Chapter

Grapevine Improvement through Biotechnology

Devaiah Kambiranda, James Obuya and Janana Snowden

Abstract

Grapevine cultivation is increasing worldwide as people realize the benefits of grape and wine consumption. To improve yield and enhance the quality of grapes, biotechnology research plays an ever-increasing role. In recent years, the sequencing of multiple grape genomes has led to increased vibrant research initiatives on grape improvement. These novel approaches include those related to the application of transgenic technology toward the improvement of grape varieties. These advancements include the development of molecular markers for valuable traits, improved plant transformation systems, genetic engineering to enhance disease tolerance in grape cultivars, and the identification of flavor and aroma components to improve the enological quality of grapes. Some of the results obtained by various researchers have direct application, whereas others are yet to gain direct application in grape quality improvement, although such techniques possess potential qualities, which can be exploited for genetic breeding of *Vitis* species. This chapter highlights selected advancements in grape biotechnology from recently reported research activities.

Keywords: grapevine, transgenic, biotechnology, *Vitis* spp., cultivars

1. Introduction

Worldwide, grapes are one of the most widely cultivated fruit crops, encompassing 6.9 million hectares of arable land from which 74.3 million metric tons were produced in 2017 [1]. From the 2017 data, grapes ranked third among crops such as bananas, apples, and oranges that produced 113.9, 83.1, and 73.3 million metric tons, respectively. Since most of the harvested grapes are usually fermented into wine, it is suggested that its economic potential is greater than those of other comparative commodity crops. For example, wine sales from California alone in 2018 generated approximately $40 billion in sales [2]. According to 2015 statistics, the California wine industry contributed $57.6 and $114 billion to both the state and the US economies, respectively. The three major uses for grapes are winemaking, fresh fruit (table grapes), and dried fruit (raisins) production. The products derived from grapes or winemaking include grape juice, jelly products, ethanol, vinegar, grape seed oil, tartaric acid, and fertilizer.

Potential health benefits of certain grape-derived antioxidant compounds (polyphenols, resveratrol) have also contributed to increased research to investigate its compounds for their nutraceutical value. Grape extracts are used food additive, cosmetic, and pharmaceutical industries. Statistics from winemaking is steeped in history and tradition—perhaps more than any other food or beverage industry.

From the soil, climate, and harvesting of grapes to the crushing and aging processes, painstaking attention to detail dictates the flavor, bouquet, and the overall sensory experience of the final product. Hence, it may not be a surprise to learn that the grape industry is increasingly looking toward biotechnology for new opportunities to improve strategies for combating crop diseases and lower production costs for producing healthier and more flavorful products.

1.1 Historical development of grape biotechnology research

Grape breeding started very early, first for wine grapes and, by the end of the nineteenth century, for table grapes. Breeding for rootstocks started toward the end of the nineteenth century after the era of *Phylloxera* devastations of European vineyards. During the twentieth century, active breeding programs for table grapes were initiated in the USA, both by the USDA and by various institutions, which resulted in many new cultivars with improved characteristics. The original cultivars released in the USA led to the proliferation of table grape industry worldwide. Since then, several breeding programs have been established in Europe, South Africa, Israel, Argentina, Chile, and several other countries.

Globally, table grape production represents 27% of the 750,000 hectares planted with this species. Although table grape production in North and South America mainly represents c.a. 18% of the total world production, North America accounts for almost 50% of the global exports. Main exporters are Chile and Italy, followed by the USA, South Africa, and Mexico. Of the thousands of existing cultivars, only about 20 are grown for fresh consumption, with "Sultanina" ("Sultani," "Sultana," "Kishmish," or "Thompson Seedless") representing about 40% of the grapes grown for fresh consumption. This cultivar has been used extensively as a parental line for the development of new cultivars, such as "Flame Seedless" and "Crimson Seedless." These varieties, together with "Red Globe" (an important seeded cultivar due to its excellent postharvest life, high productivity, and public acceptance), are some of the most cultivated worldwide. During the last decade, new biological and genetic information are available to plant breeders, particularly in the area of biotechnology.

Biotechnological tools have been incorporated into breeding programs focused on the improvement of genetic diversity [3, 4]; fingerprinting applications based on codominant markers; quantitative trait loci (QTL) mapping and identification of candidate genes linked to QTLs for quality traits; development of cDNA libraries designed for the identification of genes involved in plant and berry development and host-pathogen interactions; and finally, the establishment of a genetic transformation platform available for the introduction of genes of interest as well as for the evaluation of gene function(s) using the grapevine as a model for woody plant species. The grape genome project was started in 2005 with collaborators in France and Italy within the framework of the International Grape Genome Project (IGGP).

The grape genome is attractive to genomic research due to its diploid chromosome with a small genome size of 475–500 Mb. The economic importance of the *Vitis* family worldwide informed the initiation of the genome project since its biology was poorly understood. Although for centuries, the industry contributed to the establishment of several wine production centers worldwide, little is known on how grapevines usually responded to and/or related with their surroundings, including their ability to cope under variable environmental stressors, such as pests and diseases, as well as the prevailing environmental conditions.

2. Grapevine breeding

Most breeding programs initially were publicly funded, but nowadays many of them are privately owned. Mostly new cultivars are protected by intellectual property rights, and, hence, growers need to pay royalties for their use or they may not gain access to some of the cultivars stored in closed commercialized "entities." Due to this new scenario, many countries and companies started their own private breeding programs. In 1988, the Chilean Institute for Agricultural Research started a breeding program to develop new table grape cultivars with emphasis on seedless grapes, disease resistance, and postharvest life [5]. Since the production of seedless cultivars, crosses were made among the seedless cultivars followed by in vitro embryo rescue. Early in the program, researchers have realized that certain cultivars were more efficient for embryo rescue. For example, in "Ruby Seedless" and "Red Seedless," 68% and 40% of the embryos, respectively, could be rescued, but with "Superior Seedless" or "Black Seedless," less than 30% of the embryos could be rescued [6].

