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Title: Precision breeding of grapevine (*Vitis vinifera* L.) for improved traits

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Abstract

This review provides an overview of recent technological advancements that enable precision breeding to genetically improve elite cultivars of grapevine (Vitis vinifera L.). Precision breeding, previously termed “cisgenic” or “intragenic” genetic improvement, necessitates a better understanding and use of genomic resources now becoming accessible. Although it is now a relatively simple task to identify genetic elements and genes from numerous “omics” databases, the control of major agronomic and enological traits often involves the currently unknown participation of many genes and regulatory machineries. In addition, genetic evolution has left numerous vestigial genes and sequences without tangible functions. Thus, it is critical to functionally test each of these genetic entities to determine their real-world functionality or contribution to trait attributes. Towards this goal, several diverse techniques now are in place, including cell culture systems to allow efficient plant regeneration, advanced gene insertion techniques, and, very recently, resources for genomic analyses. Currently, these techniques are being used for high-throughput expression analysis of a wide range of grapevine-derived promoters and disease-related genes. It is envisioned that future research efforts will be extended to the study of promoters and genes functioning to enhance other important traits, such as fruit quality and vigor.

Keywords: Grapevine, Vitis vinifera, cisgenics, intragenics, genetic engineering, disease resistance, anthocyanin marker, expression analysis

Introduction

Genetic improvement of grapevine (Vitis vinifera L.) is one of the critical needs to enhance crop productivity and foster profitable wine industries throughout the world [1]. Although numerous unique hybrids were developed over the years, genetic improvement of elite hybrids, the mainstays of worldwide production, is deemed to be largely unsuccessful, especially in areas ravaged by severe disease/pest infestations and/or that require extensive chemical control to maintain. For example, the bacterial pathogen that incites Pierce’s disease, Xylella fastidiosa, has no proven method of durable control other than well-known genetic resistance and the
unsustainable mass spraying of pesticides to inhibit insect vectors, despite well over 50 million dollars expended, apparently unsuccessfully, by Federal and State governments since 1999 [2]. However, genetic resistance (tolerance) among native Vitis species was identified by 1958 [3]. The practical use of genetic resistance was subsequently confirmed through hybridization with V. vinifera cultivars to instill durable and near complete control of the pathogen [4-6]. Particularly urgent now is the introduction of specific traits for durable tolerance to diseases, pests, and abiotic stresses, while maintaining the essential quality of highly desired elite cultivars [7,8]. However, it is not possible to rely on conventional breeding to improve elite cultivars so that they can adapt to the production environment, while still meeting the strict expectations of oenophiles [9,10]. Conventional breeding cannot practically be used to add desired disease resistance traits to elite cultivars of Vitis because of a long lifecycle, severe inbreeding depression, and complex genetic control of enological qualities [11]. A majority of the relatively few elite grape cultivars currently cultivated worldwide are centuries-old and maintained primarily through a stringently managed system of vegetative propagation [12,13]. However, elite cultivars often lack other desirable traits such as durable disease and pest resistance that are demanded by today's intensive agricultural conditions. As such, producers rely on frequent use of pesticides to control diseases, particularly in areas of higher humidity; this is in spite of increasing public outcry against such practices and resulting environmental issues [14]. To mitigate such increasingly crucial agricultural and health concerns, modern biotechnology has advanced to the point where it is now possible to expedite genetic improvement of existing elite cultivars via precision breeding [7,15]. This review is intended to provide an overview of current technological advancement, particularly genomic analyses, for the development of resistance to biotic and abiotic stresses, as well as other traits, via precision breeding of elite cultivars.

