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The state-of-the-art of grapevine biotechnology and new breeding technologies (NBTs)

Lorenza Dalla Costa[1], Mickael Malnoy[1], David Lecourieux[2], Laurent Deluc[3], Fatma Ouaked-Lecourieux[2], Mark R. Thomas[4] and Laurent Torregrosa[5*]

[1] Dept. of Biology and Genomic of Fruit Plants, Foundation E. Mach, 38010 San Michele all'Adige, Italy
[2] ISVV-EGFV, CNRS, INRA, Bordeaux University, 33883 Villenave d'Ornon, France
[3] Dept. of Horticulture, Oregon State University, OR 97331, Corvallis, USA
[4] CSIRO Agriculture and Food, Hartley Grove, Urrbrae SA 5064, Australia
[5] AGAP, Montpellier University, CIRAD, INRA, Montpellier SupAgro, Montpellier, France

Corresponding author : laurent.torregrosa@supagro.fr

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ABSTRACT

Context of the review: The manipulation of the genetic basis controlling grapevine adaptation and phenotypic plasticity can be performed either by classical genetics or biotechnologies. In the last 15 years, considerable knowledge has accumulated about the grapevine genome as well as the mechanisms involved in the interaction of the vine with the environment, pests and diseases. Despite the difficulties associated with genetic mapping in this species (allele diversity, chimerism, long generation intervals…), several major QTLs (*quantitative trait loci*) controlling important vegetative or reproductive traits have been identified. Considering the huge genotypic and phenotypic diversities existing in *Vitis*, breeding offers a substantial range of options to improve the performances of cultivars. However, even if marker-assisted selection was largely developed to shorten breeding programs, the selection of improved cultivars, whether for agronomic traits or disease tolerances, is still long and uncertain. Moreover, breeding by crossing does not preserve cultivar genetic background, when the wine industry and market are still based on varietal wines.
Significance of the review: In grapevine, pioneering biotechnologies were set up in the 1960s to propagate and/or clean the material from micro-organisms. In the 1990s, the basis of genetic engineering was primary established through biolistic or *Agrobacterium* with several derived technologies refined in the last 10 years. The latest advance is represented by a group of technologies based on genome editing which allows a much more precise modification of the genome. These technologies, so-called NBTs (*new breeding technologies*), which theoretically do not deconstruct the phenotype of existing cultivars, could be potentially better accepted by the wine industry and consumers than previous GMO (*genetically modified organism*) approaches. This paper reviews the current state-of-the-art of the biotechnologies available for grapevine genome manipulation and future prospects for genetic improvement.

KEYWORDS

grapevine, biotechnologies, gene transfer, genome editing, genetic improvement

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INTRODUCTION WHAT DOES GRAPEVINE BIOTECHNOLOGY MEAN?

The term biotechnology refers to any process of cultivation, multiplication or genetic modification that uses techniques or conditions of implementation that do not exist in the nature (Torregrosa and Bouquet, 1993). This term can therefore be applied to a large number of technologies used to multiply, select elite individuals or modify their sanitary or genetic status. Biotechnologies can be classified into three categories. The first one involves all techniques used for conservation or multiplication (e.g. *in vitro* micropropagation to establish collections of varieties under aseptic conditions). The second category is related to a set of techniques that supports the sanitary (e.g. micrografting) or genetic (e.g. embryo rescue) selection of elite individuals, without causing genetic modification. The last category includes technologies that modify the genome or the epigenome (e.g. somaclonal variation or *in vitro* mutagenesis, genetic transformation or recently genome editing), which can be used to modify gene structure or functioning.

HISTORY OF GRAPEVINE BIOTECHNOLOGIES

The first reports (**Table 1**) dealing with the application of biotechnology of the vine are due to Georges Morel (1944). These early works concerned the *in vitro* culture of tissues under aseptic conditions (Torregrosa *et al.*, 2001; Bouquet and Torregrosa, 2003). As soon as the first *in vitro* culture media were developed, the first applications consisted of defining the conditions of tissue or organ development from pre-existing meristems, for propagation purposes. From the 1970s-80s, these techniques allowed the development of viral sanitation procedures and *in vitro* propagation to be used for the vegetative and genetic improvement of rootstock and scion varieties.

From the 1980s, thanks to advances in the understanding of the hormonal control of plant organogenesis, regeneration techniques were developed to induce the differentiation of shoot meristems from undifferentiated cells. The first

TABLE 1. Grapevine biotechnologies (in bold major advances).

Achievement	Reference
Aseptic tissue culture	Morel (1944)
Micropropagation	**Galzy (1961)**
Isolated meristem culture	Galzy (1972)
Somatic embryogenesis	**Mullins and Srinivasan (1976)**
Adventitious organogenesis	Favre (1977)
Axillary bud proliferation	Jona and Webb (1978)
Adventitious caulogenesis	Rajasekaran and Mullins (1981)
Engineered hairy roots	Guellec *et al*. (1990)
Transgenic vines	**Mullins *et al*. (1990)**
Particle bombardment	Hébert *et al*. (1993)
Agronomic trait manipulation	**Le Gall *et al*. (1994)**
Protoplast technology	Reustle *et al*. (1994)
L1/L2 cell layer dissociation	Franks *et al*. (2002)
Cell suspension expression	Torregrosa *et al*. (2002)
Transgenic organogenesis	Mezzetti *et al*. (2002)
Minimal cassette technology	Vidal *et al*. (2006)
Virus inducing gene silencing	Muruganantham *et al*. (2009)
Microvine transformation	Chaib *et al*. (2010)
CRISPR/Cas9 mutagenesis	**Ren et *al*. (2016)**
DNA-Free genetic edition	**Malnoy *et al*. (2016)**

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somatic regenerations were obtained by adventive organogenesis, i.e. by inducing the development of neo-buds (Barlass and Skene, 1978). In parallel, somatic embryogenesis has also been developed for a large number of *Vitis* genotypes (Martinelli and Gribaudo, 2001). The latter technology, which makes it possible to obtain thousands of somatic embryos from a few hundred mg of calli or embryogenic cell suspensions, forms the basis of genetic transformation or genome editing procedures.

The first publication mentioning the successful gene transfer in grapevine reported the recovery of calli and roots transformed by *Agrobacterium tumefaciens*-derived plasmids (Hemstad and Reisch, 1985). Baribault *et al.* (1989) obtained vegetative organs of Cabernet-Sauvignon ectopically expressing transgenes but could not achieve the regeneration of stable plants. The first transgenic vines were obtained by Mullins *et al.* (1990) who coupled the transformation through *Agrobacterium*-disarmed vectors with the regeneration by somatic embryogenesis of rootstocks. The first attempt to incorporate genes of agronomic interest occurred a few years after by Le Gall *et al.* (1994) who incorporated a gene encoding the coat protein of GCMV (Grapevine Chrome Mosaic Virus) into rootstocks and interspecific *Vitis* x *Muscadinia* hybrids.

Over the years, gene transfer technology has been improved and applied to a range of *Vitis* species (Bouquet *et al.*, 2006 and 2008; Torregrosa *et al.*, 2015). *Agrobacterium*-mediated transformation has also been developed for the microvine (Chaib *et al.*, 2010), a promising model for boosting physiology and genetics studies in grapevine (Torregrosa *et al.*, 2016). However, despite many improvements, the regeneration of non-chimeric transgenic plants remains a long and complex process in many grapevine genotypes. Nevertheless, various alternative techniques using physical vectors or viruses have been developed to obtain ectopic gene expression in individual cells, cell suspension cultures, tissues or non-caulinary organs (Vidal *et al.*, 2010).