As with other crops, plant breeders faced difficult task to develop high-vigor cultivars that would combine high yield with good quality traits. Quality in table grapes is associated with genetic factors, but also with environmental factors, most of which can be managed by different agricultural practices which can influence yield. Quality traits in table grapes are also influenced by consumer preferences, an important factor to be considered by grape breeders. Good berry quality characteristics include seedlessness, berry size, skin thickness, uniformity, aroma, firmness, flavor, texture, etc. present during harvest and after prolonged storage [7, 8]. More recently, characters such as the presence of nutritional components and nutraceutical determinants have gained increased traction. Postharvest traits of importance include resistance to prolonged storage and transport, rachis tolerance to oxidation and dehydration, low susceptibility of the berries to browning and spotting, as well as resistance to decay.

2.1 Application of biotechnology research to grapevine breeding and genetics

Research in grapevine genetics is restrained by the lack of genetic stocks, high heterozygosity, inbreeding depression, large space requirements, and the relatively long juvenile period. In 1957, De Lattin [9] summarized his work on 53 genes identified in *Vitis* sp. Research on grapevine genetics has intensified since the late 1950s, and yet until 1990 surprisingly only a few additional genes were located [10]. Molecular markers have facilitated research in *Vitis* genetics. It is now possible to map the grapevine genome and to create unique DNA profiles for each genotype. The first plant linkage maps were based on visually scored morphological markers. Later, isozymes—at least two enzymes with identical function but different structure- and DNA-based markers—which are virtually limited in number [11] were used to create densely saturated maps.

Genetic resources possessing genes for resistance to many fungal diseases were found within *Vitis* species, and, hence, the transfer of these genes to *V. vinifera* cultivars has been partially carried out [12]. However, the process takes many years, and it is rather difficult for breeding disease-resistant grapevines with commercial values from interspecific hybrids. Thus, genes that confer resistance to diseases are of special interest in improving and breeding grapevine cultivars.

2.2 Marker-assisted selection

Marker-assisted selection can be used for pyramiding genes for resistance. Genetic pyramiding is a process used for the development of new breeding lines

with homozygous resistance loci and consequently selecting new parental lines with the desired traits. To understand the potential value of molecular markers, it is imperative to identify the major markers. Isozymes have different electrophoretic mobility and, hence, can be visualized following gel electrophoresis. Over 20 polymorphic isozymes have been identified in grapes. Restriction fragment length polymorphisms (RFLPs) can be used for their rapid detection using restriction enzymes and involves cutting genomic DNA molecules at unique nucleotide sequences (restriction sites) yielding DNA fragments with varied sizes. However, identification of RFLPs requires a high concentration of DNA and could be relatively expensive to assay.

Polymerase chain reaction (PCR)-based assays are generally much less expensive and can reveal higher levels of polymorphism [11, 13]. The selection process of a DNA fragment for amplification involves "primer annealing" in which two primer pairs (5–30 bases long) complementarily bind onto genomic DNA strands in a reaction process. The primer-DNA complex is a critical step for the replication of adjacent DNA sequences by a thermostable polymerase supplied in the reaction mixture.

A commonly used PCR analysis is based on random amplified polymorphic DNA (RAPDs). These markers are based on the occurrence of an inverted pair of 9–11 base repeats (occasionally longer or shorter, as well) as within between 200 and 2000 base pairs. This is a single primer reaction that amplifies one-to-many segments of DNA through PCR. Amplified fragment length polymorphisms (AFLPs) are based on the selective amplification of restriction enzyme-digested DNA fragments. Multiple bands (50–100) are generated during each amplification reaction resulting in random DNA markers. Neither RAPDs nor AFLPs are "anchored," i.e., their primary use is within and not between crosses. On the other hand, several sequence-tagged site (STS) markers are useful as anchoring loci between crosses. The most important of these is a *microsatellite*, a simple sequence repeat (SSR) marker [11] based on the discovery of repeated sequences in the genome and usually 2–4 nucleotides in length (e.g., ... (GCC)~17~ ...). The bases flanking the repeat sequence are conserved, but the length of the repeat can vary greatly; SSR-specific primers can be readily designed. Each SSR is a single locus with multiple allele sizes.

2.2.1 RAPD markers

Genetic analyses have progressed rapidly since the discovery of polymorphic regions or loci with two or more alleles in genomic DNA [14]. Variation in location, copy number, length, and base pair sequence of these highly repetitive DNA regions provide a rich source of markers for unique identification. Random amplified polymorphic DNA analysis has been applied to several aspects of the winemaking process [15, 16]. Several investigators have attempted to discriminate between grape plant clones utilizing a variety of genetic typing techniques [17–23]. However, Regner et al. [24] utilized SSR, RAPD, and AFLP markers and were successful in detecting differences within clones of the Grüner, Veltliner, Pinot Blanc, Morillion, and Chardonnay varieties. Using RAPD markers, Moreno et al. [25] discriminated between clones of *V. vinifera* to a limited extent.

