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# Development and Use of Biotechnology Tools for Grape Functional Analysis

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Additional information is available at the end of the chapter

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## Abstract

The aim of this chapter is to provide a description of the latest scientific advances in the field of gene functional analysis in grapevine. It provides general information about the studies conducted during the past decade to understand the natural variation of this plant and how this information has been exploited for the understanding of traits of interest. Likewise, it is exposed how the use of biotechnology tools have helped to characterize the mechanisms of gene expression and its regulation, as well as the subcellular localization of proteins and their interactions with other molecules. Finally, an approximation to the new technologies of gene editing and their potential application in the functional study of grapevine has been carried out.

**Keywords:** *Vitis vinifera*, QTL, gene transfer, genome editing, CRISPR/Cas system

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## 1. Introduction

In recent years, studies of plant functional analysis are becoming increasingly relevant. Genome sequencing of a number of organisms is providing the scientific community with a vast resource of DNA sequence information that is revolutionizing the way science is being done. Thereby, progress has been made in the functional genomics of grapevine following the whole genome sequencing and assembling of *Vitis vinifera* PN40024 reference genome [1]. This has led to new interesting perspectives in genomic research and in functional analysis, providing insights into genetic regulation of grapevine genes and novel ways for isolating and characterizing genes, transcription factors, and proteins. The functional genomics methodol-

ogy is also changing the experimental strategy from a forward genetics, that is, mutant to gene approach, to a reverse genetics or sequenced-gene to mutant and function approach [2]. Although functional analyzes are very relevant to basic plant biology, an important approach for crop plant improvement and commercial applications is expected. For instance, the current development of “clean” transformation techniques intend to obtain plants without insertion of antibiotic resistance genes and non-plant sequences [3].

Nowadays, there are several tools used by reverse genetics to induce variation into a gene and then used to infer its function. Genome editing is one of them [4]. This novel technique employs engineered nucleases that cut the DNA specifically generating targeted double-strand breaks (DSBs) [5]. Starting from the already known nucleases, such as zinc finger proteins and transcription activator-like effectors, and the recent discovered technology, the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system, a precise manipulation of gene sequences as well as the addition or deletion of DNA fragments on specific locus has recently become possible for some areas. For functional analysis, this means the possibility of making specific mutations in order to discover new gene functions, different relations between them, or even a synthetic pathway.

Another interesting challenge in grapevine investigation is the identification of those genes that contribute to the natural genetic variation of specific traits as well as understanding their main functions. The frequent quantitative nature of genetic variation requires the use of quantitative trait loci (QTLs) mapping approaches to understand the genetic structure of traits [6]. Interval mapping based on DNA markers could be used to genetically localize QTLs in natural and experimental populations [7, 8]. The development of new QTL mapping strategies and DNA research tools [9, 10], as well as the successful identification of few genes responsible of simple Mendelian traits [11, 12], have reinvigorated interest in grapevine QTL analysis. The present chapter summarizes the latest scientific advances in the field of grapevine functional analysis through the development and use of different biotechnological tools. It also describes diverse methods for the discovery and modification of genes that contribute to the finding of new sources of variability.

## 2. From QTL mapping to gene function

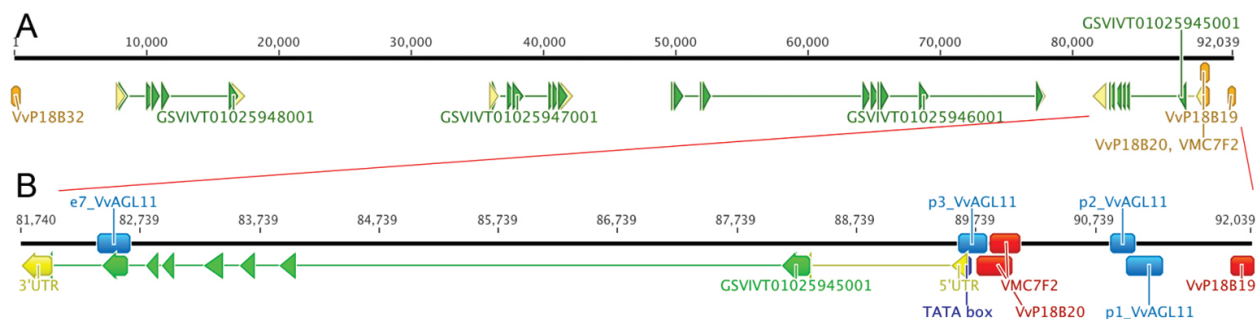
In model organisms, induced mutagenesis provides a powerful alternative for gene function discovery strategies derived from the knowledge of the phenotypic variation in plant (i.e., forward genetics). Because the prospects of gene identification are high and every gene affecting a trait is potentially a target, mutagenesis may present advantages with respect to natural, polygenic variation (i.e., quantitative trait loci) for identifying functional pathways and complex traits [13]. However, mutagenesis has not been extensively used in grapevine, although physical and chemical mutagens have been investigated [14, 15]. So far, the only published report on chemical mutagenesis of *in vitro*-grown grapevine buds comes from experiments using ethyl methane sulphonate and ethyl bromide on cultivar Pusa Seedless [16]. Likewise, the use of mutagenesis-induced variation for functional analysis has not been fully

implemented. The main limitations are related to the almost inexistence of homozygous genotypes (besides the PN40024 line [1]) and the poor germination rate of most grapevine cultivars [6].

