species, detailing their roles in plant defense systems (Vaucheret, 2008; Nakasugi *et al.*, 2013; Liu *et al.*, 2014). Plant AGO proteins can be divided into two main classes depending on the RNAi pathway in which they are involved: AGO4, 6, and 9 mediate so-called transcriptional gene silencing (TGS), and AGO1, 2, 3, 5, 7, and 10 mediate PTGS (Mallory and Vaucheret, 2012). In TGS, sRNA loaded on AGO proteins recruits DNA methyltransferases to methylate the cytosine residues of the corresponding target gene in the genome, whereas in PTGS, the sRNA-AGO complex scans the cytoplasm for complementary transcripts for cleavage and degradation. Since its discovery over 20 years ago, RNAi has been widely used in reverse genetics and functional genomics to down-regulate the expression of genes responsible for the control of abiotic stress tolerance, developmental processes, and other plant responses (Niu *et al.*, 2008; Biswal *et al.*, 2015), as well as in crop protection activities for the control of pest and pathogen resistance (as reviewed in Kaur *et al.*, 2021).

control target traits (known as host-induced gene silencing, HIGS). Despite their evident success, the use and trading of RNAi-based transgenic crops as GMOs have aroused widespread concern and criticism from the public surrounding their long-term consequences on human health and the environment. In this regard, the development of low- or non-pathogenic virus expression vectors for the application of VIGS in crops poses technical and safety challenges. The post-transcriptional gene silencing (PTGS) mechanism is still poorly explored in grapevine compared with other crops; however, these silencing mechanisms have been applied to different sides of the host-pathogen interaction, targeting both host and pathogen sequences. At the pathogen level, *B. cinerea* was observed to silence immune response genes in *V. labrusca*, producing small RNAs (sRNAs) through the DCL system to establish infection. In this regard, a PTGS strategy was developed based on *BcDCL1* and *BcDCL2* as pathogen target genes, obtaining a ‘silencing of the silencing’, which showed reduced growth of the fungi with consequent pathogenicity mitigation (Wang *et al.*, 2016). A similar approach was used for downy mildew management by targeting *PvDCL1* and *PvDCL2*, the genes responsible for the silencing machinery of *P. viticola* during grapevine infection (Brilli *et al.*, 2018).

In addition to the host-pathogen relationship determinants, an infection can also be contained by directly leveraging the molecular mechanisms underlying the vital processes of the pathogenic organism. Indeed, an emblematic case is the one carried out by Nerva *et al.* (2020) in *V. vinifera* cv. Moscato, in which three *B. cinerea* target genes were identified: *BcCYP51* (or *erg11*), *Bchs1*, and *BcEF2b. erg11* encodes a lanosterol 14 α -demethylase belonging to the cytochrome P450 monooxygenase (CYP) superfamily which controls a key step in the ergosterol biosynthetic pathway; it is a fungi-specific compound and the target of the main antifungal triazole-based products. Chitin synthase 1 is involved in chitin accumulation in the fungal cell wall, and its inactivation in *B. cinerea* results in stunted growth due to a weakened fungal cell wall (Soulié *et al.*, 2003). Finally, elongation factor 2 catalyzes ribosomal translocation and is the target gene of sordarin-derived commercial products. Independent of the application modality used, a collapse of *B. cinerea* virulence was observed in all the treatments compared with the control. From the host plant point of view, the *MLO* family contains the *S* genes to *E. necator* (Jorgensen, 1992). In monocots, the *S* genes phylogenetically belong to the IV clade (Reinstädler *et al.*, 2010) of the *MLO* family, whereas in dicots they belong to the V clade (Feechan *et al.*, 2008). *MLO S* genes seem to negatively regulate the vesicle-associated and actin-dependent defense pathways at the *E. necator* penetration site (Panstruga, 2005). Of the four *S* genes identified in *V. vinifera* (*VvMLO6*, *VvMLO7*, *VvMLO11*, and *VvMLO13*), only the last three are known to be up-regulated during powdery mildew (Feechan *et al.*, 2008). A HIGS-based knockdown strategy showed a decrease in powdery mildew symptom severity up to 77% by silencing *VvMLO7* in c