2.2.2 Microsatellites

Microsatellite genotyping requires the determination of the number of repeat units at a given locus in a given cultivar. This is achieved by electrophoretic sizing of the fragment containing the repeat region (the microsatellite allele), which was

amplified by PCR with primers situated upstream and downstream of the microsatellite DNA. The initial grapevine microsatellite study conducted by Thomas and Scott [26] at CSIRO Plant Industry, Australia, reportedly identified DNA isolated from 26 *V. vinifera* cultivars and 6 additional *Vitis* species as well as *Muscadinia rotundifolia*.

Since then researchers have accumulated microsatellite profiles of hundreds of grapevine cultivars from many different regions. The data is available in public databases (**Table 1**). Among the 19 chromosomes of grape genome from a homozygous line, PN40024, about 10,948 contained trinucleotide repeats, 4386 had tetranucleotide repeats, and 3347 had penta-nucleotide repeats [27].

2.2.3 Single-nucleotide polymorphism (SNP)

Single-nucleotide polymorphism (SNP)-based genetic markers have attracted significant attention when researchers are creating dense genetic linkage maps. SNPs are the most abundant class of polymorphisms, and they provide gene-based markers that may prove useful when identifying candidate genes of interest to be associated with quantitative trait loci. *V. vinifera* utilizes many SNP-based genetic markers and maps to them a framework of loci defined by SSR markers in the Syrah 3 and Pinot Noir cross. The markers are derived from *V. vinifera* collections of expressed sequence tags (ESTs) and bacterial artificial chromosome (BAC) end

Database name	Physical address	Internet address of public databases	Number of genotypes
European *Vitis* Database	IRZ, Siebeldingen, Germany	http://www.genres.de/eccdb/vitis/	In preparation
Grape Microsatellite Collection (GMC)	IASMA, San Michele, Italy	Not public	
Grape SSR database	Australian Wine Research Institute (AWRI)	Not public	
International *Vitis* Variety Catalogue	IRZ, Siebeldingen, Germany	http://www.vivc.bafz.de/index.php	46
SSR profiles (not searchable)	BOKU, Vienna, Austria	http://www.boku.ac.at/zag/forsch/grapeSSR2.htm	162
The Bulgarian Plant Genomics Database	Agrobioinstitute, Sofia, Bulgaria	http://bulgenom.abi.bg/AgroBioInstitute%20Selected.htm	76
The Greek *Vitis* Database	University of Crete, Heraklion, Greece	http://gvd.biology.uoc.gr/gvd/index.htm	298
The Swiss *Vitis* Microsatellite Database	University of Neuchâtel, Switzerland	http://hydra.unine.ch/svmd/	170
Ukrainian, Moldovan and Russian *Vitis* Database	Magarach Institute, Yalta, Ukraine	Not public	104
Vitis SSR database	University of California, Davis, USA	Not public	
Vitis SSR database	INRA Montpellier, France	Not public	

Table 1.
Existing public and unpublished databases of grapevine SSR profiles [28].

sequences available in the NCBI (with 149,691 EST sequences clustered into 15,194 unigenes and 30,832 BESs (http://www.ncbi.nlm.nih.gov)). In addition, SSR and AFLP markers were employed to increase the number of bridges between genetic and physical map considering specific markers used by the international grapevine community.

In grapes, polymorphic DNA loci are relatively frequent. Salmaso et al. [28] found a single SNP in every 116 bp in the coding regions of 25 genes using EST-derived primers in the analysis of seven *V. vinifera* cultivars. The high percentage of monomorphic regions (28% for EST, 19% for BES) is quite unexpected when compared with what has been reported in the literature on grape and can be explained in part by the preferential PCR amplification of one allele, which is due to mismatches between the PCR primer and the second allelic template [29]. On the other hand, coding sequences have a higher probability of being monomorphic due to a direct effect of selection in favor of sequence conservation. The addition of SNP-based markers can identify polymorphisms that are easy to locate from a database, which can be useful for evolutionary studies to significantly increase the density of the linkage map. This leads to an improved resource for high-quality mapping of quantitative trait loci, identification of candidate genes, and enhanced map-based gene isolation.

3. Grape genome sequence and its applications

The genome project was informed by the realization that the *Vitis* family is the most economically important crop worldwide due to its high value. However, its developmental biology is still poorly understood. Grape can be a potential model crop because it contains valuable genetic information that can be mined for the improvement of other fruit tree crops. As a result, an International Grape Genome Program (IGGP) was with the objective to sequence the grape genome. Research centers have been established globally in countries leading in grape production, such as France, Italy, Australia, Canada, Chile, Germany, South Africa, Spain, and the United States. Genetically, *Vitis* species have 38 chromosomes (n = 19) with fertile interspecies hybrids.

The first high-quality reference grape genome sequence was obtained from a Pinot Noir clone ENTAV 115, a variety grown in wide range of soil types to produce red and sparkling wines. The reference genome sequence information has been useful toward understanding its overall genetic organization, including the content of genes and the structural components of the DNA of the 19 linkage groups (LGs) of *V. vinifera*. A whole-genome shotgun sequencing and the Sanger sequencing method generated 12× coverage of the genome. This has been integrated with sequence reads generated by a scalable, highly parallel sequencing by synthesis (SBS) method with throughput significantly greater than capillary electrophoresis. The assembly has been improved through the addition of 4.2× coverage, including the addition of bacterial artificial chromosome end sequences that have improved the scaffolding of the sequenced contigs. The 4.2× coverage provided by SBS was crucial in the identification of polymorphic sites and resulted in closing most of the gaps between DNA contigs. This is the first project that utilized both the longer Sanger and shotgun sequence-based methods to determine the sequence of a large eukaryotic genome.