On the other hand, the extensive development of genetic resources including Amplified fragment length polymorphism (AFLP) [17], Random amplification of polymorphic DNA (RAPD) [18], Simple Sequence Repeat (SSR) [19], and single nucleotide polymorphism (SNP) [20] markers for grapevine genetics in the era before the availability of the grapevine genome [1] allowed the characterization of the genetic determinants for several grapevine traits by means of QTL mapping. The study of the genetic control of major agronomic traits in grapevine using QTL analysis allowed the elucidation of traits such as seedlessness and berry weight [12, 18, 21–23]; berry phenolic composition [24–28]; aroma [29, 30]; berry firmness [31, 32]; fertility [9, 33]; flower hermaphroditism [34]; cluster architecture [35]; pathogen resistance [34, 36–38]; plant phenology [39, 40]; and adaptation to abiotic stresses [41] and to climate change [42]. Despite several of the reports allowed the identification of candidate sequences, just few of them have finally characterized the genes responsible for a particular function or trait. Fortunately, those few paradigmatic examples are relate to two major grapevine quality traits: seedlessness and aroma.

Seedlessness, the absence of seeds in the berry [43], has been largely studied since the first genetic reports on the trait [18, 21, 44]. Those studies were mainly performed by the analysis of seed and berry size inheritance. In these works, the phenotypes segregate in experimental populations with a continuous distribution, an indicative of the quantitate nature of the trait. To date, the most accepted model proposed that seedlessness is under the control of a dominant regulator gene named seed development inhibitor (*SDI*) [18, 45, 46]. The further reports of a major QTL for seedlessness colocalizing with *SDI* on linkage group (LG) 18 confirmed the prediction of the model. These coincidental studies described a large-effect QTL by explaining between 50% and 70% of the phenotypic variation for seedlessness [18, 21, 22, 39].

The functional characterization of *SDI* begun with the identification of *VviAGL11*, a MADS-box gene putatively involved in grapevine ovule, seed, and berry development [47, 48], along with its in silico colocalization to the same contig containing the *SDI* locus in LG 18 [39]. Further, Mejía et al. [12] integrated multiple genetic, molecular, and genomic resources [1, 22, 39, 49] to elucidate the molecular basis underlying the *SDI* locus. In order to test the hypothesis for a possible role of *VviAGL11* in seedlessness, Mejía et al. [12] performed a comprehensive set of experiments providing additional genetic and transcriptional support for this hypothesis. These experiments consisted on the fine positional identification of *VviAGL11* as a candidate gene in a reduced confidence interval of 92 kb (**Figure 1**), and its additional characterization at the molecular, genetic, and transcriptional level. The authors showed that (i) the proportion of phenotypic variation in seedlessness explained by *VviAGL11* was higher than 70%; (ii) the promoter sequence comparison of the *VviAGL11* alleles at seedless and seeded genotypes showed several polymorphisms with putative functional effects, particularly two short insertions and deletions (INDELs); and (iii) the level of *VviAGL11* expression was associated with the *VviAGL11* genotype, since the homozygous genotypes for the seeded allele showed transcription 25-times higher than the homozygous genotypes for the seedless allele. In that



**Figure 1.** Annotation of the different genes found within the berry size and seedlessness QTL. (A) 92 kb window showing the structure of the four genes comprised between the VvP18B19 and VvP18B32 microsatellite markers. (B) Detailed view of the most probable candidate gene for seedlessness, *VviAGL11* (VIT\_218s0041g01880). Microsatellite and newly developed intragenic markers used for the fine mapping of the gene are indicated in red and blue, respectively. © Mejía et al.; licensee BioMed Central Ltd. 2011.

way, the genetic and transcriptional evidence suggested that seedlessness in table grapes might be due to misexpression of *VviAGL11* caused by short insertions and deletions (INDELs) in its regulatory elements. Together, all the presented information by Mejía et al. [12] pointed out to *VviAGL11* as being the major gene responsible for seedlessness.

On the other hand, it has been widely studied that aromas in grapevine arise from volatile compounds, such as terpenes, norisoprenoids, and thiols [50]. Particularly, the aroma of Muscat grapes is linked to the presence of the monoterpenes geraniol, linalool, nerol, and  $\alpha$ -terpineol [51]. In plants, all isoprenoids are formed through two different and partially independent pathways, the mevalonic acid pathway (MEP, in the cytoplasm) and the methylerythritol phosphate pathway (in plastids). However, experimental reports have shown that most species mainly use one of the two pathways. Particularly in grapevine, the dominant pathway for monoterpene biosynthesis in leaves and berries is MEP route [52].

The route to the functional characterization of the genetic basis of aroma in grapevine starts with two simultaneous reports demonstrating the colocalization of a grapevine 1-deoxy-D-xylulose 5-phosphate synthase (*VviDXS*) gene with a major QTL for terpenol content in LG 5 [11, 53]. Moreover, by the analysis of the nucleotide diversity and linkage disequilibrium within the *VviDXS* gene, and testing for association between individual polymorphisms and Muscat flavor in different genetic backgrounds, Emanuelli et al. [54] identified significant single nucleotide polymorphisms. Further analysis corroborated that all those linked polymorphisms shared a particular SNP responsible for the substitution of a lysine with an asparagine at position 284 of the *VviDXS* protein. Finally, to test the functional relationship between *VviDXS* and Muscat flavor, Battilana et al. [29] compared the monoterpene profiles of cultivar Moscato Bianco with the expression of *VviDXS* alleles throughout berry development. It is worth to mention that the cultivar Moscato Bianco is heterozygous for the SNP mutation like most of the Muscat-flavored genotypes, thus containing both a “Muscat-type” allele (284N) and a “neutral” allele (284K) [54]. By comparing the transcription profile of *VviDXS* and free monoterpene odorant variations during berry ripening, Battilana et al. [29] showed that monoterpene accumulation in Moscato Bianco berries cor-