More recently, genetic modification techniques have improved significantly with the development of genome editing approaches. These technologies, in particular those based on CRISPR/Cas9, use endonucleases that change gene structure and expression in a more specific and targeted way than previous technologies. For grapevine, a first proof-of-concept was provided by Ren *et al.* (2016) who modified the metabolism of tartaric acid by mutating the L-idonate dehydrogenase (IdnDH) enzyme of Chardonnay. Since then, several research groups have been attempting to develop the technology to induce genetic modifications without the incorporation of viral or bacterial genetic backbones. These new technologies represent interesting prospects for both functional genomics studies and for genetic improvement of grapevine (Dalla Costa *et al.*, 2017). These technologies are detailed in the next sections.

PROPAGATION AND CONSERVATION OF ORIGINAL RESOURCES

The reliable propagation of grapevine genotypes, i.e. without morphogenetic changes, cannot be achieved by somatic regeneration because of high levels of growth regulators that are required to maintain this status also can potentially cause somatic variations or mutations. In addition, by regenerating an individual from a single cell or a small number of cells, the regeneration process modifies the genetic structure of chimeric genotypes (Torregrosa *et al.*, 2011). Thus, *in vitro* propagation is best achieved by microcutting, a technique that does not need the use of plant growth regulators (Galzy, 1961). Each bud develops into a single vegetative axis producing 2-4 phytomers per month. The annual theoretical production can reach 10^3 plants depending on the varieties (**Figure 1**). Another technique so-called axillary micropropagation requires the use of cytokinins to suppress apical dominance and induce axillary shoot proliferations (Silvestroni, 1981). Because the hormonal balance imposed to obtain the axillary proliferation masses inhibits the rhizogenesis, the rooting of the shoots isolated from proliferation masses requires an additional step using an auxin-enriched media (IAA, IAB or NAA). By this technique, it is theoretically possible to obtain from a single bud more than 10^4 plants per year.

The establishment of genetic germplasms maintained by micropropagation is an alternative to the current repositories maintained in greenhouses or outdoors, exposing genotypes to biotic and abiotic risks. However, the major weakness of *in vitro* repositories is the cost of subcultures. To decrease the frequency of sub-culturing by decreasing *in vitro* plant growth,

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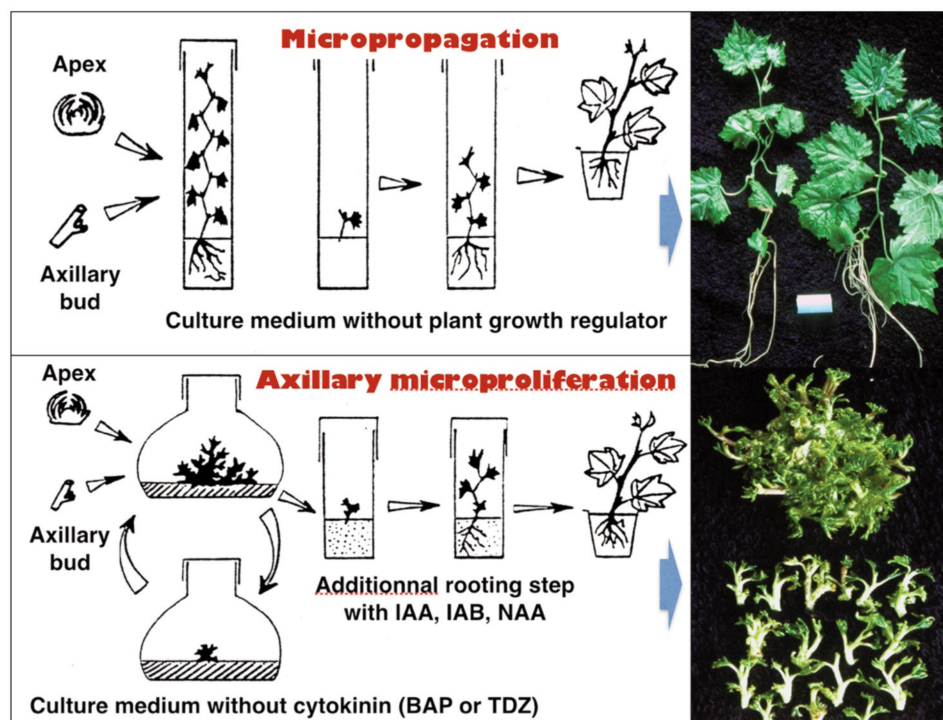

FIGURE 1. Propagation through microcuttings (top) or axillary proliferations (bottom). On the top right, a one-month plantlet developed from a microcutting. On the bottom right, a microproliferation mass before and after shoot separation.

several approaches have been tried, e.g. decreasing temperature conditions, using sugarless culture media or using plant growth inhibitors such as chlorocholine chloride (Galzy, 1985; Harst-Langenbucher and Alleweldt, 1990). All of these systems can be successfully applied to a large cultivar (genotypic) range. In contrast, cryopreservation of apex or buds which has also been investigated stays only applicable to a limited number of genotypes and with a low level of reproducibility (Plessis *et al.*, 1991; Marković *et al.*, 2015).

VEGETATIVE GENETIC OR SANITARY SELECTION

To deliver genetically homogenous and virus-free varieties, most countries perform clonal propagation (Mannini, 2000). For the elimination of viruses, *in vitro* thermotherapy was the first method proposed (Galzy, 1963). Meristem culture and somatic embryogenesis have also been reported as effective methods for the elimination of grapevine viruses (Goussard, 1981; Goussard *et al.*, 1991) and have been shown to be virus strain- and grapevine genotype-dependent. Today, the most widely used method consists of micrografting a shoot apex (200-500 µm) on *in vitro* plant internodes or on hypocotyl fragments of a zygotic or somatic embryo (Bass *et al.*, 1976; Torres-Viñals *et al.*, 2004). Thermotherapy can be performed in combination with meristem culture, apex micrografting or somatic embryogenesis to increase the efficiency of viral elimination (Gribaudo *et al.*, 2006; Maliogka *et al.*, 2015). Some authors have shown that viral load can be reduced during cryopreservation steps (Wang *et al.*, 2003), but this approach is still under investigation. Therefore, there is a range of techniques that can be implemented in clonal and sanitary programs of ancient and/or rare varieties, as a certain percentage of identified selections are systematically infected with one or more virus strains.

In vitro embryo rescue techniques (**Figure 2**) are also used to support the establishment of genetically segregating populations, especially when parents are carrying: i) the seedlessness trait, a maternal fruit character that interferes with embryo development (Ramming *et al.*, 1990) or ii) the *Vvgai1* mutation, which accentuates seed integument dormancy (Chatbanyong and Torregrosa, 2015). The different technologies to induce the development

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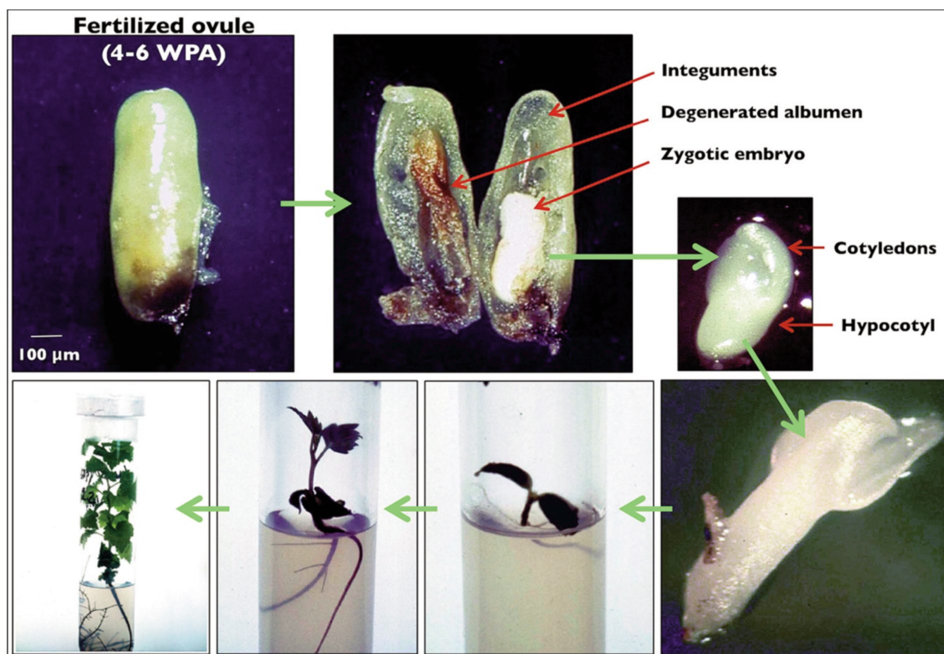