The estimated genome size of *V. vinifera* Pinot Noir clone ENTAV 115 is at least 500 Mb. Genomic sequences corresponding to 477.1 Mb were assembled in 2093 metacontigs, and 435.1 Mb were anchored to the 19 linkage groups. The number of predicted genes and pseudogenes is 28,352 of which 96.1% were assigned to

LGs. The assembled grape genome has predicted candidate genes with implicated traits relevant to grapevine cultivation, such as those influencing wine quality via secondary metabolites and those associated with extreme susceptibility of grapes to pathogens.

The NCBI taxonomy web portal for *V. vinifera* contains a summarized data for one of the common species used for wine production. The Ensembl Plants has produced a Grape Gene Index by analyzing the nucleotide sequences deposited at NCBI, and Release 12× of the index lists 29,971 unique coding genes. The gene ontology and metabolic pathway information for many of these sequences are also available at the TIGR site (http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?Species=grape).

Genetic maps have been produced [33, 61–64], and physical maps are being produced in several laboratories [65] with a consensus map in progress. A grape BAC library is available from the French National Resources Center for Plant Genomics (CNRGV). Affymetrix (http://www.affymetrix.com/index.affx) released a grape array that represents 14,000 *V. vinifera* transcripts and 1700 transcripts from other *Vitis* species that can be useful for gene expression analysis.

Qiagen (http://www1.qiagen.com) also released a new grape (*V. vinifera*) array-ready oligo set contains 14,562 probes of 70-mers representing grape gene transcripts. Probe design for the grape oligo set is based on sequence information from TIGR's Grape Gene Index (http://www.tigr.org/tdb/tgi).

4. Genetic transformation in grapevines

Genetic transformation offers new perspectives for introducing important traits like that of disease resistance into traditional *V. vinifera* cultivars. However, the most limiting factor for efficient transformation is the absence of high-yielding regeneration protocols. The regeneration of intact transgenic plants has been obtained for viticulturally important genotypes, such as Sultana, Shiraz, Dornfelder, Chardonnay, Merlot, and others [30–35]. Due to the high morphogenetic competence of embryogenic tissue, somatic embryos are often used as targets for transformation studies. Different types of explants have been tested for their ability to produce somatic embryos under inducing conditions such as anthers [36] and leaf discs [37]. The first transformation experiments with leaf tissue of grapevine cvs. Thompson Seedless and French Colombard [38] or rootstock varieties resulted in transgenic calli, which failed to regenerate [39].

Leaf disc derived embryogenic callus for grapevine cv. Koshusanjaku by Hoshino et al. [40], who subsequently established an *Agrobacterium*-mediated transformation system as one of the successful methods for genetic transformation. These researchers were able to induce the calli to regenerate embryos and intact transgenic plants. This protocol was later used for callus culture development and transformation of four important Indian *V. vinifera* cultivars by Das et al. [41]. The successful transformation of grapevine has also been reported using the *Agrobacterium*-mediated system by various researchers [42–44]. Genetic transformation of grapevine using direct DNA delivery via gene gun has also been reported [45].

4.1 Genomics and transgenic research

4.1.1 Flavor

In the past, various studies have been conducted on the origin and regulation of sugar and acid concentrations. Of these two processes, the regulation of acid levels

is probably well-understood. It is clear, for example, that the two most important acids, namely, tartaric acid and malic acid, have different origins. Tartaric acid is produced directly out of the sugar pool, while malic acid is probably formed by reactions of the Krebs cycle and phosphoenolpyruvate carboxylase (PEPcase) [46]. During ripening, malic acid is used for the synthesis of sugar and as a respiration substrate [47]. Less is known about the control of tartaric acid concentration, which is also far more slowly metabolized than malic acid. Recently researchers have identified two important wine quality genes in grapevine related to tannin synthesis. By looking at when and wherein the plant tannins are produced throughout berry development and comparing similar genes in tobacco and the model plant *Arabidopsis*, researchers have been able to pinpoint the grape genes for tannin production. Two separate genes known as *VvANR* and *VvLAR1* are responsible for the production of chemically different types of condensed tannins also known as proanthocyanidins (PAs) in grapes [48]. The discovery of these two genes has opened ways for modifying the content and composition of anthocyanins and tannins in grapes giving vine breeders the potential to control the levels of these important wine quality characteristics [48, 49].

4.1.2 Flavonoids

In grape, flavonoids are the major portion of soluble phenolics and represent the most concentrated natural antioxidants in the berry [50]. The predominant flavonoids occurring in grape berries and seeds belong to varied classes such as tannins, anthocyanins, flavan-3-ols, and flavonols [51]. These compounds in addition to phenolic acids (mainly benzoic and hydroxycinnamic acids) contribute in different ways and/or manner to organoleptic features of the wine and other by-products [52]. Flavonoids are synthesized along the general phenylpropanoid pathway by the activity of a cytosolic multienzyme complex loosely associated at the cytoplasmic surface of the endoplasmic reticulum. This pathway has largely been characterized in different plant species [53] but also in *V. vinifera* in which the expression of genes involved in flavonoid synthesis (particularly anthocyanins and proanthocyanidins (PAs)) has been well-characterized in berries and seeds of both red and white cultivars [54–56]. The patterns of gene expression show significant differences between organs and cultivars, especially for genes involved in anthocyanin synthesis. In red cultivars, all the genes are expressed in berry skin although with varied temporal patterns.