1. Advances in gene insertion technology

The methodology to insert specific genes into plants without inducing significant genetic rearrangement has been in development for over thirty years. Such technology is particularly attractive for perennial crops like grapevine that have severe genetic obstacles to conventional breeding and require multi-year evaluation for durability of desired traits due to their long lifecycle and longevity. In order to provide a reliable working platform for genetic testing,
efficient cell regeneration systems were developed [16-20]. An increasing number of scientists used such regeneration systems to document insertion of single or few genes into grapevine [11]. The precise methods of gene insertion employed either biolistic particle bombardment [21, 22] or, more commonly, *Agrobacterium*-mediated gene insertion into regenerative cells, followed by plant recovery [11, 23-25]. Both methods have been meticulously refined and optimized over the years and are now capable of producing hundreds of genetically modified plants. The majority of plants modified via the *Agrobacterium* approach tended to harbor low-gene copy number and defined gene insertion [24, 26]. A large number of modified plants is critical for identification of lines with a desirable level of gene expression and performance to meet overall improvement objectives [27]. The need to test many plant lines, as is the norm with conventional breeding, is critical in order to select outstanding individuals.

During the early years of technology development aimed towards precision breeding, it was necessary to test genetic elements from non-plant hosts, including animals and bacteria, due to the relatively primitive state of biotechnology. This approach was generally referred to as “transgenic” modification. This early discovery research was absolutely essential so that cell culture and gene insertion methods could be refined to the point of being fully functional [28]. Subsequently, as pointed out by Rommens [29], many non-plant genes and promoters with known functionality were utilized to display the technological marvel of biotechnology. This approach to crop improvement inevitably invited arguments and ongoing worries as to whether such plants with foreign genes and promoters were healthy, represented an environmental threat and/or were otherwise dangerous in some way. The use of foreign genetic material in food crops including grapevine remains to be the pivot of social and ethical public debate [7,29,30].

Along with the refinement of cell culture and gene insertion methods, the final technology required to enable precision breeding was completion of the genomic sequence of *V. vinifera* ‘Pinot Noir’ in 2007 and the relatively new-found and simplified availability of computational analysis [31,32]. It is now possible to identify grapevine genes, along with their associated genetic elements, isolate them, from sexually-compatible disease-resistant relatives, and insert them into elite cultivars. While still in its infancy, the application of precision breeding to grapevine improvement is well underway, with a number of modified plants in approved field trials and more on the way [1, 33-35].
Application of precision breeding is the logical and biologically conservative extension of conventional breeding, made possible only by long-term scientific research. Studies have suggested that application of precision breeding will boost consumer’s confidence and acceptance of improved crop products as well [36-38].

As we continue to refine precision breeding, more remains to be discovered. We require a better understanding of genome structure organization and sequence/function associations. It is estimated that grape genome contains over 30,400 genes, which is more than that found in most animals [39]. Many important agronomic traits are controlled by a complex network of regulatory sequences and factors and often influenced by dynamic sequence alterations, such as gene duplication, transposon insertion and loss- or gain-of-function mutations [40]. Environmental factors also play an important role in gene expression and interaction [41]. Thus, the actual function and sustainability of any isolated genetic material, whether a gene or a promoter, has to be confirmed within its intricate genetic milieu and then rigorously tested over a prolonged time in the environment; this is the fundamental way to determine durable structure/function relationships. Although we are making rapid progress in sequence analysis and functional annotation of the grapevine genome [31,32,42-44], progress in functional characterization of important genes/promoters is slow, creating a significant obstacle to the practical utilization of the genetic resources already available. Since precision breeding is biologically consistent with conventionally bred crops and, indeed, the entire plant lifecycle, it constitutes a technical refinement of existing breeding methodologies. The solution to accelerating the crucial functional analyses needed to both test the technology and produce improved cultivars is to not regulate evaluation of precision bred grapevine, so that individual lines can be tested in quantity and in grower fields, as has always been the manner for conventionally bred crops. As with all crop breeding, many progeny must be evaluated to find the desired individual(s) expressing the desired traits.

