

Grapevine genetics after the genome sequence: Challenges and limitations

J.M. MARTÍNEZ-ZAPATER^{1,2}, M.J. CARMONA³, J. DÍAZ-RIQUELME^{1,3}, L. FERNÁNDEZ^{1,2}
and D. LIJAVETZKY^{1*}

¹Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Cantoblanco, 28049 Madrid, Spain

²Instituto de Ciencias de la Vid y del Vino (CSIC, Gobierno de La Rioja, Universidad de La Rioja), C/Madre de Dios 51, 26006 Logroño, Spain

³Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Avenida Complutense s/n, 28040 Madrid, Spain

*Present address: Cátedra de Química Orgánica y Biológica, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo-CONICET, Chacras de Coria, M5528AHB, Argentina.

Corresponding author: Dr José Miguel Martínez-Zapater, fax 34-915854506, email zapater@cnb.csic.es, zapater@icvv.es

Abstract

The publication of the genome sequences of inbred grapevine plant PN40024 and the cultivar Pinot Noir has provided a new generation of molecular tools and has opened the way to functional genomics in grapevine. Establishing gene biological function is now a major challenge requiring the parallel development of molecular and genetic information. New massive pyrosequencing technologies will ensure no shortage of nucleotide sequence information. However, genetic analysis and genetic tools in grapevine still require additional development. Exploiting the existing natural genetic variation in *Vitis vinifera* L. and other inter-fertile *Vitis* species should be a priority to focus functional analyses on genes contributing to phenotypic variation because their genetic variation constitutes the basis for genetic improvement of classical cultivars and for the development of new ones. In this review, we discussed the current molecular and genetic tools available in grapevine and considered those that need to be developed to exploit natural genetic variation in the analyses of gene function. We also reviewed the scarce information on the genetic and molecular structure of relevant grapevine traits and proposed future directions.

Keywords: *grapevine genetics, genetic resources, genetic tools, natural genetic variation, somatic variation*

Introduction

One major challenge for viticulture this century will be maintaining a sustainable production of high quality grapes in a changing environment. Strategies to meet this challenge in a knowledge-based society will come from a deeper understanding of the biology as well as the interactions of *Vitis vinifera* and its close relative species, with the physical and biological environment of the vineyard. Furthermore, understanding the genetic and molecular basis of existent natural genetic variation within the genus *Vitis* will provide the information and tools for the genetic improvement required to cope with new threats (e.g. new pathogens or pests) while maintaining specific berry composition. Fortunately, this challenge has currently been met by a set of new opportunities that derive from the rapid development of molecular biology technologies and their application to grapevine and other interacting organisms. As a consequence, in the last decade, there has been a rapid increase in the genomic resources that are available for grapevine research (This et al. 2006, Troggo et al. 2008), including the recent

publication of the first genome sequences (Jaillon et al. 2007, Velasco et al. 2007). This is expected to change experimental approaches and speed up the acquisition of new biological information in grapevine.

As other woody perennial species, grapevine is not as easy a genetic system as Arabidopsis or rice. Grapevines require large growing facilities and experimental fields and their generation time varies between 2 and 5 years depending on genotype and growing conditions. In addition, grapevine genotypes are highly heterozygous and the relevance of near-homozygous lines was not considered till recently because of the need to generate a high quality reference sequence (Jaillon et al. 2007) as well as more efficient genetic systems (Boss and Thomas 2002). High heterozygosity results from the dioecy of wild grapevine plants and has been maintained in cultivated plants through vegetative propagation. However, most cultivated genotypes are hermaphroditic self-fertile as a result of selection for fruit production and can be easily out-crossed (This et al. 2006). For genetic analyses, vegetative reproduction compensates for the length of generation

time by allowing the immortalisation of genotypes and mapping populations, and facilitating their distribution and analysis in different environments.