In berry pulp their expression is low, and phenylalanine ammonia-lyase (*PAL*) and UDP glucose: flavonoid 3-*O*-glucosyl transferase (*UFGT*) genes are not expressed [56]. These two genes code for enzymes involved in the first and in the last step of the anthocyanin pathway, respectively, whereas PAL allows the hydrolysis of ammonia from phenylalanine, and UFGT catalyzes the glycosylation of anthocyanidins to produce the anthocyanins (colored and stable products). The absence of *UFGT* has been reported in seeds [56]. On the contrary, studies concerning the expression of genes involved in flavonoid synthesis in white cultivars were performed only from berry skin. It was demonstrated that *UFGT* was not detectable and the expression of other associated genes was low in the skin of red cultivars especially during the early stage of berry development.

Anthocyanins are responsible for the red and white color in grapes. Grapes are primarily distinguished based on the level of anthocyanin in berry skin. Geneticists discovered that the grape skin color is controlled by two *MYB* genes (*VvMYBA1* and *VvMYBA2*). Although either can dictate the berry skin color, it was determined that mainly the *VvMYBA1* gene can activate anthocyanin biosynthesis in red grapes. The *MYB* gene (*VvMYBA2*) allele, present in white berries, is a mutant of the latter and

contains two distinct amino acid substitutions. Sequence analyses of the *VvMYBA2* gene found 55 white grape varieties. All grape varieties contained the same double mutations, suggesting they originated from a single common grape ancestor. Vvmyb5a, a cDNA from grape cv. Cabernet Sauvignon encoding an R2R3-MYB protein, has been cloned. Phylogenetic analysis has shown that Vvmyb5a protein belongs to a different group from VlmybA2 protein. The expression of *VlmybA* has been detected mainly in berry skin and flesh at the late stage of berry development, whereas the expression of *Vvmyb5a* was detected in both vegetative and reproductive plant tissues [57].

Vvmyb5a was overexpressed in tobacco under the control of the CaMV 35S promoter for use as a visual marker, an alternative to antibiotic markers in the screening of transgenic plants [58]. The overexpression of *VlmybA2* in tobacco seems to be promising for the visual identification of transformants. A versatile gene for anthocyanin production could be a good candidate for a simple and nondestructive visual marker during plant transformation [59, 60] and may replace controversial antibiotic marker genes. As VlmybA2 shows higher potential than other anthocyanin regulatory genes previously tested, its potential should be exploited in the genetically modified plant production process starting from the efficient recovery of transformants during transformation up to the monitoring of transgenic plants at the field level for risk assessment [61].

Until now, the models of flavonoid transport have been mainly based on genetic approaches where this process has been correlated to the expression of several specific genes in reproductive organs during development or in response to environmental factors. Limited information is available for direct identification and characterization of proteins involved in the uptake and accumulation of these metabolites. Therefore, it is crucial that future research should be more focused on the understanding of the biochemical mechanisms responsible for flavonoid transport and regulation [62].

4.1.3 Nutraceutical value

Muscadine (*Muscadinia rotundifolia*) is a native crop across the southern United States; it has natural adaptability, including resistance to diseases and insect pests, and has long vine life. Generally, it is underutilized due to its potential as a local flavor; for example, muscadines have a characteristic aroma and sweetness that makes them acceptable as table wines. In addition, it has high levels of polyphenols with potential benefits to human health. Resveratrol (3, 4, 5-trihydroxystilbene) is one of the important phenolic compounds found in muscadine grapes. Most red wines contain measurable concentrations of resveratrol; however, their concentration varies among the cultivars [63]. Resveratrol is considered a biochemical precursor of viniferin, a major stilbene phytoalexin [64]. Resveratrol is known to be synthesized by stilbene synthase (STS), a condensation enzyme with numerous biological properties in muscadines. The enzyme utilizes 4-coumaroyl-CoA (or another phenylpropanoid CoA-ester) and undergoes a three-step condensation process with malonyl-CoA, resulting in an enzyme-bound tetraketide intermediate during resveratrol synthesis.

Previous [65] study conducted at the Center for Viticulture and Small Fruit Research, Tallahassee, Florida, which involved the analysis of metabolites in local grape varieties with high-performance liquid chromatography (HPLC), determined a high phenolic content in muscadines as compared to bunch and Florida hybrid bunch grapes [65]. Grape seed extracts from some muscadine grape cultivars showed high anticancer activity. Characterization of these compounds confirmed the presence of resveratrol. One other advantage of resveratrol is its *trans*-isomer

state in muscadines, which is considered the most active form. Six isoforms of the stilbene synthase gene have been isolated from the muscadine grape cv. "Regale." Out of the six isoforms, four were found to be unique to muscadine, with more than 40% sequence dissimilarity with *Vitis* STS. In silico analysis of one of the isoforms revealed that the deduced protein sequence has a signal peptide [66]. Further analysis using reverse transcription and quantitative real-time PCR determined that one (MS 1) of the four unique stilbene isoforms has expressed at high levels during the different stages of muscadine berry development. Further characterization of the gene encoding stilbene synthase will help determine fingerprints of muscadine cultivars with higher resveratrol content and will also help in the development of muscadine cultivars with high expression of resveratrol.

4.1.4 Disease control

Currently, *Vitis vinifera* is the major species cultivated due to its high quality for wine production. However, *V. vinifera* is susceptible to diseases and pests. Fungal infestation is a major problem in grapevine production worldwide. Two fungal pathogens, causing powdery and downy mildews, respectively, are a major threat in grape production. The fungal pathogens spread to Europe during the nineteenth century along with infested accessions of the American wild *Vitis* species in which they were endemic [67]. In general, fungal infestation leads to the decreased yield and impacts berry and wine quality through the reduction in plant vitality and productivity or by the direct infection of berries. Disease control can be achieved by the application of fungicides. However, the economic costs and negative environmental impacts associated with these applications have informed the impetus in current research for alternative strategies involving the understanding of how to manipulate host defense mechanisms. Grapevines with improved disease resistance would be welcomed, especially if other traits were not altered. The reduction of pesticide sprays by between one and two percent in a year would cut the cost of production and is also beneficial to the environment [68]. Major pathogens that infect grapevine are listed **Table 2**.