2. Development of a grape gene-based marker system

2.1 Traditional marker genes
Marker genes are utilized to facilitate identification and selection of engineered cells; which is akin to finding a needle in a haystack. Such genes may provide growth advantages for modified cells to outgrow the large number of non-modified cells and/or signify the successful integration of new genetic elements [45]. Presently, the markers in use are not derived from the grapevine genome. Among these genetic marker genes, some confer resistance/tolerance to antibiotics or cytotoxic chemicals such as herbicides. Others encode selectable metabolic enzymes such as visible green fluorescence protein (GFP) and the assayable β-glucuronidase (GUS) enzyme [30, 46, 47]. These marker genes were also successfully tested in grapevine [21, 25, 48]. Thus far, the majority of previous genetically modified grape plants were generated primarily by using the \textit{NPTII} gene for kanamycin-based selection and GFP for visual selection; this allowed for the identification and selection of modified cells, which typically are present in very low numbers.

Although current regulatory guidelines recognize \textit{NPTII}, among other microbe-derived marker genes used in modified plants, to be safe, they are no longer needed and their use continues to remain an issue causing polarized public debate and governmental regulation [45].

Recently, a co-transformation system was tested to achieve marker-free genetically modified grapevine [26]. In this system, two strains of \textit{Agrobacterium} were used to treat target explants: one strain harboring a binary vector containing the target gene expression unit only, whereas the other strain contained a binary vector with a dual gene \textit{codA/nptII} selection marker expression unit. By using a brief exposure to kanamycin, modified cells with the target gene are encouraged to grow along with cells containing the dual gene cassette. Upon applying negative selection based on the \textit{codA} function, the \textit{codA/nptII}-containing cells cease to grow and the only survival is from target gene-containing cells. Although the efficiency of plant recovery needs further improvement, this system offers a working example of marker gene elimination for perennial plants such as grapevine.

2.2 Grapevine gene-derived anthocyanin marker system

A grapevine gene, \textit{VvMybA1} isolated from ‘Merlot’ was recently tested as a new visible reporter marker for non-destructive and quantitative analysis of gene expression [49, 50]. \textit{VvMybA1} belongs to the MYB transcription factor superfamily functioning in the grapevine anthocyanin biosynthesis pathway. It activates the expression of UDP-glucose flavonoid 3-O-
glucosyltransferase (UFGT), an enzyme catalyzing the final reaction in the modification and stabilization of anthocyanin [51]. The ectopic expression of VvMybA1 results in production of anthocyanin, an easily discernible coloration in otherwise non-pigmented cells/tissues. VvMybA1 can serve as visual reporter to identify modified cells and subsequent regenerants, which was first demonstrated by the recovery of stably transformed grapevine without relying on the use of NPTII expression and kanamycin selection [49]. However, the VvMybA1-expressing grapevines ('Thompson Seedless') that were recovered lacked vigor and did not persist in the field environment. These plants produced intensely pigmented, curly and highly brittle leaves as a result of the over-accumulation of non-recyclable anthocyanin in cell vacuoles [49]. Such undesirable phenotypic consequences of VvMybA1 as a reporter marker now are being mitigated by employing promoters with tissue-specific and/or developmentally regulated expression capability to avoid unwanted hyper-accumulation [52]. Recently, VvMybA1 was placed under control of a late embryogenesis abundant protein Dc3 gene promoter of carrot (Daucus carota) and shown to be capable of rendering pigment production exclusively in embryos but not in vegetative tissues of citrus [53,54]. The tissue-specific expression strategy may provide a solution to utilize VvMybA1 as an indicator marker. Current endeavors in the discovery and mining of grapevine promoters should take such findings into consideration. An effective plant-based marker system eliminates reliance on microbe-derived genes and/or complicated and inefficient gene elimination technologies for the development of genetically modified grapevine [46].