FIGURE 2. Zygotic embryo rescue to recover individuals from crosses between seedless varieties.

of a zygotic embryo extracted from a fertilized ovary are known as embryo rescue (Sharma *et al.*, 1996; Ramming *et al.*, 2000). The effectiveness of embryo rescue can be optimized by selecting specific developmental stages of the fruit after anthesis (Pommer *et al.*, 1995), the type and level of growth regulators (Agüero *et al.*, 2000) and the progenitors (Hewstone *et al.*, 2006). These technologies, which are now commonly used in table grape breeding programs, provide germination percentages of up to 50% (Hewstone *et al.*, 2006). Applied to crosses involving microvine parents, embryo rescue results in a very high rate of germination, in particular when embryos are extracted at an early stage of berry development (Chatbanyong and Torregrosa, 2015).

CELL AND ORGAN TRANSIENT OR STABLE TRANSFORMATION

1. Cloning strategies and tools

Molecular cloning is a fundamental step of genetic engineering. It refers to the process by which DNA/RNA molecules are produced and transformed into a host organism. It typically consists of the following components: i) the DNA fragment of interest to be inserted, and ii) the accepting vector/plasmid backbone that contains all the components for replication in the host. Each component (insert and vector) will go through a series of preparative steps necessary to generate the final cassette (insert + vector) to be transferred into a host organism *via* a transformation procedure. There are several strategies available to either conduct one insertion event or the assembly of several DNA modules (**Table 2**).

Traditional cloning usually refers to the use of restriction endonuclease to generate DNA fragments with complementary end sequences that can be joined together with a DNA ligase enzyme. This usually involves preparing both the insert and the vector by cutting with two unique restriction enzymes. The use of two restriction enzymes that generates two non-compatible ends results in a directional cloning of the insert into the vector, thereby lowering the transformation background for self-ligation events of the vector. The discovery of the *Polymerase Chain Reaction* (PCR) has expanded the use of restriction cloning to ***PCR cloning*** by the introduction of restriction sites necessary for the ligation reaction with the vector at the end of PCR-amplified insert.

In the recent years, the ***seamless cloning*** method has gained scientific attention because it enables sequence-independent insertion of one or more inserts into the vector. The workflow involves PCR to amplify the gene of interest, an exonuclease generating cohesive ends for the insert and vector, and either a ligase or a

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recombinase to join the insert to the vector. The ability to join 5 to 10 fragments in a predetermined order, without any sequence restrictions or artifacts, is a very appealing and powerful tool in the light of synthetic biology. The most popular method is the **Gibson assembly** method developed by Gibson *et al.* (2009). This *in vitro* recombination is a one-step isothermal reaction employing a 5' exonuclease (generation of overhang ends), a polymerase to fill in the gaps of the annealed single-stranded regions, and a DNA ligase to seal the nicks.

The ***Golden Gate assembly*** is another approach of seamless cloning that exploits the use of Type IIS restriction enzymes to cleave DNA outside of the recognition site (Engler *et al.*, 2008 and 2009). There are several advantages to this methodology. First, the overhang sequence created is not dictated by the restriction enzyme, and no additional DNA is introduced. Second, the fragment-specific sequence of the overhangs enables the assembly of multiple fragments in a specific sequential order. Third, the restriction site is eliminated from the ligated product, so the digestion and ligation reactions can take place simultaneously. Both methods (Gibson and Golden Gate Assembly) were recently reported for grapevine as part of a cloning strategy aimed to generate a TAS3-resistant *ARF4* gene construct and an amiRNA (artificial microRNA) targeting the endogenous *ARF4* gene (Gouthu *et al.*, 2018).

For the past decade, ***recombinational cloning*** has become very popular in genetic engineering of plants using either integrases or recombinases that enable transfer of a DNA fragment from one vector to another without the use of restriction enzymes and ligases. Multi-round Gateway technologies (recombinase) can produce the delivery of multiple transgenes through multiple reactions (Vemanna *et al.*, 2013) but they leave a 21-bp DNA artifact between building blocks. Yet, the combination Golden Gate Entry Vector along with a Gateway Recombination system is becoming extremely useful as part of multiplexed Plant Genome Editing and Transcriptional Regulation approaches employing CRISPR/Cas9 (Lowder *et al.*, 2015).

As generally conceived, the Golden Gate technology was not reusable and thereby not useful for multigene engineering (***modular cloning***). Two new strategies for DNA assembly were created to enable the reusability for instance of the Golden Gate cloning scheme: Moclo (Weber *et al.*, 2011) and GoldenBraid (Sarrion-Perdigones *et al.*, 2011). Both strategies use the Golden Gate Property to build transcriptional units (TUs) and design destination vectors that enable the TUs to be assembled among them. Those two strategies are advantageous for the development of modular assembly systems in plant synthetic biology as long as common standards are adopted by the community so that the building blocks can be shared by as many users as possible (Vazquez-Vilar *et al.*, 2017).

Finally, ***Ligation Independent Cloning*** (LIC) is an alternative technique to restriction enzyme/ligase cloning (Aslanidis and de Jong, 1990). Inserts are PCR amplified and vectors are

TABLE 2. Summary of the physical and chemical DNA delivery methods (from Cunningham *et al.*, 2018).

Delivery method	Adverse effects	Targets	Cargo types	Limitations
Physical methods				
Biolistic or particle-mediated delivery	• Damage to target tissue & cargo • Low penetration depth • Random integration	Calli, embryos, leaves	DNA, siRNA, miRNA, ribonucleoproteins, large size	• Effectiveness highly species and cultivar specific • Targeting leaves limits time to observe delivery effects • Targeting embryos requires laborious regeneration procedure
Electroporation	• Damage to target tissue • Non-specific transport of material through pores • May lead to improper cell function	• Protoplasts • Meristems • Pollen grains	Nucleic acids (DNA, siRNA, miRNA)	Limited cargo-carrying capacity
Chemical methods				
PEG-mediated delivery	High density can induce cytotoxicity.	• Species amenable to protoplast regeneration • Protoplasts	Nucleic acids (DNA, siRNAs, miRNAs)	Regeneration is inefficient for most species in transient studies

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made linear either by restriction enzyme digestion or by PCR. The use of 3’ → 5’ exonuclease activity of T4 DNA Polymerase creates overhangs with complementarity between the vector and insert. The incorporation of dGTP in the reaction limits the exonuclease processing to the first complementary C residue, which facilitates the generation of sticky ends PCR products complementary to the vector. Joined fragments (insert+vector) have four nicks that are repaired by *E. coli* during transformation. This technique allows efficient creation of recombinant plasmids without the introduction of DNA artifacts. One particular variation of the ligation independent cloning - the SLIC method for *Sequence and Ligation independent cloning* - has been adopted by many researchers. As many as five inserts can be assembled in one reaction simultaneously with great efficiency (Li and Elledge, 2012). More marginal but still efficient is the *Uracil-Specific Excision Reagent Fusion* (Geu-Flores *et al.*, 2007), which consists of the use of PCR primers that contain a single deoxyuridine residue near the 5’ end. Subsequent treatment of the PCR products with deoxyuridine-excision reagents generates long 3’ overhangs designed to complement each other and thereby facilitating their fusion. The combination of this approach with improved cloning techniques has led to the development of an efficient toolbox for transformation in cereals (Hebelstrup *et al.*, 2010).