ombination with *VvMLO6* and *VvMLO11* in cv. Brachetto, with no pleiotropic effects observed (Pessina *et al.*, 2016). Another work on *S* genes involved targeting the *VvLBDIf7* grapevine gene, which encodes an LOB domain (LBD)-containing protein (Canaguier *et al.*, 2017). The product of this gene is the putative ortholog of an LBD TF, a repressor of jasmonate-mediated defense mechanisms in Arabidopsis roots during *Fusarium oxysporum* infection. The disrupted function of this gene leads to enhanced resistance to pathogens (Thatcher *et al.*, 2012). A PTGS procedure on *V. vinifera* (cv. Pinot noir) gave satisfying results, with a significant decrease in the growth and sporulation of *P. viticola* (Marcianò *et al.*, 2021).

Due to the strong restrictions on the main genetic engineering techniques, research has recently focused on the development of methods that do not involve modification of the host genome and, therefore, do not lead to the production of varieties considered genetically modified by the current legislation. For these reasons, exogenous RNAi-based approaches have emerged as a widely accepted and environmentally friendly strategy relying on the direct exogenous application of RNA molecules (dsRNAs and/or sRNAs) to improve crop qualities, with the potential to trigger RNAi in various plants regardless of their genetic backgrounds (for a more detailed description of the advantages/disadvantages of this approach, see Box 5). Following the results that emerged in the application of various RNAi methods for the development of resistance by plants, the evidence of how the exogenous application of polynucleotides can influence the mRNA levels of important virulence-related genes of pathogens or plants has proven to be pivotal in the context of the development of new techniques and strategies for crop protection (Dubrovina and Kiselev, 2019). In detail, spray-induced gene silencing (SIGS) allows the absorption of dsRNA by cells and plant tissues, with the RNAi machinery being created by the host plant or directly conveyed at the cellular level of the pathogen, thus triggering gene silencing through the RNAi machinery of the pathogen itself (Sang and Kim, 2020). An experimental SIGS trial demonstrated how the exogenous application of dsRNA specific for the transcripts of the *B. cinerea* *DCL1/2* genes, which regulate the expression of Dicer proteins essential for the production of sRNA, on the leaf surface of *V. vinifera* led to effective absorption by the necrotrophic fungus, thus enhancing plant protection. Moreover, local application of *B. cinerea* vacuolar protein sorting 51 (*VPS51*), dynactin (*DCTN1*), and suppressor of actin (*SAC1*)-targeted dsRNA particles to grape berries led to stunted mycelial growth and reduced susceptibility in the plant (Qiao *et al.*, 2021). Using SIGS, encouraging results against *B. cinerea* were observed by Nerva *et al.* (2020) with post-harvesting berry application, as well as in the above-mentioned work on *PvDCL1* and *PvDCL2* (Haile *et al.*, 2021) and on *VvMLO* (Marcianò *et al.*, 2021).

However, in the development of dsRNA-based RNAi strategies, several aspects must be considered: (i) the choice of the target gene; (ii) the delivery methods; and (iii) the

formulation of the exogenous ‘inoculum’. In addition to the above-mentioned genes, members of different classes of transcripts can be identified as promising RNAi targets (Table 4). Among them is the ever-growing class of plant *S* genes, which are recessively inherited and less suitable for canonical breeding programs for resistance. *S* genes belong to different functional classes according to the role they play in plant-pathogen interactions (van Schie and Takken, 2014), such as TFs or genes catalyzing catabolic reactions. The major class of secreted effectors in *P. viticola* are the RxLR cytoplasmic effectors, which act inside the cell of the host plant (Tofolatti *et al.*, 2020). Among them, a few have been identified as programmed cell death inhibitors shown to suppress the immune response in *Nicotiana benthamiana*. Something similar was observed when investigating the role of some *E. necator* RNase-like effector proteins expressed in haustoria (RALPH) effectors. Exploring these gene classes as well as those already scavenged in RNAi-targeted research represents a promising strategy. Moreover, regarding the target tissue, there are some variations in SIGS delivery methods, such as petiole absorption and trunk injection, with each having different efficiencies and resistance persistence; however, they still function and give promising results (Nerva *et al.*, 2020).