In addition to its potential as a visible selectable marker, VvMybA1 currently is used for real-time monitoring of transgene expression at the whole plant level. Quite often the expression of previously-used marker genes varied greatly between plants and throughout the entire course of plant development due to factors associated with gene integration and environmental interactions. Using multiple modified plant lines with a consistent level of gene expression, facilitates the accurate assessment of trait analyses. Reporter markers such as GUS and GFP are widely utilized to monitor transgene expression [55, 56]. However, there has been no suitable marker for expression analysis at the whole plant level that does not require special equipment, detection reagents or destructive assays, and that can be readily adapted to high-throughput experiments involving selection of hundreds of genetically modified grapevines.
Anthocyanin is a self-revealing pigment that can be easily discerned with the naked eye due to its vibrant pink-to-red color. It also remains relatively stable in vivo and accumulates in insoluble anthocyanic vacuolar inclusions (AVIs) [57]. As illustrated previously, pigmented inflorescences as a result of VvMybA1 expression were consistently observed in modified tobacco [49,50]. This unique utility demonstrated that VvMybA1 provides a highly efficient and reliable monitoring system for gene activity in grapevine. Currently VvMybA1 has been incorporated into transformation vector platforms controlled by a constitutively active grapevine-derived ubiquitin gene promoter for high throughput analysis of genes and promoters [50].

3. Promoter mining and functional analysis

3.1 A non-destructive anthocyanin-based promoter assay system

Promoters play an indispensable role in the regulatory process of gene expression [58]. The employment of appropriate native promoters for precision breeding dictates the final outcome of trait development and durability. In recent years, extensive analysis of the genome and transcriptome uncovered a plethora of functional genes. For instance, the sequence identity of at least 18,725 grapevine genes have been confirmed [44]. The functional annotation of these genes also provided a significant opportunity to evaluate their promoters, which direct gene expression in response to developmental, environmental and regulatory network cues. For better utilization, it is critical to acquire a thorough understanding of the important features of these promoters including their sequences, activation capacity, regulatory interaction and stability throughout plant development. However, over the years, progress in functional analysis of promoters of grapevine has lagged behind other academically or economically important plant species such as Arabidopsis and rice. Some major reasons for such stagnation include the limited ability to initiate and maintain a large quantity of suitable explants and the lack of reporter systems for efficient expression analysis. Currently, only a few labs worldwide are capable of transforming grapevine, but many are still facing major obstacles in efforts to expand the efficacy to the level comparable to that in other model plant species. Furthermore, use of non-grapevine-derived reporter markers like GFP and GUS are hampered by requirement of a large quantity of explant materials and by laborious destructive assay procedures.
In order to expedite progress, the nondestructive reporter marker \textit{VvMybA1} in combination with an efficient Clonase cloning system (Life Technologies, CA, USA) significantly facilitates characterization of promoters from grapevine [49]. The amount of anthocyanin accumulation in grapevine or tobacco explants due to \textit{VvMybA1} expression correlates with the expression activity of a controlling promoter. Such correlation is readily determined using a non-destructive, quantitative color histogram analysis method in both transient and stable expression materials [49]. The expression reporting capability of \textit{VvMybA1} conforms to the common criteria for previously used quantitative reporter markers [45,59]. The \textit{VvMybA1}-based expression assay system offers an efficient and versatile alternative for high-throughput promoter analysis in grapevine and other plants.

3.2 Constitutively active promoters

Early studies inserted useful genes into grapevine under control of constitutive viral promoters to provide high levels of gene expression at the whole explant/plant level [18,23,55,56]. These promoters were used to drive the expression of either marker genes for identification of genetically modified plants or of target genes for trait development, mostly aimed at increasing disease resistance in modified plants. To eliminate the need for such constitutive viral promoters, a number of putatively constitutive ubiquitin gene promoters were identified and their expression capabilities characterized using the anthocyanin-based assay method [50]. The study revealed that among 7 \textit{VvUb} promoters analyzed, many were inactive and appeared to be older in evolutionary lineage, with a higher level of sequence abnormality, whereas two highly active promoters, \textit{VvUb6} and \textit{VvUb7}, were identified that possessed fewer nucleotide deletions or substitutions between cultivars and contained more \textit{cis}-acting elements, which are commonly involved in up-regulation of gene expression. These latter promoters supported an activity level equivalent to or higher than that of the commonly-used double-enhanced CaMV 35S promoter in both transient and stable expression analyses [50]. Such constitutively active grapevine promoters provide a key tool needed to add durable traits via precision breeding technology.