4.2. Physical and chemical delivery methods

Biolistic, also called particle bombardment (**Table 3**), is a physical method developed in the 1980s intended to deliver into plant cells microparticles (gold or tungsten) coated with a genetic cargo containing the genetic information of interest (Klein *et al.*, 1987). Unlike *Agrobacterium*-mediated transformation, biolistic delivery can result in transformation of nuclear, plastidial, or mitochondrial genomes. However, its effectiveness is highly dependent on the species/cultivars, making its impact very limited. The nature and the size of the genetic cargo have evolved along with technology advances of genetic engineering. DNA, siRNAs (small interfering RNAs), miRNAs, ribonucleo-proteins (RNPs) have been demonstrated to be efficiently transferred through biolistic methods although plasmid DNA remains the most common genetic material transferred through this mode.

Biolistic experiments were conducted in grapevine in the late 1990s and early 2000s. In 1996, Kikkert and colleagues reported on the first generation of transgenic grapevine plants using the *Vitis* Hybrid Chancellor. Later on, Vidal *et al.* (2003) adapted a reliable and efficient system for stable transformation and regeneration of ‘Chardonnay’ plants using microprojectile bombardment. In grapevine, the biolistic method was also found to be extremely popular for transient assays in various plant materials (cell suspension culture, leaf sections, and somatic embryos). Promoter analyses in Cabernet Sauvignon suspension cells were successful in studying the effect of anaerobiosis on the regulation of *VvAdh* gene expression (Torregrosa *et al.*, 2002). Co-transformation experiments using GFP (*green fluorescent protein*), GUS (*beta-glucuronidase*) and Luciferase for the validation of protein-DNA interactions were routinely conducted to examine the trans-activation of MYB and MYC transcription factors on promoters of several flavonoid-related genes (Bogs *et al.*, 2007; Walker *et al.*, 2007; Deluc *et al.*, 2008; Czemmel *et al.*, 2009; Hichri *et al.*, 2010). More recently, promoter deletion analysis of *VitViGIP1* was performed on leaf discs and somatic embryos and revealed the core promoter of this gene and several other cis-acting regulatory elements (Joubert *et al.*, 2013).

Electroporation - Initially developed for protoplast transformation, this technology was expanded to intact plant cells in rice and wheat (Shimamoto *et al.*, 1989; He *et al.*, 1994). The principle requires the application of a strong electric field to cells that creates temporary pore in cell membranes, thereby facilitating the uptake of the genetic cargo into the cell. In the past decade, electroporation protocols for plant transformation have been optimized and standardized for several plant species (including tobacco, rice, wheat, and maize) using commercially available electroporators (Barampuram and Zhang, 2011). Although the methodology is fast and inexpensive, it has some constraints such as the thickness of the cell wall, and the impact of the strong electric field pulse that can damage the structure of the delivered gene by creating inaccurate translational end products (Rakoczy-Trojanowska, 2002). To date, there is no study using this method in grapevine genetic studies.

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TABLE 3. Advantages and disadvantages of cloning and assembly methods.

Cloning method	Advantage	Disadvantage
Traditional	Low cost Versatile Directional cloning	Sequence constraints due to presence and/or translation of restriction site
PCR	High efficiency Amenable to high throughput	Limited vector choices Higher cost Lack of sequence control at junction Directional cloning may be difficult
Seamless	The overhang sequence not dictated by the restriction enzyme Allows for orderly assembly of multiple fragments simultaneously	Low cloning efficiency may require the use for ultracompetent chemically competent cells
Recombinational	Allows high-throughput vector creation Widely available ORF collections	Relatively more expensive compared to traditional methods Vector sets defined by supplier
Ligation-independent Cloning	Low cost Many different vector choices	Some type of sequence modifications not possible

PEG-mediated transformation uses an inert hydrophilic polymer of ethylene oxide that can help the transfer of DNA into protoplasts. In this approach, DNA molecules are directly incubated with protoplasts and the transfer is initiated by the addition of divalent cations to the mixture. The addition of PEG to the protoplast mixture destabilizes the permeability of the plant membrane and allows free DNA to enter the plant cytoplasm. On the one hand, the lack of an efficient technique and methodology to regenerate whole plants from protoplasts renders the use of PEG-mediated delivery system for mature plant transformations marginal. On the other hand, it has been extensively used for transient assays in plants to validate gene function. In grapevine, PEG-treated protoplasts from Cabernet-Sauvignon cell suspension cultures were used to study protein subcellular localization (Hichri *et al.*, 2010), promoter analysis (Saumonneau *et al.*, 2012), protein/protein (Saumonneau *et al.*, 2008) and DNA/protein interactions (Marchive *et al.*, 2013). More recently, PEG-mediated transformation of Chardonnay protoplasts was exploited as preferential method for the direct delivery of purified CRISPR/Cas9 RNPs (Malnoy *et al.*, 2016).

3. *Agrobacterium*-derived technologies

Among the different plant genetic transformation methods, *Agrobacterium*-mediated technologies are described as powerful tools for delivering genes of interest into a host plant. Despite being technically challenging, these *Agrobacterium*-mediated approaches are still preferred for transgenic plant production, as they present several advantages: they allow i) the transfer of large and intact DNA segments, ii) the insertion of simple transgenes with defined ends and low copy number, iii) stable integration and inheritance, and iv) consistent gene expression over generations (Barampuram and Zhang, 2011).

Agrobacterium-mediated transformation methods are divided into two categories: stable transformation and transient transformation. Stable transformation occurs when the DNA transferred inside the plant nucleus is inserted into the plant genome for inheritance to subsequent generations. Alternatively, transient transformation refers to the situation in which the foreign DNA transiently remains in the nucleus without being integrated into the plant genome but is transcribed to express the genes of interest. In grapevine, these *Agrobacterium*-mediated gene transfer methods were developed in the early 1990s (Baribault *et al.*, 1989) and underwent continuous improvement to allow the characterization of the function and regulation of several genes (**Table 4**).

The main *Agrobacterium* strain used for grape transformation is *A. tumefaciens*, but the use of

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TABLE 4. Examples of Agrobacterium-mediated transformation assays (modified from Jelly *et al.*, 2014).

Method	Tissue	Cultivar	Strain	Application	Genes/seq.	References
Syringe infiltration	Leaves of *in vitro* plantlets	Superior seedless	AGL1, GV3101	Gene silencing	hpRNA against *VvPDS*	Urso *et al.* (2013)
Vacuum infiltration	Leaves of *in vitro* plantlets	Thompson seedless	C58C1 (pCH32)	Overexpression	D4E1 (synthetic AMP)	Visser *et al.* (2012)
		Cabernet Franc, Syrah, Zinfandel	EHA105	Viral vector engineering	GLRaV-2 cDNA	Kurth *et al.* (2012)
	Detached leaves of *in vitro* plantlets	Cabernet Franc	GV3101	Gene silencing	hpRNA against *VvPGIP1*	Bertazzon *et al.* (2012)
		Cabernet-Sauvignon, Cinsault, Muscat, Carignane	C58C1 (pCH32)	Overexpression	*VvVST1*	Santos-Rosa *et al.* (2008)
			GV3101	Overexpression	*VpGLOX*	Guan *et al.* (2011)
				Overexpression	*VpPR10.2*	He *et al.* (2013)
				Promoter analysis	*VpSTS*	Xu *et al.* (2010)
			LBA4404	Overexpression	*VpPR10.1*	Xu *et al.* (2014)
	Leaves	Grenache	GV2260	Viral vector engineering	GLRaV-2 cDNA	Liu *et al.* (2009)
		Syrah	GV3101	Overexpression	*VvNPR1*	Le Henanff *et al.* (2009)
Agro-drenching	Roots of *in vitro* plantlets	Prime, Thompson seedless	EHA105	Viral vector engineering	GRSPaV cDNA	Meng *et al.* (2013)
		Prime	EHA105	Viral vector engineering	GVA cDNA	Muruganantham *et al.* (2009)
Co-cultivation	Cell suspension	Gamay Red	EHA105	Promoter analysis	*VvDFR*	Gollop *et al.* (2002)
	Somatic embryos	Chardonnay	GV3101	Ami-RNA validation (cotransformation)	amiRNAs against *Grapevine fanleaf virus* and GUS sensor CaMV35S, CsVMV,	Jelly *et al.* (2012)
		Thompson seedless	EHA105	promoters testing	*At* ACT2 promoters	Li *et al.* (2001)
		Thompson seedless	EHA105	promoter testing	BDDPs with CaMV35S, CsVMV promoters and enhancers	Li *et al.* (2004 ; 2011)
					31 grapevine	Li *et al*. (2012)
		Freedom	EHA105	Overexpression	*VvCBF4*	Tillet *et al.* (2011)
					VvSK1	Lecourieux *et al.* (2010)
		41B	EHA105	Overexpression	*VvCEB1*	Nicolas *et al.* (2013)
					VvABF2	Nicolas *et al.* (2014)
		Chardonnay, Thompson seedless	EHA101	Overexpression	Pear *PGIP*	Agüero *et al.* (2015)
		Thompson seedless	GV3101	Overexpression	*VpSTS*	Fan *et al.* (2008)
					VpPUB23	Zhou *et al.* (2014)