Conclusions

The co-evolutionary interaction between host and parasite is often characterized by strong reciprocal selective pressures exerted by the two antagonists upon one another. If the interaction is long lasting, then it may lead to fast evolutionary change (Woolhouse *et al.*, 2002). Although this phenomenon is common in plant-pathogen interactions in wild populations, it is not common in agriculture or viticulture. In fact, while the pathogen is subject to constant evolution, determined by the selective pressure exercised by chemical defense treatments, the plant host is not exposed to any evolutionary process due to strictly correlated commercial and propagative reasons. The absence of co-evolution in viticulture led to a phenomenon of genetic stagnation at the level of elite cultivars, making them increasingly subject to attack by pathogens. Consequently, viticulture, while occupying 3% of the entire cultivated area, uses ~65% of all fungicides applied in agriculture, thus creating strong issues relating to the environmental, economic, and social sustainability of the wine sector. In this scenario, plant breeding for genetic improvement can be extremely helpful for the development of new sustainable viticulture through the development of genotypes resistant to pathogens by exploiting the existence of resistance genes or the presence of genes for susceptibility in the cultivars of interest. However, at least for grapevine, the pursuit of this goal cannot be approached by means of the traditional methods of hybridization and selection alone, not only for purely technological reasons but also

Box 5. Technical, biological, and social issues associated with conventional and biotechnological breeding techniques

Conventional breeding. This approach is based on several backcrossing generations, introgressions, induced mutagenesis, and somatic hybridization. Genes of interest are found in crossable, sexually compatible organisms. Although this technology has a high consumer acceptance as a non-GMO approach and has no biosafety concerns, it presents several side technical issues. First, the timing needed for breeding programs is quite long and requires deep knowledge and the availability of genetic resources. Newly developed genotypes are similar to the original clones but are not exactly the same, a less appreciated factor in a highly conservative discipline such as viticulture. Moreover, if resistance to diseases is provided by the insertion of dominant *R* genes, it will be easily overcome by the onset of new pathogen strains (Myles, 2013).

Transgenesis/cisgenesis. Both approaches are based on genetic transformation. The main difference between them is the origin of the target gene: transgenesis involves the overexpression of genes from non-sexually compatible organisms and the presence of sequences from non-compatible organisms (promoters, terminators, and selective markers), while cisgenesis only considers genes (including introns) and their regulatory regions (promoters and terminators) from sexually compatible plants. These technologies have the advantage of bypassing linkage-drag phenomena and take less time to produce (1–2 years). Nevertheless, they are considered GMOs, which presents several side effects or technical issues, such as the release of genes with different origins into the environment, the expression of new protein products with possible allergen/toxic effects (transgenesis), and the scarce availability of efficient cisgenic selectable marker genes. Furthermore, the expression of unknown proteins and the use of antibiotic- or herbicide-resistant markers make transgenesis unpopular with consumers. In contrast, cisgenic plants may partially solve the current biosafety problems regarding the presence of foreign DNA in the host genome, increasing consumer acceptance.

Genome editing is based on both genetic transformation and plasmid-free protoplast transformation. The advantage of this technique lies in the fact that it is extremely precise and can introduce mutations at the single nucleotide level without changing the genomic background, thus preserving clonal identity. Technical issues are related to the fact that when applying plasmid-based genetic transformation, segregation is required from T_0 plants, an extremely disadvantageous procedure in a clonally propagated plant with a highly heterozygous genetic background. In contrast, plasmid-free protoplast transformation bypasses the aforementioned problem but requires *in vitro* regeneration from protoplasts, which can be tricky since many grapevine varieties are recalcitrant to regeneration.

dsRNA-based SIGS has the advantage of not being considered a transgenic approach since it is not based on recombinant DNA technology and can be implemented in a few months. Compared with many of the techniques discussed, the exogenous application of sRNA molecules is characterized by a much higher targeted precision during intervention and even reduced pesticide use following intervention, which fits in an integrated crop protection strategy by being able to down-regulate exogenous or endogenous gene expression. On the other hand, it is necessary to evaluate how the effectiveness of gene silencing depends on the efficiency and specificity of the RNA molecule sequence and the degree of absorption feasible by the plant host and pathogen cells (Gebremichael *et al.*, 2021). Likewise, the RNAi sequence must be carefully selected to avoid any possible off-target and pleiotropic effects. The onset of potential negative effects on the ecosystem and human health from the use of this technique must be investigated in more detail. A multilevel study that includes every single layer and all reciprocal interactions would feed-back on the wide complete integrated model to predict the effect of a treatment on every single stakeholder and on the entire system.