3.3 Tissue-specific, inducible and developmentally regulated promoters
Tissue-specific promoters activate gene expression only in particular types of cells, tissues, or organs, whereas inducible and developmentally regulated promoters are activated in response to specific cues and at certain stages of plant development, respectively. The development of a novel trait using precision breeding necessitates the availability of plant regulated promoters. For instance, engineering a fruit quality trait requires the use of a fruit-active promoter such as the *V. vinifera* thaumatin-like protein (*VvTL-1*) gene promoter [60]; likewise, improvement of root growth requires root-specific promoters. Thus far, only an extremely limited number of regulated promoters in grapevine are known. They include a seed-specific 2S albumin gene *VvAlb1* promoter, two alcohol dehydrogenase gene promoters; a stilbene synthase gene promoter and a number of PR1 gene promoters [50,52,61-63]. The lack of characterized regulated promoters limits the deployment of grapevine regulatory elements via precision breeding and inevitably attracts the use of non-grapevine materials that are not biologically consistent with the *Vitis* lifecycle [64]. Therefore, the discovery and functional verification of candidate grapevine promoters is crucial to further progress in precision breeding [65-68].

4. Resistance/tolerance genes to biotic and abiotic stresses

4.1 Antifungal genes

The development of highly reproducible genetic engineering protocols for a wide array of grapevine cultivars and rootstocks now allows identification and screening of grapevine-derived genes for introducing desirable traits, particularly disease resistance. A number of field studies for screening precision-bred grapevines are currently in advanced stages of testing prior to commercialization [see 69 for a complete list].

Grapevines are affected by a number of fungal pathogens worldwide and require intensive fungicide spray regimes for sustainable crop production [70,71]. Major improvement efforts have been directed towards enhancing fungal-disease resistance in table and wine grape cultivars [69]. A number of pathogenesis-related (PR) proteins were screened for their response to fungal pathogen infection. Genetically modified ‘Neo Muscat’ and ‘Pusa Seedless’ grapevines constitutively expressing rice chitinase genes exhibited enhanced resistance to anthracnose and powdery mildew [72,73]. However, no resistance to powdery mildew was
observed in ‘Seyval Blanc’ plants expressing barley chitinase genes [74]. Enhanced resistance to *Eutypa lata* was observed in ‘Richter 110’ grapevines that constitutively expressed a *Vigna radiata* eutypine detoxifying gene (*Vr-ERE*), which converts eutypine toxin produced by the pathogen to non-toxic eutypinol [75]. Stilbene synthase genes encoding resveratrol were isolated from several *Vitis* species and engineered for constitutive expression to improve fungal resistance [76, 77]. A *VvTL-1* gene previously reported to inhibit *Elsinoe ampelina* spore germination and hyphal growth was constitutively expressed in ‘Thompson Seedless’ grapevines [35, 78]. Enhanced resistance to foliar fungal diseases and decreased incidence of sour bunch rot in berries was observed in greenhouse and field tests. Constitutive expression of a *V. vinifera* WRKY1 (*VvWRKY1*) transcription factor gene in ‘41B’ rootstock plantlets resulted in activation of regulatory genes in the jasmonic acid pathway and upregulation of several defense related proteins. A reduction in downy mildew symptoms caused by *Plasmopora viticola* was observed on *in vitro*-derived genetically modified plant leaves compared to the controls, when inoculated under similar conditions [79]. Other non-grapevine derived genes such as lytic peptides encoding magainin and polygalactouranase inhibiting proteins (PGIP) were demonstrated to improve fungal disease resistance [80,81].