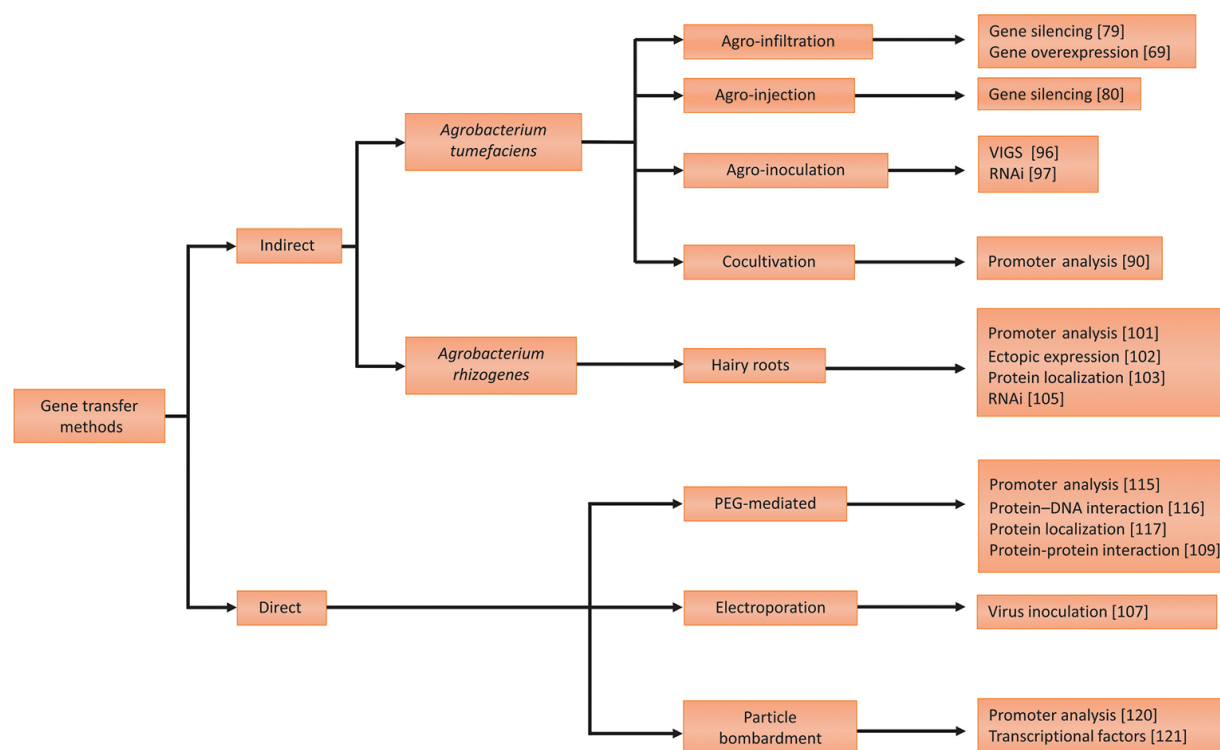


relates with *VviDXS* expression (at both the gene and the allele level). Despite all these accumulation evidences, the most dramatic demonstration of functional effect of the *VviDXS* gene polymorphisms were the biochemical experiments showing that the amino acid non-neutral substitution (K284N) influences the enzyme kinetics by increasing the catalytic efficiency. The kinetic analyses performed by Battilana et al. [29] clearly showed that the substrate affinities of the proteins encoded by the *VviDXS* alleles were similar but a major difference was found in the catalytic efficiencies of the enzymes, being *VviDXS* N284 twice as efficient as *VviDXS* K284.

Despite the robustness of the two presented examples for seedlessness and aroma, forthcoming gene functions determination in grapevine will be boosted by the use of new and more efficient tools. Recently, the availability of next-generation sequencing and whole-genome sequence information allowed the generation of a SNP-based genotyping array [55]. This array, developed for the grapevine international community, was built after the resequencing of 43 *V. vinifera* ssp. *vinifera*, four *V. vinifera* ssp. *sylvestris*, three *V. cinerea*, three *V. berlandieri*, three *V. aestivalis*, three *V. labrusca*, one *V. lincecumii*, and five *Muscadinia rotundifolia* genotypes using Illumina platforms. The use of this powerful tool in combination with the microvine model system [56] allowed an innovative study in grapevine genetics [9]. This work achieved the identification of 10 robust and stable QTLs for vegetative and reproductive traits as well as the first berry acidity QTLs reported so far in grapevine [9]. Additional initiatives like the recent development of large diversity panel including 279 cultivars from different uses (table and wine grapes) and geographical origin (eastern and western), and including most of the major founders of modern cultivars [10], will certainly increase the power and value of the 18K SNP chip for genome wide association studies and to gain more insight into the genetic control of many agronomic traits and their interaction with the environment.

### 3. Gene transfer technologies as a tool for functional analysis

In the plant biotechnology community, the term transformation is used to describe the insertion of engineered gene sequences into a plant cell, leading to a change in the genetic makeup of the target cell and its derivatives (i.e., reverse genetics). The foreign molecule can function for a short time in the nucleus as an extrachromosomal entity (transient transformation) [57], or the integration into the genetic material of the target cell can be necessary for long-term functionality and expression (stable transformation) [58, 59]. Therefore, transient expression has been used to evaluate factors that influence the stability or consistency of gene expression [60]. The final determination of factors that modulate transgene expression must ultimately be made only following introduction to plant cells for stable transformation. Methods for molecule transfer in grapevine involve biological methods (*Agrobacterium*-mediated transformation) for indirect gene transfer or chemical/physical methods (biolistics, electroporation, and protoplasts) for direct gene transfer to plant cells [61, 62] (Figure 2).



**Figure 2.** Schematic summary of direct and indirect transformation methods assayed in *Vitis vinifera* L. References regarding each functional analysis are presented in brackets.