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A. rhizogenes strains to transform roots (hairy roots) also provides an interesting system to perform functional studies (Hu and Du, 2006). The most frequently *A. tumefaciens* strains used for grapevine transformation are EHA105 (Hood *et al.*, 1993), GV3101 (Koncz and Schell, 1986) and C58C1 (Hamilton *et al.*, 1996).

Stable transformation methods **-** In grapevine, stable transformation is a long and sometime difficult process. Its efficiency strongly depends on the genotype, the explant source, the medium composition and the transformation method used. Co-cultivation of somatic embryos with *A. tumefaciens* is the most commonly used method to stably transform grapevine. It was largely exploited to achieve proof-of-concepts (Torregrosa *et al.*, 2002; Chaib *et al.*, 2010; Ren *et al.*, 2016) and functional characterization of putative key genes (Lecourieux *et al.*, 2010; Li *et al.*, 2012; Nicolas *et al.*, 2013, 2014; Pessina *et al.*, 2016; He *et al.*, 2018; Sun *et al.*, 2018).

Because regeneration of transgenic plants from stably transformed embryos is long, gene function can also be addressed using certain plant organs. In this regard, transgenic hairy roots induced by *A. rhizogenes* provide a powerful system for functional studies (Hu and Du, 2006; Gomez *et al.*, 2009; Terrier *et al.*, 2009; Höll *et al.*, 2013) as their production takes only a few weeks (Torregrosa and Bouquet, 1997).

Non-embryonic cell cultures can also be stably transformed by *Agrobacterium*. This was shown to be particularly interesting for the production of bioactive compounds (Martínez-Márquez *et al.*, 2015). To improve *Agrobacterium*-mediated transformation efficiency of dedifferentiated *V. vinifera* cv Monastrell cells, Chu *et al.* (2016) used the Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT) method that was previously developed for soybean transformation (Trick and Finer, 1997). This method significantly increases transformation efficiency (Chialva *et al.*, 2016).

Transient transformation methods **-** To rapidly investigate unknown gene function, several transient transformation systems have been developed. These assays are faster and convenient alternatives to the time-consuming stable transformation method. They mainly include *Agrobacterium*-mediated transient transformation and direct gene transfer by biolistic bombardment, electroporation or protoplast transfection. Janssen and Gardner (1990) showed that a short period of co-cultivation with *A. tumefaciens* led to the active transcription of many copies of the transgene in the mesophyll cells. In grapevine, this technology was successfully used to monitor elicitor-induced calcium changes by overexpressing apoaequorin in *V. vinifera* cv. Gamay cells (Vandelle *et al.*, 2006; Aziz *et al.*, 2007). Other methods exist: i) *A. tumefaciens* can be infiltrated into plant leaves using a needleless syringe to inject the *Agrobacterium* suspension through the stomata (Zottini *et al.*, 2008), or ii) the leaves (or entire plant) can be flooded with the bacterial suspension under vacuum pressure to facilitate liquid penetration into the cells. This second method has the advantage of allowing gene expression in the whole leaf (or plant). A number of studies have reported the successful use of vacuum-infiltration experiments either with detached leaves (Santos-Rosa *et al.*, 2008; Zottini *et al.*, 2008; Le Henanff *et al.*, 2009; Xu *et al.*, 2010; Guan *et al.*, 2011; Bertazzon *et al.*, 2012; He *et al.*, 2013; Xu *et al.*, 2014) or with entire plants (Kurth *et al.*, 2012; Visser *et al.*, 2012). The success of agroinfiltration experiments was shown to be cultivar dependent (Santos-Rosa *et al.*, 2008).

For some particular cases, other methods were developed. An ***agrodrenching method*** was developed using roots of young *in vitro* grapevine plantlets to deliver an infectious viral cDNA clone of GVA that could not be inoculated using leaf agroinfiltration (Muruganantham *et al.*, 2009). This method has been validated for Prime and Thompson seedless grapevine cultivars (Muruganantham *et al.*, 2009; Meng *et al.*, 2013). As the production of transgenic fruits after grapevine transformation takes time, berry agro-injection was developed to shorten the time needed to study gene function in fruits. This method is derived from the transient methodology successfully applied to tomato fruit (Orzaez *et al.*, 2006) based on *Agrobacterium* cultures injection through the fruit stylar apex using a syringe with needle. Using this transient overexpression technique in the "Kyoho" grape fruit, Gao *et al.* (2018) showed the involvement of the ABA Receptor VlPYL1 in the regulation of anthocyanin accumulation in grape berry skin. Similarly, Sun *et al.* (2017) showed that *VvVHP1;2* (*vacuolar H^+-PPase 1*) over-expression promoted anthocyanin accumulation

TABLE 5. Potential plant virus-based vectors suitable for VIGS in grapevine.

Virus	Virus type / Genus	Agroinoc. of viral vector	Infectivity / Recomb. virus	*V. vinifera* varieties	Reporter gene	VIGS Transcription	Reference
Grapevine virus A (GVA)	RNA / *Vitivirus*	Agro-drenching of roots	Yes / Leaves	Prime	*GFP*	*PDS* silencing	Muruganantham *et al.* (2009)
Grapevine leaf rool-associated virus-2 (GLRa-V2)	RNA / *Closterovirus*	Vacuum agroinfiltration of the whole plantlets	Yes / Leaves	Infectivity with Syrah and Cab. Franc.	*GFP and GUS*	*PDS* and *ChlI* silencing	Liu *et al.* (2009) Kurth *et al.* (2012)
Grapevine rupestris stem pitting-assoc. virus (GRSPaV)	RNA / *Foveavirus*	Agro-drenching of plantlet's roots	Yes / Roots	Prime Thomson	*GFP*	nd	Meng *et al.* (2013)
Grapevine Algerian latent virus (GALV)	RNA / *Tombusvirus*	Vacuum agroinfiltration of plantlets	Yes / Leaves	Syrah Brachetto Nebbiolo Sultana Corvina	*GFP* (*using N. benthamania*)	*ChlH* (using *N. benthamania*)	Lovato *et al.* (2014) Park *et al.* (2016)

in berry skins. These reports underline the possible application of the transient overexpression technique for studying grapevine fruit development.

4. Virus-derived technologies

An alternative approach to perform functional studies in grape is the use of viral vectors (**Table 5**). For a long time, modified plant viruses have been used for several purposes including the transient expression of heterologous proteins (with protein size limitations according to the viral system used) or the silencing of host genes (known as Virus Induced Gene Silencing or VIGS).