### 4.2 Antibacterial genes

Significant efforts have been directed towards improving grapevine resistance to bacterial crown gall disease (*Agrobacterium vitis*) and Pierce’s disease (PD) (*Xylella fastidiosa*). A number of non-plant antimicrobial peptides from various sources were tested for their in vitro efficacy against *A. vitis* and *X. fastidiosa* prior to functional analyses [82, 83]. Genetically modified grapevines expressing the animal-derived lytic peptide, magainin, and truncated *Agrobacterium virE2* protein, exhibited decreased crown gall symptoms caused by *A. vitis* [81,84]. Other lytic peptides such as cecropin, mellitin and their hybrid derivatives, LIMA1 and LIMA 2, were inserted into elite table and wine grape cultivars [85-89]. Lytic peptides were detected in such genetically modified grapevines using ELISA, and enhanced PD resistance was observed in repeated greenhouse trials and field tests for several years [69]. A chimeric peptide to incorporate bacterial resistance was developed by fusing a pear-derived bacterial cell surface recognition domain with a cecropin-derived lytic domain [90]. A significant reduction in
bacterial colonization and leaf scorching symptoms associated with PD were observed in modified ‘Thompson Seedless’ and ‘Chardonnay’ grapevines in the greenhouse environment, however resistance in the field was not reported.

The possibility of imparting PD resistance to unmodified scions grafted on genetically modified rootstocks expressing lytic peptide genes also was studied [91]. Non-modified ‘Cabernet Sauvignon’ scions grafted onto modified ‘Thompson Seedless’ plants expressing an animal-based synthetic lytic peptide were used as an experimental rootstock [92]. Lytic peptide could be detected in xylem sap of unmodified scions at levels similar to that of genetically modified rootstocks. In other studies, a non-grapevine PGIP protein could be detected in xylem sap of unmodified scions grafted onto modified ‘Thompson Seedless’ and ‘Chardonnay’ vines constitutively expressing PGIP [80]. Factors including protein size that can be transmitted from a modified rootstock to a grafted unmodified scion via the xylem and its threshold concentration necessary to impart disease resistance in grafted vines are being evaluated [93]. Conferring PD resistance using a mobile rootstock-derived anti-bacterial peptide would eliminate the need for creating genetically modified versions of multiple scion cultivars.

4.3 Antiviral genes

Virus resistance was obtained using the strategies of parasite derived resistance and RNA interference [94,95]. The coat protein gene of several viral pathogens including Arabis mosaic virus (ArMV), Grapevine fanleaf virus (GFLV), Grapevine chrome mosaic virus (GCMV), Grapevine virus A and B (GVA and GVB) were expressed in modified grapevines [96-98]. Unmodified scions grafted onto modified rootstocks expressing GFLV CP exhibited no visual disease symptoms after 3 years of natural infection in two vineyard sites [99]. No viral recombination between modified and native GFLV isolates was observed in field tests, thus demonstrating the safety of this approach. In other studies, an inverted repeat construct and artificial microRNAs (amiRNA) were inserted into grapevine plants to evaluate GFLV resistance using RNA interference [100, 101].

4.4 Abiotic stress tolerance genes
Phenotypic plasticity may cause the same genotype to exhibit significant variability in fruit yield and quality in different environmental conditions [102]. The availability of the sequenced grapevine genome combined with high-throughput expression profiling technologies such as microarrays, differential display and RNA sequencing now makes it possible to analyze gene expression in several *Vitis* species under varying environmental conditions. Transcriptome analysis was successfully applied to identify genes involved in vegetative and reproductive growth and to study vine response to abiotic and biotic stress factors. It has also been proposed to be an alternative to reference genome sequencing for studying variability that might exist among cultivars with respect to enological characteristics [103].

Transcriptome analysis of grapevine tissues at various developmental stages revealed significant differences in gene expression between actively growing, green/vegetative tissues and mature woody tissues [104]. Differential gene expression was observed during tissue maturation as a result of coactivation of pathways that were not expressed in actively growing tissues. Such transcriptome reprogramming during maturation has been specifically observed in woody plants. Transcriptome analysis of berry tissues during various stages of development and ripening has provided a significant amount of information on the expression of transcription factors and genes involved in the production of organic acids, tannins and other secondary metabolites [105].

Drought, salinity and temperature fluctuations adversely affect grapevine production worldwide. These stresses reduce crop yield and quality, although irrigation management strategies may be used to concentrate pigments and flavor of wine grapes and improve enological qualities [106]. Response to abiotic stress primarily occurs through altered physiological processes, which ultimately affects vegetative and reproductive growth patterns [107]. Grapevine species exhibit significant differences in transcriptome and protein patterns under conditions of abiotic stress; such information may be useful to elucidate underlying differences in phenotypic response and ultimately improve performance under adverse growing environments.