### 3.1. Indirect gene transfer methods: *Agrobacterium*-mediated transformation of grapevine

Indirect transformation methods introduce plasmids, that is, independent circular molecules of DNA that are found in bacteria, into the target cell by means of bacteria capable of transferring genes to higher plant species [63]. The most popular used microorganisms are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. As wild-type bacterium, they can transfer a crown gall disease-inducing plasmid to its host, promoting tumor formation (Ti plasmid) [64], or a hairy root disease-inducing plasmid (Ri plasmid) promoting the formation of proliferative multibranched adventitious roots [65], respectively. However, the disease-inducing genes have been removed from current (disarmed) vectors and thus, they are not able to cause disease anymore. In their place, any genetic construction or gene of interest can be placed and those constructions introduced into plant cells.

Although other methods are suitable for plant transformation, such as protoplast or biolistic transformation, the *Agrobacterium*-mediated transformation is preferred since plants bearing single transgene copy can be more easily obtained (e.g., see [66]). The system is cheap and has been shown to work effectively in a variety of plant species [67]. However, it features some drawbacks. For instance, the host tissue must experiment some physical damage, the vectors are normally designed to infect the nucleus, the bacteria need to be eliminated using antibiotics, and the host range must be sensible to infection. Moreover, the presence of *Agrobacterium* may alter the activity of several plant proteins [68]. So, this aspect should be considered when using

*Agrobacterium*-mediated system to study stress signaling components [69]. In addition, this method is often associated with a high level of nonspecific autofluorescence and is difficult to be used for fluorescence-based analysis, such as subcellular localization [70]. In addition, the waxy cuticles of some plants organs can limit observations using a fluorescence microscope.

*A. tumefaciens* is a soilborne bacterium and a plant pathogen causing crown gall disease in angiosperms and gymnosperms [71]. *Agrobacterium*-mediated gene transfer methods were developed in grapevine in the early 1990s. Baribault et al. [72] first succeeded in the transformation of cell suspension cultures of Cabernet Sauvignon. Presently, transformation methods based on the Ti plasmid of *A. tumefaciens* are the predominantly employed protocols for grape transformation worldwide [73] and are compatible with the regeneration of transgenic plants from a variety of cultivars [74]. It has been widely used as a versatile tool for development of stably transformed model plants and crops. However, the development of *Agrobacterium*-based transient plant transformation methods attracted substantial attention in recent years.

The most significant applications of transformation with *A. tumefaciens* include functional genomics by reverse genetics. In grapevines, this technology has been recently applied to analyze the function of several genes such as *VviAdh* [75], *VviPIP2* [76], *VviCCD1* [77], and *VviWRKY33* [78]. Likewise, this method has been proved to be very useful for transgenic complementation [79–81], technical studies [82–87], promoter analysis [88–91], subcellular localization analysis [92], and protein production [69, 93] in grapevine. Nonetheless, the genetic background and plant growth conditions are key factors in performing successful *Agrobacterium*-mediated transformation. For instance, the compatibility of *A. tumefaciens* strains with the plant species represents an important variable to be considered in this kind of assay. Thus, the strains most efficiently used for gene transfer into grapevine are probably C58C1 (pCH32) [69, 78, 86] and EHA105 [75, 77, 82, 83, 85, 88, 90], which contain extra copies of *vir* genes that make them hypervirulent [61].

### 3.1.1. Agroinjection and agroinfiltration

Transformation and regeneration of grapevine plants have been achieved via organogenesis [84], embryogenesis [83, 93], or from nonembryogenic cell cultures [86, 94], demonstrating that grapevine is not recalcitrant to *Agrobacterium* infection but this approach is time consuming and takes several months to produce transgenic plants suitable for analyses. In some cases, the production of whole transgenic organisms may not be needed if a large number of cells within a plant can be uniformly and consistently transformed. Indeed, direct *Agrobacterium*-mediated transformation at the plant-organ level has proven very useful when the recovery of transgenic plants is not required [95]. This is particularly interesting for species like grapevine, where the regeneration of transgenic plants is difficult.

*A. tumefaciens* can be infiltrated into plant leaves using a syringe or vacuum, allowing different origins of the target material (greenhouse-grown plants, plantlets grown *in vitro*, green cuttings, or *in vitro* shoots). The bacteria enter the intercellular air spaces within the leaf and transform a very large percentage of the internal mesophyll cells. The agroinjection method involves a needleless syringe that can be filled with the bacterial suspension and then pressed against the underside of a leaf to infiltrate the suspension by active pushing through the



stomata [92]. This method can be used to rapidly and simply generate a chimeric plant, where a large number of leaf cells contain the gene of interest. Using this technique, Urso et al. [80] developed an efficient agroinjection-based gene silencing assay of specific genes likely to be involved in resistance to powdery mildew in grapevine leaves of *in vitro* plantlets.

Similarly, the agroinfiltration method consists of plunging detached leaves [69, 79] or whole plants [81] into the bacterial suspension. As the flow of bacterial suspensions through stomata and across the epidermis is impeded by the high surface tension of aqueous solutions, transient transformation is obtained through the rapid release of a vacuum to introduce the bacterial suspension into the mesophyll cells. Nowadays, this method represents an easy and noninvasive technique that allows gene expression in the whole leaf [67]. Agroinfiltration is usually performed on tissues of young plantlets grown *in vitro*, as greenhouse grown plants have often been described as recalcitrant to this technique [67, 92]. Santos-Rosa et al. [69] transiently overexpressed stilbene synthase genes in detached grapevine leaves in order to study the influence of stilbenes on downy mildew infection. Bertazzon et al. [79] assayed the transient downregulation of a grapevine defense-related gene by the agroinfiltration of the constructs for the expression of dsRNA. Interestingly, Ben-Amar et al. [81] established a protocol to agroinfiltrate leaves of greenhouse-grown plants using a vacuum device. In their work, they delivered the first evidence of GFP gene silencing in grapevine achieved for the first time using *in planta* agroinfiltration method.