VIGS requires engineered infectious viral clones that generate double-stranded RNA resulting in post-transcriptional silencing of a target gene as part of the plant's own defense response against invading virus (Baulcombe, 1999). VIGS is one of the most widely used genomic tools, displaying several advantages such as allowing easy and rapid gene silencing, with no need for stable plant transformation, and enabling both forward and reverse genetic approaches in many crop species (Senthil-Kumar and Mysore, 2011). VIGS has been successfully used to better define the role of genes involved in various plant processes including fruit development (Fernandez-Moreno *et al.*, 2013) or abiotic stress tolerance (Ramegowda *et al.*, 2014).

The majority of the available plant virus-based vectors displays a host range mainly restricted to annual or herbaceous plants. In this context, several grapevine infectious viral vectors were engineered, including those based on the *Vitivirus Grapevine Virus A* (GVA) (Muruganantham *et al.*, 2009), the *Closterovirus Grapevine leafroll-associated virus-2* (GLRaV-2) (Kurth *et al.*, 2012), and the *Foveavirus Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Meng *et al.*, 2013). Generation of recombinant GFP-expressing viral cDNA clones inserted into binary plasmids allowed the agroinoculation of different grapevine cultivars and highlighted the infectivity of these viral constructs by GFP visualization. Four weeks after agroinoculation, the virus was detected in the *V. vinifera* infected plantlets regardless of the viral-derived vector used. GVA-GFP and GRSPaV-GFP fluorescent signals were detected in plantlet leaves and roots, respectively (Muruganantham *et al.*, 2009; Meng *et al.*,

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2013). In the case of GLRaV-2, infected cells appeared first in the stem phloem and then colonized all plant organs including berries, thus revealing a systemic virus expression propagating through the phloem. Interestingly, the GLRaV-2-derived vector infection was shown to be transmitted by grafting to a wide range of grape varieties and to be genetically highly stable within infected plants that express the exogenous transmitted sequence up to 3-years post-inoculation (Dolja and Koonin, 2013). GVA- and GLRaV-2-mediated transcriptional gene silencing efficiency was demonstrated using derived vectors harboring fragments of targeted host genes. Thus, virus-induced silencing of endogenous *PDS* (*phytoene desaturase*) or *ChlI* (*subunit I of magnesium-protoporphyrin IX chelatase*) was observed in infected grapevines, displaying leaf bleaching symptoms resulting from the loss of chlorophyll (Muruganantham *et al.*, 2009; Kurth *et al.*, 2012). The capacity of such grapevine specific virus vectors to silence genes in particular organs (i.e. roots and berries), or in specific tissues (i.e. fruit skin) remains an open question.

This challenge may explain, at least in part, the lack of functional studies using these grape infectious viral vectors. Other viral vectors like *Tobacco rattle virus* (*TRV*) and *Apple latent spherical virus* (*ASLV*) vectors can provoke VIGS in many plant species (Senthil-Kumar and Mysore, 2011) but not in grapevine, as recently confirmed by Gao *et al.* (2018) who failed to silence *VlPYL1* (*pyrabactin resistance/PYL* (*PYR-like*)) using the TRV-mediated silencing in "Kyoho" grapes. To the best of our knowledge, only one viral-derived vector was successfully used to perform functional studies in grapevine. In 2007, Peretz and co-workers engineered the IL-60 system derived from the geminivirus *Tomato yellow leaf curl virus* (TYLCV). IL-60 was defined as a universal DNA plant vector system providing efficient expression or silencing in numerous plant species (Peretz *et al.*, 2007). Ten years later, Sun *et al.* (2017) successfully exploited the IL-60 viral transient expression system to overexpress *VvVHP1 (vacuolar H^+-PPase 1)* in "Kyoho" berries. However, the efficiency of IL-60 system to silence grape genes remains to be demonstrated.

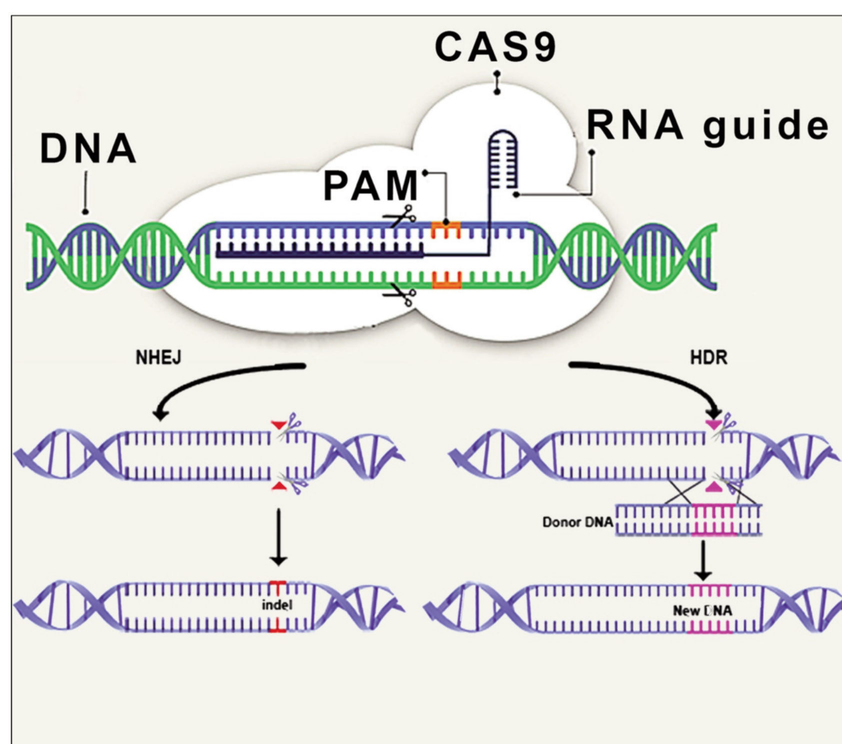

FIGURE 3. Scheme showing the functioning of CRISPR-Cas9 nuclease. PAM=protospacer adjacent motif; NHEJ=non-homologous end joining; HDR=homology directed repair.

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Recently, viral-based vectors have been developed as promising tools for plant genome engineering including the use of genome editing (Zaidi and Mansoor, 2017). Gene targeting (GT) efficiency relies on the delivery method of GT reagents (sequence specific nucleases and repair templates) to plant cells. In this way, autonomously replicating virus-based vectors have been demonstrated as efficient means of delivering GT reagents into plants. Plant RNA viruses like the *potato virus X* (PVX) and the *tobacco rattle virus* (TRV) were used for delivering ZFN nucleases (Marton *et al.*, 2010) or the CRISPR guide RNAs (gRNA) (Ali *et al.*, 2015). Using the CRISPR-Cas system combined with the *bean yellow dwarf virus* (BEyDV) rolling circle replicon, Dahan-Meir *et al.* (2018) optimized a method to target mutagenesis and to replace genes in tomato, without any selection marker or reporter genes. The single viral-derived construct described in this study contains both the CRISPR-Cas9 and a gemini viral replicon system, as well as a guide RNA and a donor fragment that can be exchanged in a modular manner. This makes the present viral-derived vector an interesting tool that could potentially be adapted to grapevine.

5. Genome editing

The term 'New Breeding Technologies (NBTs)' comprises several techniques having different purposes and methodologies. The best known and the one which can allow precise genetic changes is genome editing. The application of programmable nucleases for genome editing has been one of the greatest scientific breakthroughs in the field of plant genetic engineering (Kim and Kim, 2014). These nucleases are enzymes which cut the DNA at specific desired sites in the genome. The induced double stranded breaks (DSBs) are then repaired by the cell natural repair mechanism, either non-homologous end joining (NHEJ), which may introduce nucleotide variation, or homologous recombination (HDR) when a donor DNA with homologous arms is present. The main achievable outcomes are gene knockout or gene replacement (**Figure 3**).