Causal agent	Properties of pathogen	Disease	Specific characters of disease
Uncinula necator	Obligate biotrophic fungus	Powdery mildew	The most economically important disease of *Vitis vinifera* worldwide
Plasmopara viticola	Obligate biotrophic oomycete	Downy mildew	Affects *V. vinifera* worldwide
Botrytis cinerea	Necrotrophic fungus	Grey mold rot	One of the most common and widely distributed grapevine diseases
Elsinoe ampelina	Non-obligate fungus	Anthracnose	Affects *V. vinifera* and its hybrids in tropical and subtropical regions
Phomopsis viticola	Non-obligate fungus	Phomopsis cane blight and leaf spot	A wood disease
Fusicoccum aesculi	Non-obligate fungus	Excoriosis	A wood disease
	Ascomycete fungus	Eutypa dieback	A major grapevine disease in many countries that infects the vine stock; a wood disease

Table 2.
The major widespread and economically important pathogens affecting grapevines worldwide [69].

Knowledge and experience in the field of genetically engineered grapevines have increased enormously. Several ongoing projects are aimed at the improvement of transformation efficiency, allowing its use as a standard strategy for various purposes. The great interest in the transgenic approach is due to its capability to establish disease tolerance or resistance in both elite grapevine varieties and rootstocks without changing their genotype-specific traits. Progress made in grapevine genomics along with the availability of reference genome sequence obtained from Pinot Noir [70] has made the transgenic approach attractive for both basic research and functional genomics. Included herein is a list of major transgenic disease-tolerant plants, which are currently in field trials in the last 5 years for improved bacterial and fungal resistance (**Table 3**).

Institution	Received	Status	Gene(s)	Phenotype(s)	Release location	Acreage
Cornell University	07/21/09	Issued	Coat protein—donor: Grapevine fan leaf virus resistant	Grapevine Fan leaf Nepovirus Resistant	CA, USA	4
University of Florida	09/27/07	Acknowledged	*Aequorea victoria/E. coli* lytic peptide gene for bacterial resistance	*Xylella fastidiosa* Resistant	FL, USA	1.1
University of Florida	09/27/07	Acknowledged	Lytic peptide gene for bacterial resistance *E. coli* Endogenous gene for fungal resistance	Powdery Mildew Resistant BR—*X. fastidiosa* Resistant	FL, USA	1.1
University of Florida	09/27/07	Acknowledged	Endogenous gene for fungal resistance—grape lytic peptide gene for bacterial resistance	Powdery Mildew Resistant BR—*X. fastidiosa* Resistant	FL, USA	1.1
University of Florida	09/27/07	Acknowledged	Lytic peptide gene for bacterial resistance	*X. fastidiosa* Resistant	FL, USA	1.1
University of Florida	09/27/07	Acknowledged	Lytic peptide gene for bacterial resistance	*X. fastidiosa* Resistant	FL, USA	1.1
University of Florida	09/13/06	Acknowledged	Synthetic lytic peptide gene Grape thaumatin-like protein gene	Fungal Resistant, Bacteria Resistant	FL, USA	
University of Florida	09/13/06	Acknowledged	Neomycin phosphotransferase (NPTII)* Synthetic lytic peptide gene cercopin of Silkworm Grape thaumatin-like protein gene	Fungal Resistant, FR—Fungal Resistant, —Bacteria Resistant	FL, USA	

Institution	Received	Status	Gene(s)	Phenotype(s)	Release location	Acreage
University of Florida	09/13/06	Acknowledged	Synthetic lytic peptide gene cercopin of Silkworm Grape thaumatin-like protein gene	Bacteria Resistant, Fungal Resistant	FL, USA	
State University of New York	08/02/06	Acknowledged	Lignan biosynthesis protein from peas	Powdery Mildew Resistant	NY, USA	1
Cornell University	03/03/06	Acknowledged	Antimicrobial peptide from *Amaranthus caudatus* Magainin from *Xanopus laevis* NptII*	Pathogen resistant	TX, USA	0.1
State University of New York	04/11/05	Acknowledged	Lignan biosynthesis protein	Powdery Mildew Resistant	NY, USA	1

Source: http://www.isb.vt.edu/cfdocs/fieldtests2.cfm

Table 3.
Transgenic grape plants in field trails resistance to major pathogens.

4.1.4.1 Downy mildew

Different strategies and genes have been used in genetic engineering to enhance resistance to major plant pathogens [71]. Expression of a fungal endochitinase gene in cv. "Chardonnay" led to reduced symptoms of powdery mildew and *Botrytis* bunch rot [72]. Another strategy, in this regard, involves the use of antimicrobial peptides (AMPs). These are natural defense compounds found in many organisms ranging from bacteria to humans and plants, which protect the host from pathogens. Among these compounds include magainins [73] isolated from the skin of an African clawed frog, *Xenopus laevis*. Magainins and their analogs are small (c.a. 21–26 amino acids long) cationic peptides with α-helical structure with broad-spectrum antimicrobial activity, which was demonstrated in vitro and led to the inhibition of growth of bacteria and fungi [74, 75], including major grapevine pathogens such as *A. tumefaciens* [76]. Magainins have a strong affinity for microbial membranes due to the high concentration of anionic phospholipids in the outer bilayer of their leaflet. They confer very low toxicity to animal and plant cells due to the presence of cholesterol or other sterols in the host membranes [74]. Magainins can disturb cell membrane function either by forming ion channels or by depolarizing the membrane leading to leakage of metabolites and cell death [77]. The selective activity of magainins and their synthetic derivatives on microbial membranes as well as their simple amino acid sequence enables them to be potential candidates for genetic engineering for disease-resistant plants [78]. Recently, transgenic plants with expressed high levels of magainin peptides exhibited significant resistance to a broad range of fungi and bacteria, including pathogens causing botrytis and powdery mildew diseases [79].