### 3.1.2. Cocultivation

This method is used to introduce bacterial suspension into intercellular spaces within plant tissue, simply by submerging above-ground parts of the plant into an *Agrobacterium* solution for a few seconds or inoculating the plant material (cell culture) with bacteria cocultivated on a solid medium. The technique involves the preparation of a diluted bacterial solution that incorporates the addition of a surfactant, the preparation of the explant, the dip of the material in the bacterial solution, and the cocultivation in the presence of the antibiotic. The method facilitates high-throughput transformation at ambient pressures and considers that vacuum infiltration or syringe pressure is unnecessary as long as a suitable surfactant is used. For instance, Lizamore and Winefield [87] used the organosilicone surfactant Silwet L-77 to increase transient transformation in grape without the need for vacuum- or syringe-based method for infiltration of leaves. The transformation efficiency was achieved by measuring red pigmentation of cells, transiently transformed with the transcriptional activator of anthocyanin biosynthesis, *VviMybA1*.

Cocultivation with *A. tumefaciens* is normally used to perform transient expression assays [87]. Likewise, it is the most common method to obtain stably transformed grapevines [83, 86, 88, 90, 93]. The cocultivation of cell suspension cultures of Gamay Red with EHA105 strain of *Agrobacterium* was used for studying the expression of the grape dihydroflavonol 4-reductase gene (*VviDFR*) and the analysis of its promoter region [88]. In another work, Li et al. [90] cocultivated somatic embryos of Thompson Seedless with EHA105 strain of *Agrobacterium* harboring a construct of *VviMybA1* as a reporter gene and a vast number of grapevine constitutive promoters from various genotypes. Cocultivation has been the transformation

method chosen for stable transformation of embryogenic and nonembryogenic cell cultures as well. Cheng et al. [93] used proembryogenic masses of the grapevine cv. Thompson Seedless for genetic transformation via *Agrobacterium* with a gene involved in the defense system. Martínez-Márquez et al. [86] applied the same method to stably transform nonembryogenic cell cultures of two *V. vinifera* cell lines with the GFP gene under the control of pCaMV35S. This study was relevant for those interested in bioactive metabolite production.

### 3.1.3. Agroinoculation

Agroinfiltration was classically used for introducing gene constructs driven by a Ti plasmid. Additionally, this technique has enabled the introduction of virus-derived vectors into several *V. vinifera* cultivars [96, 97]. Agroinoculation, first developed as a simple tool to study plant-virus interactions, is a popular method of choice for functional gene analysis of viral genomes. It also serves as a mean for disease control via RNA interference (RNAi)-enabled vaccination against pathogens or invertebrate pests. It uses a live virus to attain desirable traits via either expressing a protein of interest or knocking down gene expression via RNAi. The latter is an attractive approach of virus-induced gene silencing (VIGS). Muruganantham et al. [96] developed a VIGS vector based on the *Grapevine virus A* (GVA) that is a member of the genus *Vitivirus*, family *Flexiviridae*. They described an *Agrobacterium*-mediated method for inoculating *in vitro*-propagated *V. vinifera* plantlets via their roots with the GVA-derived vector for silencing the endogenous phytoene desaturase (PDS) gene. Similarly, Kurth et al. [97] generated a virus-derived gene expression and regulation vector based on *Grapevine leafroll-associated virus-2* (GLRaV-2). This relatively benign virus of the family *Closteroviridae* is spread throughout grape-growing areas worldwide. The GLRaV-2 vector expresses recombinant proteins in the phloem tissue that is involved in sugar transport throughout the plant, from leaves to roots to berries. This avenue provided a tool to track virus infection through the entire pathway of sugar transport. Furthermore, the vector provides a powerful RNAi capability of regulating the expression of endogenous genes via virus-induced gene silencing for disease protection.

### 3.1.4. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT)

A number of grapevine cultivars have been stably transformed using *Agrobacterium*-mediated procedures and most progress has been achieved using embryogenic cell masses [82, 83, 85, 93]. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) was first reported by Trick and Finer [98], who used this technique for the production of transgenic soybean (*Glycine max*) and Ohio buckeye (*Aesculus glabra*) plants from SAAT-treated embryogenic cell suspensions. Chu et al. [94] recently reported the effect of different times in a sonication bath while infecting dedifferentiated nonembryogenic grapevine suspension cell cultures with *A. tumefaciens*. Plant tissue damaged by sonication allows the tissue to be much more easily transformed by *A. tumefaciens*. This pioneering protocol significantly increased the number of cells expressing the reporter gene that consistently produces transgenic microcalli that can be converted into transgenic cellular lines.

### 3.1.5. Hairy roots

*A. rhizogenes* is a soil pathogen that elicits adventitious and genetically transformed roots. This leads to the production of so-called “composite plants” comprising a transgenic hairy root system attached to nontransformed shoots and leaves. While grapevine roots have been successfully transformed with *A. rhizogenes* [99], the regeneration of transgenic plants via *A. rhizogenes*-mediated transformation was only obtained using embryogenic calluses [100].