5.1. State-of-the art of the NBTs

Three major classes of programmable nucleases for precision genome editing have been used over the past 15 years: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspersed short palindromic repeats (CRISPR) with the associated nuclease Cas9. The CRISPR/Cas9 system is the most powerful gene editing technique now available due to its simplicity of application and effectiveness (Puchta and Fauser, 2014). Originally discovered as a part of adaptive immunity in *Streptococcus pyogenes*, CRISPR/Cas9 technology has been exploited to introduce desired mutations in genes of interest in medical, plant and animal studies. It operates through guide RNAs composed of a spacer, complementary to a desired DNA sequence, and a scaffold forming complex with Cas9 (van der Oost *et al.*, 2014). The Cas9/guide RNA complex scans the genome searching for complementarity by unwinding double stranded DNA. The unwinding process, prerequisite for checking and finding the complementary target site, is allowed by the protospacer-adjacent motif (PAM) recognized by the nuclease. Once the right target has been found, the nuclease can generate a DSB with the effect of introducing insertion/deletion (INDEL) mutations in the specific gene sequence. In a recent paper, Jones and colleagues (2017) evaluated the kinetics of Cas9 nuclease by measuring the timeframes needed for unwinding double stranded DNA and for checking complementarity to the guide RNA in living *E. coli*. They found that each potential target - each region with a PAM site (for SpCas9 is the triplet NGG) - is bound for less than 30 milliseconds allowing to find a specific single target sequence in about six hours.

The application of these new technologies may be particularly useful in grapevine since they produce minimal and precise modifications in selected genotypes of interest, such as elite cultivars sought-after by the wine market, without altering the genetic background as happens in traditional breeding. CRISPR/Cas9 technology has been successfully applied to generate edited grapevine plants (Osakabe *et al.*, 2018). Two different delivery systems have been used: one based on the stable integration of the genetic components in the genome by means of *A. tumefaciens* gene transfer and the second one based on the direct delivery of purified Cas9 protein and gRNAs (**Figure 4**). Ren *et al.* (2016) transformed 'Chardonnay' embryogenic cell masses to gain point mutations in the L-idonate dehydrogenase gene. They were able to regenerate plants with an altered production of tartaric acid and vitamin C. In 2017, 'Neo Muscat' somatic embryos were transformed with

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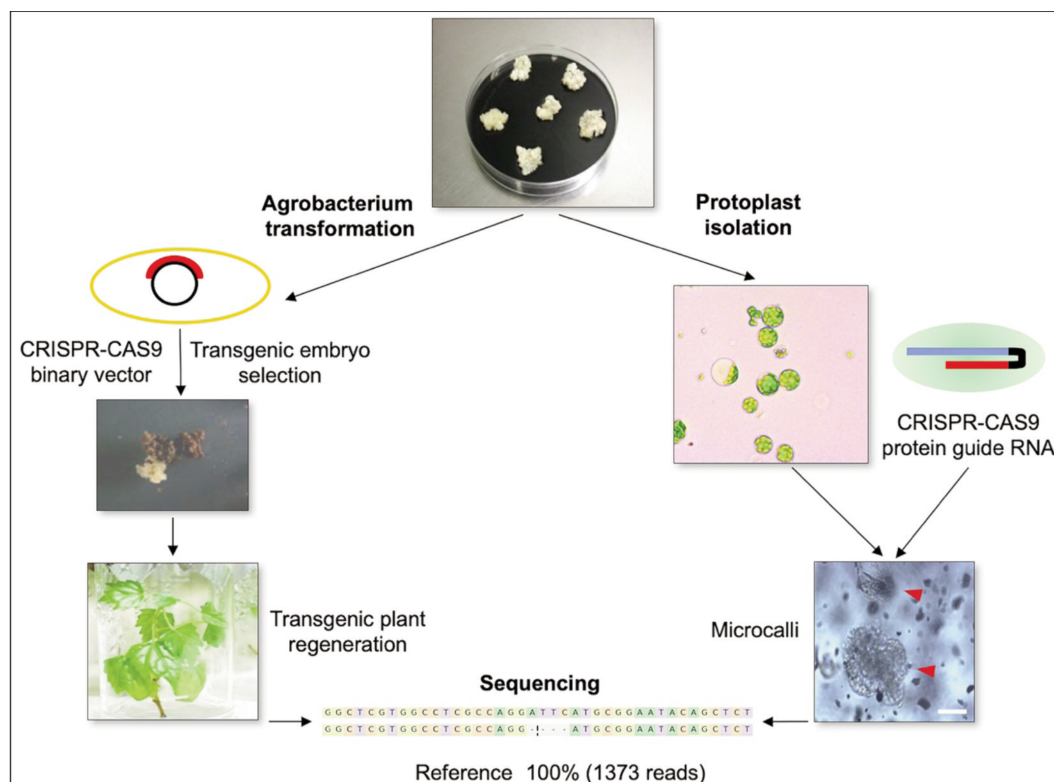

FIGURE 4. Current options to perform genome editing with grapevine using CRISPR-Cas9 nuclease.

a CRISPR/Cas9 editing construct targeting the phytoene desaturase gene and plants with albino leaves were produced (Nakajima *et al.*, 2017). Transgenic 'Thompson Seedless' plants have also recently been produced with mutated versions of the WRKY52 transcription factor gene under both mono- and bi-allelic conditions (Wang *et al.*, 2018). In an attempt to produce non-transgenic edited grapevines, Malnoy and colleagues (2016) directly delivered the purified Cas9 and gRNAs into 'Chardonnay' protoplasts generating edited protoplasts, but not whole plants. However, the rate of protoplasts presenting a mutation was relatively low, around 0.5%.

5.2. Technical challenges for NBTs

The main issues limiting the potential of the commonly used *S. pyogenes* Cas9 are: i) the occurrence of off-target editing which are highly undesired, ii) the requirement of a specific PAM site (NGG) adjacent to the target site which limits the number of potential targets and iii) the large size of the nuclease (approximately 160 KDa) which may hamper the delivery of the editing machinery via endonuclease complexes or by viral particles. Accordingly, technological improvements of SpCas9 have been focused on three main goals: increasing accuracy and specificity of targeting, expanding the set of PAM sites recognized by the nuclease, and reducing the molecular size. At present, many Cas variants more specific, accurate and able to recognize different PAM sequences have been obtained by mutagenizing key amino acids of SpCas9. As well, several SpCas9 orthologues were found in other bacterial species which show different PAM site specificities and smaller size (Cebrian-Serrano and Davies, 2017).

Another line of research is the methodological improvement for the delivery of CRISPR/Cas machinery in plant cells. This is an important line of research since the availability of methods which avoid the incorporation of foreign DNA in the host genome could be crucial in paving the way for the exemption of genome editing products from GMO legislation. Recent developments and techniques in the field of DNA-free genome editing are summarized in Metje-Sprink *et al.* (2019) and they rely mainly

on the delivery of protein-RNA complex (RNP) to protoplasts by electroporation, polyethylene glycol (PEG) vesicles, biolistics (see paragraph 4.2). The main pitfalls of these approaches in comparison to classical transformation system based on *A. tumefaciens*, are the low editing efficiency, the lack of a selection phase to enrich for positively edited plants and the need of sequencing for screening. On the other hand, new approaches are being evaluated for the elimination of exogenous DNA when classical *Agrobacterium*-mediated transformation is used. In grapevine, if the cultivar genome is to be preserved then backcrossing and screening of the progeny is not a feasible strategy and methods for site-specific DNA cassette elimination should be considered and tested (Dalla Costa *et al.*, 2019).