The expression of synthetic magainins, such as Myp30 [80] and MSI99 in transgenic plants via either the chloroplast genome [81] or in the nuclear genome [82], led to enhanced resistance against bacterial and fungal pathogens. The studies

in "Chardonnay" (*V. vinifera*) have shown stable transformation and expression of either the natural magainin-2 (mag2) or the synthetic derivative (MSI99) gene under the control of *Arabidopsis* ubiquitin-3 promoter. Some transgenic lines exhibited enhanced resistance to crown gall and powdery mildew diseases in the greenhouse. Data suggested that the expression of magainin-type genes can confer resistance to the bacterial disease, crown gall, and moderate symptom reduction in the fungal disease, caused by powdery mildew pathogen.

4.1.4.2 Pierce's disease

The most devastating diseases in the southeastern United States include Pierce's disease commonly present on bunch grapes and anthracnose that infects Florida hybrid bunch grapes (**Figure 1**) [83]. Pierce's disease, caused by the bacterium *Xylella fastidiosa*, is a serious bacterial disease of grapevines. It is spread by certain types of xylem feeding leafhoppers (Cicadellidae) known as sharpshooters. The bacterium is an obligate pathogen that lives in the xylem tissue of a wide variety of plants. The bacteria, *X. fastidiosa*, are limited to the xylem or water-conducting vessels of plants. Symptoms begin to develop about midsummer as the bacteria block these vessels leading to dry or scorched leaves. Leaves become chlorotic along the outer edges or adjacent to the dead tissue. The drying or scorching of the leaf continues for a few days to weeks until the leaf eventually falls off, leaving only the petiole attached to the cane. Petioles gradually die back and fall. Maturing canes are tan in color with green islands along the infected sections. When new vegetative growths occur on infected canes, they are delayed and are usually stunted. Leaves on stunted shoots have a yellow mottling color between the major veins. Depending on the grape variety, death of the entire vine usually occurs in 1–5 years.

A study was conducted at the Center for Viticulture and Small Fruit Research, Tallahassee, Florida, USA, to understand the molecular basis of Pierce's disease tolerance by employing subtractive hybridization (SH) and real-time PCR for the detection and characterization of transcripts, which are differentially expressed in the xylem tissue challenged by PD bacterium. Results obtained from the SH analysis of 300 partial cDNAs indicated high to moderate expression patterns in PD-tolerant *cv*. "Blanc du Bois" (Florida hybrid) and "Zinfandel" (bunch grape) subtracted with PD-susceptible bunch cv. "Pinot Noir." The expression patterns of selected genes and their potential association with PD tolerance were analyzed using real-time PCR. Research showed that enolase expression, an enzyme that has been associated with bacterial activity, was high in PD-susceptible cultivar. The PD-susceptible

Figure 1.
Common diseases of grapes. Note: The picture of a downy mildew infection is from Ya Li Zhang, China Agricultural University, Beijing, China.

cultivar was severely infected by *X. fastidiosa.* The expression of defense-related enzymes, such as chalcone synthase, s-adenosyl-L-methionine synthase, chitinase, PR genes, adenosine kinase, quinine reductase, and translationally controlled tumor protein, was observed in PD-tolerant grape cultivars. Also, PD-tolerant cultivars showed high expression of protease inhibitor and stilbene synthase mRNA, which suggested that the presence of *X. fastidiosa* had influenced the expression levels of transcripts associated with signal transduction and host defense more significantly in PD-susceptible cultivars than in PD-tolerant cultivars. Early expression of these genes in PD-tolerant cultivars postinfection with *X. fastidiosa* may have modulated tolerance in the grape cultivars.

Transgenic Pierce's disease-resistant plants have been developed using polygalacturonase-inhibiting proteins (PGIPs) and antimicrobial peptides. PGIPs are plant cell wall proteins that specifically inhibit fungal endo-polygalacturonases (PGs) that contribute to an aggressive decomposition of susceptible plant tissues. The inhibition of fungal PGs by PGIPs suggested that PGIPs have a role in plant tolerance to fungal infections, and this has been confirmed in transgenic plants expressing PGIPs. The bacterium, *X. fastidiosa*, the causal agent of Pierce's disease (PD) in grapevines, contains genes encoding cell wall-degrading enzymes, including a putative PG enzyme. Research hypothesis suggested that PGIP expression can confer tolerance against both *X. fastidiosa* and *Botrytis cinerea*. This hypothesis was tested by transforming cvs. "Thompson Seedless" and "Chardonnay" with *V. vinifera.* The transformants had a pear fruit PGIP-encoding gene (*pPGIP*) under the control of the CaMV 35S promoter. Results determined substantial pear PGIP (*pPGIP*) activity in crude extracts of grape leaves and xylem exudates of transgenic lines obtained from independent transformation events but were not present in untransformed controls. Also, *pPGIP* activity was detected in the xylem exudate of untransformed scions grafted on to transgenic rootstocks expressing *pPGIP* [84].