Hairy root technology has already been used in several functional studies relative to grapevine. Secondary metabolism investigation associated to flavonoid analysis of hairy roots overexpressing *VviMybA1-2* determined that this transcription factor is specifically involved in the last steps of anthocyanin biosynthesis and transport [101]. Moreover, ectopic expression of either *VviMybPA1* or *VviMybPA2* in grapevine hairy roots induced qualitative and quantitative changes of the proanthocyanidin profile [102]. Localization studies, also using this technology, showed that anthoMATE transporters play their role in the tonoplast [103]. Regulation studies of stilbene biosynthesis demonstrated that ectopic expression of MYB15 in grapevine hairy roots resulted in increased stilbene synthase gene expression and in the accumulation of glycosylated stilbenes in planta [104]. Other studies dealing with pathogen interaction used hairy root methodology to enhance nematode resistance in transgenic grape. For this purpose, a biotech-based solution was designed for controlling root-knot nematodes (RKNs) by introducing RNA interference to silence RKN effector gene [105].

## 3.2. Direct gene transfer methods in grapevine

Due to the difficulty of transforming plants through the use of *Agrobacterium*, direct gene transfer methods were developed based on chemical, physicochemical, and mechanical procedures. The principle of passing DNA molecules through large pores or holes in the cell wall or membrane are very efficient in the introduction of DNA but inefficient for the recovery of transgenic plants. Taking this into consideration, direct gene transfer methods in plants require one of the following techniques for transformation: permeation of protoplast membranes by a chemical (PEG) treatment or by electroporation to allow direct DNA uptake, or cell bombardment of plant tissues with microparticles coated with the DNA of interest.

### 3.2.1. Polyethylene glycol (PEG) treatment and electroporation of protoplasts

Due to the rigid cellulose wall, it has been relatively difficult to handle plant cells. Several methods based on mechanical removal of cell walls and on the use of solvents have been used to degrade cell wall for the obtention of protoplasts. However, the methods involving the use of hydrolytic enzymes have been the most popular ones [106]. The protoplasts used for transformation are usually isolated by enzymatic digestion of mesophyll cells from leaves [107–109], berry mesocarp [106, 110], roots [111], stems [112], embryogenic tissue [108, 113, 114], and from fast-grown suspension-cultured cells [115–117].

Although the generation of transgenic lines represents a powerful research tool for characterizing plant gene function, protoplast-based protocols for grapevine stable transformation have some drawbacks. The production of polyphenols and phytoalexins is induced at a high level

during the digestion process and the corresponding genes remain activated during the culture of *Vitis* spp. protoplasts, diminishing its viability [118, 119]. Despite this, some progress has been reached in obtaining whole plants from protoplast regeneration [113, 114]. However, plant protoplasts constitute a versatile system for transient gene expression and have been widely used for the functional characterization of genes [109], virus inoculation [107, 108], protein subcellular localization [109, 117], promoter analysis [115], protein-DNA interaction [116], and protein-protein interaction [109].

PEG-mediated transient assay utilizing protoplasts has become a powerful tool for rapid gene functional analysis that can be readily carried out using ordinary lab supplies and usually has high transformation efficiency. Wang et al. [117] described a rapid and efficient transient expression system for PEG-mediated transformation of protoplasts derived from grape berry suspension-cultured cells. The system was applied for subcellular localization studies of flavonoid biosynthesis enzymes using GFP as a reporter gene. In the same way, Zhao et al. [109] reported a simplified and highly efficient method for the isolation of mesophyll protoplasts from grapevine leaves and a modified transfection protocol using PEG. This transient transformation of protoplasts was developed to characterize the function of a heterologous plant defense gene through its gene expression, and was regarded as suitable for the study of protein expression, protein subcellular localization, and protein-protein interaction.

In electroporation, cells are permeabilized by the application of very short, high-voltage electric pulses to introduce DNA into cells. As mentioned before, the use of electroporation is restricted to stable transformation in species whose protoplasts are regenerable. Thus, the primary application of electroporation to plants has been for DNA uptake for studies of transient gene expression. As an example, protoplast electroporation has been used for virus inoculation [107, 108]. Valat et al. [107] used the mesophyll protoplast electroporation as a rapid screening technique of transgenic grapevine clones expressing the viral capsid gene or the movement protein gene of grapevine fanleaf virus (GFLV) to identify material that reduces or inhibit the accumulation of viral proteins at the cell level.

### 3.2.2. Particle bombardment (biolistics)

The most commonly used method for direct DNA uptake (or naked DNA introduction) is particle bombardment. Also known as biolistics, the technique consists on the acceleration of high-density carrier particles covered with genes that pass through the cells, leaving the DNA inside. Although it is mainly reported as a stable transformation method, it is also convenient for transient expression assays. However, it requires expensive equipment and causes severe tissue damage, and usually yields low transformation efficiency. The advantages of this technique are that it has no limitation on species ranges, genotypes, or subcellular organelles. In grapevine, it has been assayed with the use of circular plasmids [89, 91, 104, 120] and minimal cassettes [121, 122]. Moreover, it has let cotransformation with multiple genes [61, 121]. Likewise, particle bombardment has allowed the manipulation of organ sections such as leaves [91], embryos [91], and suspension-cultured cells [89, 104, 120–122] as targets for grape transformation.



Due to its versatility, the technique has been applied for several functional studies such as promoter analysis [89, 91, 120] and the regulatory function of some transcription factors [104]. In their work, Höll et al. [104] demonstrated via transient gene reporter assays that the cotransformation of cell suspensions with transcription factors (MYB14 and MYB15) and promoter sequences specifically activate the promoters of *STS* genes. Without neglecting the technical aspects, the work of Vidal et al. [121] is worth mentioning. In their work, they compared the efficiency of the method when an embryogenic cell suspension culture was cotransformed via biolistics using a minimal gene cassette and a traditional circular plasmid. The stability of the plant phenotype compared to nontransgenic lines after its regeneration, confirmed the effectiveness of the minimal cassette technology for genetic transformation of grapevine cultivars. Later studies [122] demonstrated the importance of 3'-end cassette protection for successful protein expression using the minimal cassette technology. Protection of the minimal cassette upstream promoter and downstream terminator may be necessary due to the nuclease activity of target plant material.