for commercial reasons. In fact, although the application of conventional breeding methods has already led to the development of improved cultivars from an oenological point of view that are sometimes characterized as having resistance to certain plant diseases, these represent completely new genotypes that have lost their commercial names and may possibly be subject to important existing regulations. If it is true that the expertise of grapevine breeders and conventional breeding methods will never be totally replaced by grapevine biotechnologists and biotechnological methods, it is also true that next-generation technologies available for molecular selection and genetic

improvement will become increasingly important for the development of superior and resilient grapevine cultivars. The potential use of new biotechnological approaches for either loss-of-function and gain-of-function applications, such as cisgenesis (for conspecific gene transfer or introgression), genome editing (for endogenous gene knockout and gene editing or replacement), and RNAi-based methodologies (for targeted gene silencing), will assume strong relevance in the future of viticulture, as these methods would allow direct intervention at the genomic level in any variety without changing its genetic background. All these genome-wide biotechnological

Table 4. List of candidate and validated (in bold) target genes for RNAi conferring resistance towards *E. necator* (PM), *P. viticola* (DM), and *B. cinerea* (GM)

Gene name	Gene ID	Organism	Disease	Function	Reference
VvMLO6^a	Vitv10g00509 ^b	<i>V. vinifera</i>	PM	Trans-membrane protein, negative regulator of cell wall appositions.	(Pessina <i>et al.</i> , 2016)
VvMLO7^a	Vitv13g00578 ^b				
VvMLO11^a	Vitv10g01055 ^b				
VvCSN5	Vitv10g00286 ^b			COP9 signalosome complex subunit 5 (CSN5), involved in protein degradation via the ubiquitin-proteasome pathway.	(Cui <i>et al.</i> , 2021)
VvDMR6-1	Vitv16g01336 ^b			SA 5-hydroxylase, involved in SA catabolism.	(Pirrello <i>et al.</i> , 2022)
VvDMR6-2	Vitv13g01119 ^b		DM	TF, stabilized by <i>PvRXLR11</i> effector.	(Giacomelli <i>et al.</i> , 2022)
VvWRKY40	Vitv13g00189 ^b			LBD protein, repressor of JA-mediated defense.	(Ma <i>et al.</i> , 2021)
VvLBD1f³	Vitv13g00549 ^b			Lanosterol 14 α -demethylase, primary target of sterol demethylation inhibitor (DMI) fungicides.	(Marcianò <i>et al.</i> , 2021)
BcCYP51^c	BcDW1_10539 ^d	<i>B. cinerea</i>	GM	Chitin synthase, contributes to cell wall composition. β 1-tubulin promoter.	(Nerva <i>et al.</i> , 2020)
Bchs1^c	BcDW1_7822 ^d			Dicer-like (DCL) gene, effector.	(Wang <i>et al.</i> , 2016)
BcEF2^c	BcDW1_2938 ^d			Vesicle trafficking pathway genes.	(Qiao <i>et al.</i> , 2021)
BcDCL1^c	BcDW1_481 ^d			Dicer-like (DCL) gene, effector.	(Haile <i>et al.</i> , 2021)
BcDCL2^c	BcDW1_2215 ^d			Cell death inhibitor, effector.	(Lei <i>et al.</i> , 2019)
BcVPS51^c	BcDW1_6998 ^d			Cell death inhibitor, effector.	(Xiang <i>et al.</i> , 2016)
BcDCTN1^c	–			Cell death inhibitor through interaction with <i>BRI1</i> kinase inhibitor 1 (<i>VvBK17</i>), effector.	(Lan <i>et al.</i> , 2019)
BcSAC1^c	–			INF1-triggered programmed cell death and defense-related gene expression inhibitor, effector.	(Liu <i>et al.</i> , 2021)
PvDCL1^c	PvITv1_T038441 ^e	<i>P. viticola</i>	DM	Candidates for secreted effector proteins (CSEPs) RALPH effector, plant immunity inhibitors.	(Zhang, 2021)
PvDCL2^c	PvITv1_T003331 ^e				
<i>PvRXLR159</i>	–				
<i>PvRXLR28</i>	–				
<i>PvRXLR131</i>	–				
<i>PvRXLR53</i>	–				
<i>EnCSEP56</i>	–	<i>E. necator</i>	PM	Candidates for secreted effector proteins (CSEPs) RALPH effector, targets <i>VvADC</i> suppressing ROS production.	(Mu <i>et al.</i> , 2022)
<i>EnCSEP65</i>	–				
<i>EnCSEP115</i>	–				
<i>EnCSEP087</i>	–				