AMPs are particularly effective against bacteria since they disrupt cell membranes. To date, transgenic plants with antimicrobial peptides have been generated. The plants included 76 "Chardonnay" lines and transformed with two magainin-type genes, mag2 and MSI99, as well as a PGL class gene. The primary objective of the research was to study the potential resistance to Pierce's disease of magainin- and PGL-producing vines. A newly designed antimicrobial peptide was developed based on natural antibacterial toxins. For example, shiva-1 peptide was designed with a significantly different sequence from natural cecropin B (46% homology) [85, 86]. A more advanced generation of lytic peptides was based on the synthesis of newly designed antimicrobial peptides instead of its natural antibacterial toxin [86]. The design of synthetic antimicrobial peptides with predetermined structures and properties led to improved stability of these gene products and enhanced their protection property against proteases in the transformed plants.

In another research with the objective to control Pierce's disease, Aguero et al. [87] studied transgenic plants of grape cvs. "Chardonnay" and "Thompson Seedless" by expressing the pear polygalacturonase protein (pGIP). They reported a delayed development of Pierce's disease in some of the transgenic lines. The lines had reduced leaf scorching, lower titers of *X. fastidiosa*, and better regrowth after pruning than untransformed control lines. Two US patents have been granted to Scorza and Gray [88, 89] for the method of production of transgenic grapevine cv. "Thompson Seedless" and expression of the lytic peptide cecropin B and shiva-1 peptides that improved resistance to the bacterium *X. fastidiosa*. The authors suggested that the expression of magainin-type antimicrobial peptides in grapevines was likely more effective toward the control of bacteria than fungi.

4.1.4.3 Anthracnose

Genetic resources possessing genes for resistance to many fungal diseases have been found in *Vitis* species, and therefore, the transfer of these genes to susceptible *V. vinifera* cultivars has been successfully carried out to a certain extent [12]. However, the process can take several years, and it is rather difficult to breed disease-resistant grapevines with the commercial value from such interspecific hybrids. It has been revealed that plants have innate defense mechanisms that involve pathogen-related proteins, e.g., chitinase [90, 91] and β-1,3-glucanase [92], to control pathogen infection. Genes encoding hydrolytic enzymes, such as chitinase, which can degrade fungal cell wall components are potential candidates for the improvement of disease resistance. The rice chitinase *RCC2* gene could be utilized as a genetic source for disease resistance, leading to breeding and improvement of resistance in grape species. Transgenic grapevines with the *RCC2* gene were tested for resistance to the fungus, *Elsinoe ampelina*, which causes anthracnose disease. Fungal conidiophores were unable to germinate at the initial phase of infection, but transgenic plants exhibited severe symptoms during the later stages of incubation [93]. Conversely, rice chitinase *RCC2* was unable to confer resistance to anthracnose disease.

Recently, researchers at the Center for Viticulture and Small Fruit Research, Tallahassee, Florida, USA, successfully identified genes/gene products from Florida hybrid grape that are uniquely expressed in response to anthracnose infestation postinoculation with pure cultures of *E. ampelina* isolated from field-grown grape plant cv. "Blanc du Bois." Differential display RT-PCR analysis identified several unique genes induced upon *Elsinoe* infection in anthracnose-tolerant hybrid bunch grape. Sequencing and characterization of these genes revealed their similarity with chitinase III, PR 4 and 10, chalcone synthase, and stilbene synthase. Further, real-time PCR analysis revealed that the expression patterns of gene-encoding enzymes, such as chitinase and stilbene synthase, were higher than that of other genes during *E. ampelina* infestation [94]. These genes are known to play an important role in plant defense against fungal pathogens. Expression of these genes was rapid in anthracnose-tolerant Florida hybrid bunch grape cultivars upon *Elsinoe* infection as compared to anthracnose-susceptible cultivars. Chitinase gene (antifungal) expression was completely absent in susceptible cultivars upon *Elsinoe* infection but showed a low-level expression level of the stilbene synthase gene. In contrast, the tolerant cultivars appeared to have maintained the expression of these defense-related genes in order to overcome the adverse effects of infection. Further study of these uniquely expressed genes should inform the understanding of the basis of their specific role in anthracnose tolerance. A great deal of effort over the past several years has been made to understand the plant-pathogen interaction. This has provided an excellent framework for the identification of over 100 genes associated with disease resistance which has been incorporated in numerous ongoing transgenic research initiatives.

5. Conclusion

The grape industry must maintain and expand grape production despite increasing constraints caused by pests, diseases, and abiotic stressors. Biotechnology represents one of the most promising approaches that can bridge the knowledge gap that exists since it is crucial for the introduction of single gene determinants with defined phenotypic traits. In addition to aiding the production of grapevine varieties (disease-resistant and stress-tolerant), biotechnology has also contributed to the

modification of numerous quality traits, such as color, flavor, ripening characteristics, and modulation of specific metabolites with potential health benefits. Multiple genes for disease resistance and/or modification of quality traits should be inserted simultaneously into grape cultivars. However, researchers are still concerned that the product of a single gene could readily be overcome by virulent pathogens. New genes are being sought from grapevines and other close relatives with an attempt to create a comprehensive genetic gene pool for the improvement of grapevines. Genetically altered vines should be subjected to stringent field testing to assure the public that ultimately the technology will be safe and will not alter essential traits of both the vine and the fruit. These advantages can only be realized if concrete strategies are put in place to overcome potential technical hurdles. Strategies should be put in place that involve a comprehensive regulatory framework to improve the general public acceptance of such biotechnology-derived foods.

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Author details

Devaiah Kambiranda*, James Obuya and Janana Snowden
Plant and Soil Science, Southern University Agriculture Research and Extension Center, Baton Rouge, LA, USA

*Address all correspondence to: devaiah.kambiranda@gmail.com

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