## 4. New tools for genome editing

The dominant genome editing tools before 2013 were zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) [4]. Both are artificial fusion proteins comprising an engineered DNA-binding domain fused to the nonspecific nuclease domain of the restriction enzyme *FokI*. While these nucleases have generated efficient targeted mutagenesis and other genome editing applications [123, 124], the design and construction of large modular proteins are both laborious and expensive. For instance, the utility of ZFNs is limited by their long synthesis time and their high rate of failure [125]. These nucleases generate the introduction of targeted DNA double-strand breaks, stimulating cellular DNA repair mechanisms. Two different DSBs repair pathways have been defined: nonhomologous end joining (NHEJ) and homologous recombination (HR) [126]. Subsequent cellular DNA repair process generates desired insertions, deletions, or substitutions at the loci of interest. The newest technology for genome editing is based on RNA-guided engineered nucleases, which seems to have a great future due to their simplicity, efficiency, and versatility.

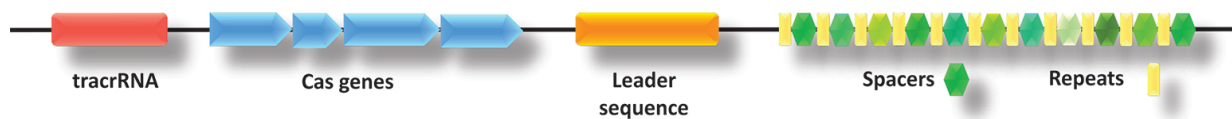
### 4.1. The CRISPR/Cas9 system

Clustered regularly interspaced short palindromic repeat-associated Cas is an adaptive bacterial and archaeal immune system that uses antisense RNAs to control invasions of phages and plasmids [127]. CRISPR loci are short variable spacers separated by short repeats that are transcribed into noncoding RNAs. The noncoding RNAs form a functional complex with CRISPR-associated Cas proteins and guide the complex to cleave complementary invading DNA [128] (**Figure 3**).

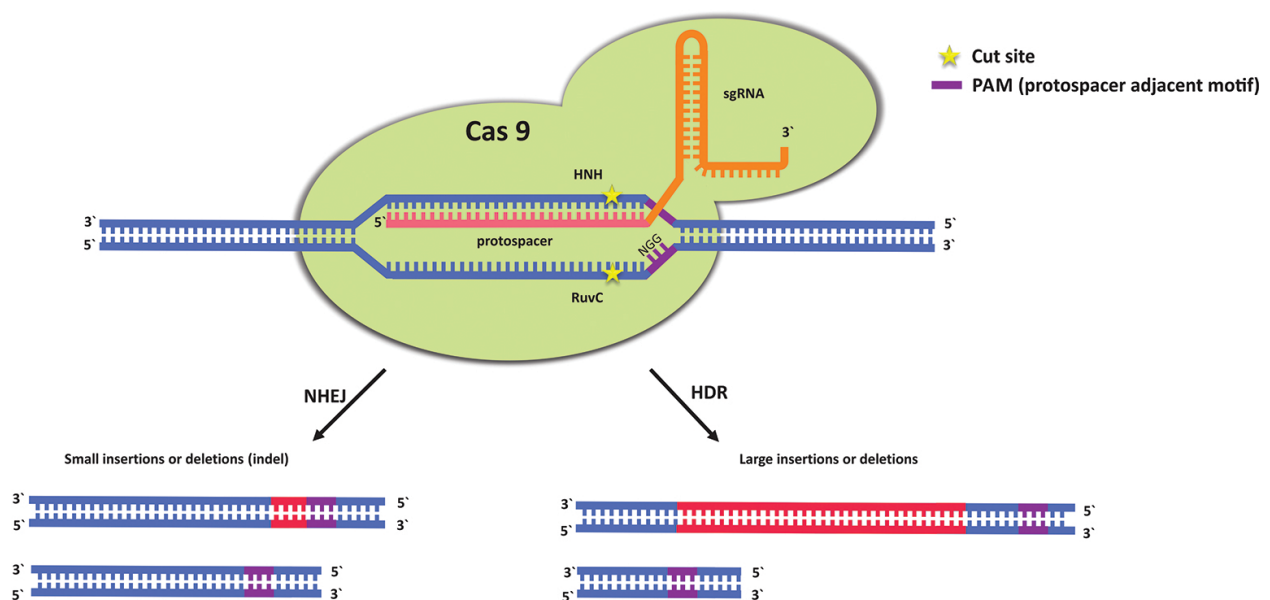
There are three CRISPR/Cas system types (I, II, and III) that uses distinct molecular mechanisms to achieve nucleic acid recognition and cleavage [129]. In genome editing, type II CRISPR/Cas system has been developed as a new gene-targeting tool. The Cas9 endonu-



clease, from *Streptococcus pyogenes*, forms a complex with two short RNA molecules called CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) that guide the nuclease to cleave DNA on both strands at a specific site. A prerequisite for cleavage is the presence of a conserved protospacer adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5'-NGG-3' [4, 130, 131]. The dual tracrRNA:crRNA was then engineered as a single guide RNA (sgRNA). This structure maintains the 20-nucleotide sequence at the 5' end of the sgRNA, which determines the DNA target site by Watson-Crick base pairing (crRNA), and the double-stranded structure at the 3' side of the guide sequence, which binds to Cas9 (tracrRNA) [131]. Given the sequence specificity conferred by a 20-nt sequence in the sgRNA, CRISPR/Cas system can be retargeted to cleave virtually any DNA sequence by redesigning the sgRNA. These techniques could induce several DSBs and generates genomic modifications such as deletions, insertions, or gene replacement [132–135]. These genomic modifications will depend on the repair pathway. If NHEJ is taken, small deletion or insertion will occur; if homology-directed repair (HDR) is chosen, DNA seg-



**Figure 3.** A typical structure of CRISPR/Cas9 type II locus. It includes a tracrRNA section (pink box), a family of Cas genes (light blue boxes), a CRISPR section that is an array of alternating nonrepetitive spacer (green hexagons), a short palindromic direct repeats (beige rectangle), and a leader sequence (orange box) that is an AT-rich not conserved sequence. The leader sequence always precedes the clustered regularly interspaced short palindromic repeats.