Together with pathogen target genes, we also reported several grapevine susceptibility target genes.

^a Experiment conducted by means of host-induced gene silencing (HIGS).

^b Available at JBrowse <https://urgi.versailles.inra.fr/browse/gmod/browse/> (Buels *et al.*, 2016).

^c Experiment conducted by means of spray-induced gene silencing (SIGS).

^d Available at GrapeGenomics.com http://www.grapegenomics.com/pages/P_Bcinerea/ (Blanco-Ulate *et al.*, 2013).

^e Available at NCBI https://www.ncbi.nlm.nih.gov/assembly/GCA_003123765.1 (Brill *et al.*, 2018).

Box 6. Rules and disputes relating to the use of transgenic, cisgenic, intragenic, and edited plants in agriculture and food in the European framework

Despite the potential ability of the application of genetic transformation techniques to control the main phytopathogens, the use of transgenic varieties in agriculture is at present severely limited in the EU. The community regulatory context, based on the precautionary principle, was first defined by Directive 2001/18/EC of the European parliament ([EU Parliament and Council, 2001](#)). This law replaces Council directive 90/220/EEC and stipulates the basic rules for the approval of a new GMO. Second, there are two regulations ([EU Parliament and Council, 2003a, b](#)) that control the authorization and labeling/traceability of food and feed consisting of or derived from GMOs. Third, the Commission Recommendation of 23 July ([EU Commission, 2003](#)) indicates the guidelines for the co-existence of GM and conventional crops, to which national and regional regulations must refer. Finally, the EC Directive 2015/412 ([EU, 2015](#)) integrates the previous EC Directive 2001/18 by delegating ownership to national states to allow, limit, or prohibit the commercial cultivation of GM plants. The action taken by EU countries must comply with EU legislation and be reasonable, proportional, and non-discriminatory. EU countries where GMOs are grown must take measures in border areas to avoid possible contamination of neighboring EU countries where the cultivation of those GMOs is prohibited. Basically, the cornerstone of European standards is the great attention given to the assessment of all potential risks, based on the precautionary principle, and the fact that all authorizations are granted for a limited period of time, during which environmental and health effects must be carefully monitored. The utmost precaution toward the authorization and cultivation of transgenic plants by the EU derives from a series of concerns raised by public opinion related to the transfer of selection markers expressed by transgenic plants to pathogenic microorganisms in the gastrointestinal tract or soil, bringing resistance to herbicides or antibiotics. This problem could be overcome by the cisgenic approach, which, however, carries with it a series of critical issues: (i) insertion of the donor sequence in a random position, which can potentially influence DNA methylation and regulation; (ii) potential occurrence of mutations at the insertion site, with the possibility of rearrangements and translocations in the flanking regions; (iii) small portions of T-DNA remaining as 'junk DNA' and not being able to determine any type of phenotypic effect; and (iv) donor sequences not being replaced with an allelic sequence but instead added to the recipient genome ([Schouten *et al.*, 2006](#); [Rommens *et al.*, 2007](#); [Jacobsen and Schouten, 2008](#); [Telem *et al.*, 2013](#)). Many of the questions raised by public opinion on cisgenics could also be raised about plants obtained from traditional breeding. Indeed, according to EFSA, the product obtained by means of a cisgenic approach is not dissimilar to that obtained by traditional breeding [[EFSA Panel on Genetically Modified Organisms \(GMO\), 2012](#)]. Nonetheless, at present, according to the community legislation in place, cultivars obtained by cisgenesis and intragenesis are considered GMOs. Breeding innovations consist of known, controllable, and predictable procedures. With the development of new molecular techniques for crop improvement, new legislative issues need to be resolved. As testified to by the sentence released by the European Court of Justice on 25 July 2018 ([Vives-Vallés and Collonnier, 2020](#)), organisms obtained through techniques or methods of mutagenesis must be considered GMOs. The sentence includes in the scope of application of the EC Directive 2001/18 organisms obtained by means of new techniques or new methods that have emerged after the adoption of the Directive in question (i.e. CRISPR/Cas9-driven genome editing) and, consequently, excluding from its scope only organisms obtained through techniques or methods conventionally used in various applications with a long tradition of safety (such as organisms obtained with traditional methods of random mutagenesis by ionizing radiation or exposure to chemical mutagenic agents). On the one hand, this ruling condemns genome editing techniques to confinement in the legislation on GMOs but, on the other hand, it generates paradoxes that require the European legislator to review the entire discipline. Indeed, the current legislation is based on the regulation of organisms mainly on the basis of their production process (e.g. chemical/physical mutagenesis, conventional genetic transformation, or genomic editing); however, it is essential to start a discussion that also takes into consideration the nature of the final product, since some induced genetic modifications can be considered equivalent to those obtainable with natural selection mechanisms. For example, the same plant genotype could be derived from a spontaneous random event, from a mutagenesis experiment with ionizing radiation, or from site-specific mutagenesis implemented by CRISPR, and, by sequencing the genome of the organism, there would be no possibility of determining the source of the genetic variation, thus making any type of regulatory framework impossible. Releasing the organisms obtained via new technologies from natural/unnatural dualism of this regulation would allow for the simplification and real effectiveness of the procedures for risk assessment, which, at that point, would be applied to individual cases with the intervention of adequate legislation and regulatory bodies ([ISAAA, 2021](#)). As a signal that the European legislative bodies and public opinion are moving in this direction, the EC has recently started a public consultation to prepare a policy initiative on plant products aimed to provide an adequate regulation that guarantees the protection of the environment and human health with the contribution to innovation given by the plants obtained with new genomics techniques ([EU Commission, 2022](#)).