Future prospects for grapevine genetic improvement by means of the CRISPR/Cas system may concern the optimization of the targeted insertion of donor DNA at the cleavage site driven by homology (knock-in strategy). At the moment the knock-in approach remains very challenging compared to gene knock-out (nucleotide mutations at the cleavage target site) but would be very useful for targeted gene insertion or allele replacement. The donor DNA can contain an allelic variant with a loss or gain of function compared to the targeted allele or could be a gene deriving from wild relatives which can confer specific pathogen resistance. A visionary objective is the introgression in elite cultivars of multiple disease resistance genes or polygenic quality traits as well as the modulation of important metabolic pathways by using multiple guide RNAs and multiple donor DNA simultaneously but this ambitious goal will require a long time and strong technological progress. Finally, CRISPR/Cas technology may also be used to produce genetic variability for studying plant biology and for obtaining new allelic variants with agronomic/qualitative advantages.

6. Regulatory framework and prospects for NBTs

Development in genome editing technologies can decrease the cost and time required to improve agronomic as well as horticultural crops in the future, but the broad adoption of NBT requires government support in setting up an updated regulatory framework (Ma *et al.*, 2018). National policies concerning NBT products for feed and food will probably play a key role in determining their success in the foreseeable future. Two opposite attitudes towards green biotechnologies may be observed: one is focused on the final product while the other pays more attention to the process through which a specific product has been generated. The first approach is followed by USA, Argentina, Australia and Brazil which have established that if no foreign genes or genetic material is present in a genome-edited variety, then it will not be subject to additional regulatory oversight and risk

TABLE 6. Main principles underpinning the European legislation on GMO.

Principle	Legislative act	Requirement
Authorization	Dir. 2001/18/EC Reg. (EC) No 1829/2003 Dir. (EU) 2015/412	The applicant must present a request for GMO authorization to the Member State which will transmit it to EFSA (European Food Safety Authority) for the risk assessment. EFSA's opinion will be submitted to European Commission for the final decision. Authorizations are valid for a maximum of 10 years (renewable) Member States have the possibility to restrict or prohibit GMO cultivation on their territory
Traceability	Reg. (EC) No 1830/2003	GMOs and products produced from GMOs must be traced at all stages of their placing on the market. The information "the product/ingredient consists of or contains or is produced from GMOs" must be transmitted to all the operators receiving the product along the production and distribution chains GMOs information must be recorded in a public available register
Labelling	Reg. (EC) No 1829/2003 Reg. (EC) No 1830/2003	Specific product labelling is required when GM material > 0.9% of the food ingredient/feed material while labelling is not compulsory when GM material ≤ 0.9%, provided that this presence is adventitious or technically unavoidable

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assessment as in the case of GMO (Eriksson *et al.*, 2019). The second approach has been historically adopted by Europe, where, on 25th July 2018, the Court of Justice of the European Union ruled that organisms obtained by mutagenesis (including genome editing) are GMO within the scope of the GMO European Directive 2001/18/EC (http://curia.europa.eu/juris/document/document.jsf?text=&docid=204387&pageIndex=0&doclang=EN&mode=req&dir=&occ=first&part=1&cid=747443). This decision strongly reaffirms the precautionary principle and forces gene-edited plants with small and targeted nucleotide modifications to go through the very long and expensive regulatory process intended for classical transgenic plants and defined by the Regulations (EC) 1829/2003 and (EC) 1830/2003. This legal framework relies mainly on the safety assessment of the highest possible standards, full traceability throughout the production/distribution chain and labelling requirement (**Table 6**).

A crucial point concerns the requirement of an analytical method for the unambiguous identification of the specific genetically modified organism. In the case of genome edited organisms, nucleotide mutations produced by a gene-editing mechanism might be indistinguishable from the naturally occurring ones (or from those induced chemically or by irradiation) by means of the current analytical techniques available (Grohmann *et al.*, 2019). It follows that a method allowing to unequivocally trace the origin of this mutation cannot be provided as it cannot be excluded that the identical DNA alterations occurred already spontaneously, were introduced by random mutagenesis or were/will be created in an independent editing experiment. This uncertainty will have consequences for enforcement of the GMO legislation (ENGL, 2019).

The scientific community (upon the initiative of VIB, a life science research institute based in Belgium) expressed its opposition to the ruling of the Court of Justice, to safeguard genome editing from GMO legislation. A hundred of European research centers (University and public and private institutes) have signed an important letter which has been submitted to the President of the European Commission, Jean-Claude Juncker on 24th January 2019 (www.vib.be/en/news/Documents/Position%20paper%20on%20the%20ECJ%20ruling%20on%20CRISPR%2012%20Nov%202018.pdf). In this document, scientists claim that regulating genome editing as GMOs will have negative consequences for agriculture, society and economy in Europe and hope that organisms containing small genetic alterations and which do not contain foreign genes will not be subject to the provisions of the EU GMO Directive but instead will fall under the regulatory regime that applies to classically bred varieties. As observed by Tyczewska *et al.* (2018) the EU has incurred large investment costs in research as well as in NBTs that should promote competitiveness of European agriculture. The huge development of analytical tools over the last years could likely help to get out of the Manichaean diatribe "product vs. process". At present, we have the technology and the knowledge to deeply evaluate the plant that has been endowed with new trait, at both phenotypic and genomic level. Regarding this last aspect, next generation sequencing (NGS) platforms and bioinformatics can make whole genome sequencing possible in short times and with low costs, allowing to select the proper and safer plant material to be propagated and commercialized. Such scientific data should guide the actions of politicians and regulators to valorize innovations for the benefit of society.

CONCLUSION WHAT PERSPECTIVES FOR GRAPEVINE BIOTECHNOLOGIES AND NBTS ?

Biotechnologies cover a very large number of applications to multiply or improve sanitary or genetic of grapevine varieties. Some of these technologies are now so integrated in the selection of rootstock and scion varieties that they are no longer debatable. Thus, without the use of apex culture or micrografting, that makes possible the elimination of pathogenic viruses or bacteria, it would not be possible to provide the industry with healthy clonal material. As a reminder, 100% of the rootstocks distributed in France are certified clones, the majority of which have been sanitized by thermotherapy and micrografting. This is also true for a significant proportion of scion clones. In terms of varietal innovation, the use of embryo rescue is widespread in the breeding of seedless table grapes, leading to a very innovative range of varieties.

The latest biotechnologies, genetic transformation developed in the 1990s, and genome

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editing still under development, allow targeted modifications of the genome. These technologies that have been validated in functional genomics studies are still only tentatively considered for breeding, whereas this is the only possible approach to maintain the identity and agronomic characteristics of the varieties so dear to the sector. The first issue concerns the traditional image that winegrowers have attached to their activities, a notion that is not very compatible with varietal innovation in general and NBTs in particular. Another reason limiting the use of NBTs for genetic improvement is the complexity of the architecture of some agronomic traits (plant growth, yield, fruit composition…). Moreover, studies on vine response to abiotic constraints showed that traits of adaptation (tolerance to temperature, water use efficiency…) are determined by several quantitative trait loci (QTLs), each QTL only explaining a fraction of the phenotypic variability in a complex genetic interaction network. Even though few applications could be considered for fruit quality traits regulated by major QTLs (e.g. berry skin variations, seedlessness), the current potential of NBT remains limited.

Actually, the first promising contribution of NBT to breeding would be in disease tolerance manipulation as some of these traits are already subjected to pyramidal selection through marker-assisted selection. However, although some genetic markers are mapping major disease resistance QTLs, most of the causal genetic sequences and their functioning are not identified. Moreover, very little is known about minor traits of disease tolerance, while they could play a decisive role in resistance sustainability. Finally, because *V. vinifera* is being susceptible to a range of fungi, the construction of multi-resistant genotype will still be quite complicated.

So, for the breeding of grapevine varieties able to both limit inputs (pesticides, water, minerals) and cope with climate changes and abiotic stresses, NBTs will require significant technical progress and genetic knowledge to replace or complement classical breeding approaches.

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