**Figure 4.** The mechanism of genome editing using CRISPR/Cas9. The genomic DNA target must lie adjacent to a protospacer adjacent motif. Cas9 protein associates with the sgRNA and binds to the target sequence, cleaving both strands of the DNA upstream of the PAM. Cleavage results in a DSB that becomes substrate for endogenous cellular DNA repair machinery that could catalyze nonhomologous end joining or homology-directed repair.

ments with sequences homologous to the break site will be inserted (large insertion) or gene replacement could happen [136] (**Figure 4**).

## 4.2. Applications in plants

Although CRISPR/Cas system has not been applied on *V. vinifera*, it could be harnessed to achieve a great progress in grapevine functional genomics. This technique is an interesting alternative tool to induce DSBs in plant genomes. In 2013, several reports were published discussing the first applications of CRISPR/Cas9-based genome editing in plants [137, 138]. Different species were used for these studies such as *Arabidopsis thaliana* [139], tobacco [140], rice [141], wheat [132], maize [142], sorghum [133], and tomato [143]. Jiang et al. [133] demonstrated that three slightly different versions of the Cas9/sgRNA system delivered by *A. tumefaciens* or polyethylene glycol-mediated transfection are functional in four plant types: *A. thaliana* and tobacco (dicots), and rice and sorghum (monocots). Jia and Wang [144] made the first report on targeted genome modification in citrus using the Cas9/sgRNA system. They used agroinfiltration to deliver Cas9 along with a synthetic sgRNA targeting the CsPDS gene into sweet orange. Another important finding was made by Woo et al. [145]. In their studies, they achieved the edition of plant genomes without the introduction of foreign DNA into cells. Instead, they transfected protoplasts of *A. thaliana*, tobacco, lettuce, and rice with preassembled complexes of purified Cas9 protein and guide RNA.

With CRISPR/Cas9 system, efficient NHEJ-mediated targeted mutagenesis was detected in *A. thaliana* and tobacco protoplasts. Positive HDR events were documented in tobacco protoplasts as well [146]. Rice and wheat protoplasts were also studied exhibiting efficient mutagenesis frequencies [137, 138]. Besides of being a very promising tool for generating modifications to the genome, the CRISPR/Cas9 system could generate genome modifications that could be present in the germ line and be segregated normally to the next generation of plants without new mutation or reversion [139, 143, 147]. This encourage the system to be a very promising tool for generating modifications in the genome that can be present in the germ line and be segregated normally to the next generation of plants without new mutation or reversion [139, 143, 147].

The fact that the CRISPR/Cas system allows multiplexing gene editing is particularly valuable [141, 148]. Xing et al. [149] developed a system where several sgRNA could be generated from one construct. Subsequently, simultaneous multiplex mutageneses were analyzed in maize protoplasts, transgenic maize lines, and transgenic *Arabidopsis* lines exhibiting targeted mutations. This kind of studies could be very helpful in the future to understand different gene functions or biosynthetic pathways. An additional potential use for CRISPR/Cas9 system is to confer molecular immunity against DNA plant viruses [150, 151].

The CRISPR/Cas9 system can be used for several purposes in addition to genome editing. Disabled nucleases (catalytically inactive version of Cas9, dCas9) can still bind to their target DNA sequence, so they can be expressed as a fusion protein with the transactivation or transrepression domain of a transcription factor and be used to regulate gene expression [152, 153].

Besides all the great applications of this system, a major concern when using an RNA-guided Cas9 is the off-site target activity. Off-site targeting is defined by the tolerance of Cas9 to mismatch in the RNA guide sequence and it is dependent on the number, position, and distribution of mismatches throughout the entire guide sequence [154–156]. The technical decision of using a 20-nucleotide motif complementary to the target DNA in the sgRNA may leads to the possibility that it cross-hybridizes to highly similar DNA sequences in other genomic regions. So, the use of genome-specific designing tools for guide RNAs [157] in CRISPR-Cas experiments is a strongly desirable prerequisite.

Although the CRISPR/Cas9 system has been thoroughly investigated in the past three years, there are no experiments made on grapevine yet. The first advances need to mention that Wang et al. [157] computationally identified and characterized five different types of CRISPR/Cas9 target sites and developed a user-friendly database for upcoming editing projects of grapevine genomes. These novelties provide an encouraging future perspective for genome editing by the use of the CRISPR/Cas9 system.

## 5. Conclusions

A major challenge for grapevine research is the identification of genes and gene variants responsible for important agronomic traits and to assign biological function to annotated sequences. Demonstration of biological function requires genetic approaches that deal with genetic variation. During the last two decades, different molecular techniques have allowed the fine characterization of the natural genetic variation underlying QTLs for traits of agronomic interest, and in few cases, identified the responsible genes. Along with QTLs, the development of new methodologies of gene editing such as CRISPR/Cas and gene transfer methods, the detailed genomic, transcriptomic, and proteomic studies have been performed. Nowadays, these techniques are constantly evolving and becoming more and more simple, efficient, and precise. These set of tools will soon help promoting the progress in knowledge, both in functional genomics and biotechnology, for its subsequent application.

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