strategies represent rapidly increasing precision breeding methods that are potentially useful for developing cultivars genetically improved for single traits while preserving all other remaining traits. In addition, these methods have the benefit of reducing the time needed to breed a certain varietal genotype coupled with the advantage of avoiding any genetic recombination or transfer of undesirable genetic material. These are aspects common to all tree species as well as grapevines, and such technologies have been successfully applied in woody crops. Furthermore, in the coming years, it seems possible that biotechnological approaches could allow not only the transfer of cisgenes or the editing of genes of interest but also the control of whole biosynthetic pathways and regulatory networks of certain plant genomes, making the improvement of grapevine cultivars achievable by intervening in the development or composition of specific tissues and organs. Assuming that an accurate risk and environmental impact assessment is carried out for each individual genetic improvement intervention implemented through the application of new plant breeding techniques, the use of these methods in viticulture could concretely solve specific phytosanitary problems and, therefore, guarantee the safeguarding and competitiveness of grape-related products. To conclude, the economic and environmental sustainability of viticulture requires the design and development of programs that include conventional breeding and the most recently advanced breeding technologies in a synergistic way. A fundamental step to achieve this goal is the recognition of the indispensability of new plant breeding techniques by the national institutions responsible for information on organic farming and competent ministerial bodies (Box 6). Since their purpose is to guarantee a productive rural network that is less invasive from an environmental, economic, and social point of view, the potential use of these new technologies is absolutely in line with their goals.

Supplementary data

The following supplementary data are available at [JXB online](#).

Table S1. List of the eight most cultivated winegrape resistant varieties worldwide.

Author contributions

AV: conceptualization; CP, AV, GM, FP, AM, and SF: writing; GB and ML: supervision of the final manuscript. All authors contributed to editing the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. This manuscript reflects only the authors'

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