Microarray analyses of salt and water stressed ‘Cabernet Sauvignon’ grapevines demonstrated that more than 2000 genes were differentially expressed, and expression was influenced by both drought and ABA [108]. Significant differences as well as common attributes were observed in gene response to varying stress factors. These included transcription factors, genes involved in signal transduction and carbohydrate metabolism, which may ultimately contribute to enhanced abiotic stress tolerance [109]. Water deficit imposed by drought appeared
to have a more severe effect than that imposed from salinity. Differences in protein expression were observed between *V. vinifera* cultivars Chardonnay and Cabernet Sauvignon exposed to drought and salinity stress. Decrease in growth parameters were correlated with corresponding reductions in the amounts of proteins involved in photosynthesis [110].

A number of cold-inducible transcription factors recently were isolated from grapevines and shown to act as master switches for stress-induced activation of several genes [67,111,112]. Transcriptome analysis of *V. amurensis* and *V. vinifera* ‘Hamburg Muscat’ grapevines indicated large differences when vines were exposed to cold-stress [113]. A higher number of unidentified sequences were obtained in cold-hardy *V. amurensis* when compared with the reference genome, possibly due to the large phylogenetic distance between the two species.

Results of genetic engineering of grapevine to improve abiotic stress tolerance and qualitative traits are just emerging. For instance, modified grapevines expressing a *Medicago sativa* ferritin gene and *Arabidopsis* CBF1 transcription factor were shown to exhibit improved abiotic stress tolerance [114,115]. Enhanced freezing tolerance was observed in *Vitis* interspecific hybrid ‘Freedom’ that constitutively expressed a *V. vinifera* CBF4 transcription factor [116]. Genetically engineered grapevines with improved fecundity and reduced berry browning currently are being evaluated [117,118]. Nevertheless, with the tremendous amount of information being accumulated through genome mining and expression analysis, more critical genes/promoters will be revealed and become available for use in grapevine improvement.

4.5 Precision bred plants under field testing

The majority of previous attempts to increase stress resistance in grapevine employed non-grape genes or promoters. However, in ongoing research, a number of grapevine-derived genetic elements, including several pathogenesis-related genes were isolated from disease resistant, sexually-compatible *V. vinifera* hybrids and plants overexpressing these genes were placed under field conditions [1,119]. These plants contain genes encoding the PR1 variants [119], VvTL1 (PR5) [35], VvAlb1 [52], homologues of VvAMP1 and VvAMP2/defensin [120] and an orthologue of Snakin-1 [121]. It is expected that results from the long-term, real world tests will enhance understanding of gene function and expand our ability to engineer resistant grapevine using only genetic materials from sexually-compatible grapevines.
Conclusion

Significant advancements in cell culture, gene discovery and gene insertion technologies were only recently merged to fully enable precision breeding for the genetic improvement of grapevine. It should be noted that the results of precision breeding are fully in sync with the developmental biology of grapevine, but provide a significantly more efficient and predictable method of genetic improvement compared to that of conventional breeding. With precision breeding, only genetic elements from grapevine are used, but certain key obstacles, including inbreeding depression (resulting in the inability to “self” desirable lines), that stifle improvement via conventional breeding are completely overcome. The results obtained by precision breeding are more predictable than that of conventional breeding, lowering the risk of unintended outcomes. With precision breeding, a major goal is to create new versions of elite cultivars that not only maintain their desirable traits, but are able to be managed with less-to-no chemical intervention. Such precise modification cannot be obtained through conventional breeding within any practical timeframe. Currently, prototype plants harboring putative grapevine resistance genes and promoters are in the field to test for trait expression. Plants containing only genetic elements from grapevine will begin to be planted for testing within the next year. However, more widespread and robust evaluations, as is the norm for conventional breeding, must occur to confirm the utility of cultivars produced by precision breeding.

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