Like in other fruit tree species, vegetative propagation of exceptional individuals was used during domestication of grapevine as a way to fix the desired phenotypes and evade genetic segregation. This strategy maintains the high heterozygosity level of the original plants (Zohary 2004) and nowadays still conditions breeding practices. In recent decades, classical table grape breeding has been very effective in selecting new cultivars fitting the quality expectations of consumers as well as the requirements of growers and commercial networks. On the wine side, the selection of new wine cultivars requires one additional time consuming step: winemaking and evaluation. A major goal in winegrape breeding is the introduction of resistance traits to pathogens and pests from other *Vitis* species (Alleweldt and Possingham 1988), facing the constraint of maintaining the berry composition features of classical cultivars. In spite of these difficulties, breeding has been shown possible and several commercially successful pathogen resistant cultivars are under production. Still, the improvement of specific features of classical wine cultivars mostly relies on the exploitation of somatic variation and the possible future utilisation of genetic engineering that could allow the incorporation of single monogenic or oligogenic trait on a given genotype. Unfortunately, somatic variation is a random process depending on spontaneous or induced mutations, and the appearance of new resistance functions seems extremely unlikely. On the other hand, genetic engineering needs further information on resistance genes and their function and a more propitious social environment.

The information derived from the application of genomic approaches in grapevine as well as in related and interacting organisms is expected to provide new tools to cope with the challenges of viticulture in this century. Two main areas of application can be identified: (i) crop management systems that will benefit from the acquisition of more precise information on the crop and its behavior under different physical and biological environmental conditions; and (ii) plant breeding, where new technologies and information will speed up the process of improvement of current cultivars or the selection of new ones. A better understanding of the grapevine genome and the genetic structure of quality and resistance traits can provide new tools to accelerate classical breeding technologies for both cultivars and rootstocks, an approach known as genome assisted breeding (Varshney et al. 2005). Furthermore, this information can also help to make a more efficient use of somatic variation or to identify those gene sequences that could be useful in genetic engineering strategies based on *Vitis* genes.

With the exception of genetic engineering strategies that can use genes and gene variants outside the range of the target species, most breeding strategies exploit the natural genetic variation of target and inter-fertile species. This is the reason why a major challenge in grapevine genomics, as in other crops species, is the identification of genes and alleles that are responsible for phenotypic

variation within the species (Morgante and Salamini 2003). In this review, we focussed on the interaction between grapevine genomics and genetics to understand the biology of grapevine. We will describe first how genome sequence information is changing genetic analyses in grapevine, and the possibilities offered by the use of nucleotide diversity as a tool for functional genomics in this species. Second, we will review the genetic variation available in grapevine as well as the possibilities to generate it, as a required tool in the establishment of gene biological function. The use of genetic transformation in gene functional analyses based on the phenotypic characterisation of transgenic plants was covered in an accompanying review and will not be addressed here (Vidal et al. 2010). Finally, we will review the limited information on the genetic structure and control of agronomic traits currently available in grapes.

Implications of genome sequence on grapevine genetics

Availability of the grapevine genome sequence is causing a rapid acceleration of grapevine genetics research by providing a new research framework and genetic tools. Approaches such as genetic mapping, genetic identification or genetic diversity studies that have generated hundreds of published works will be re-dimensioned and undertaken at a larger scale. On the other hand, the so far scarce genetic analyses of gene biological function based on natural or induced genetic variation will be supported by a large set of additional tools.

The genome sequence is the ultimate genetic map. The genome sequence of the near-homozygous line PN40024 (Jaillon et al. 2007) has provided a reference genome sequence for grapevine; while those of the highly heterozygous cultivar Pinot Noir (Velasco et al. 2007) offer a first genomic view of sequence polymorphism in grapevine. With the annotated genome sequence in hand, there is a reference sequence for nearly every gene, and gene variants can now be described with respect to this reference. Specific gene primers and probes can be designed at any position and gene-specific molecular markers can be rapidly generated based on sequence comparison between a sequence variant and the reference one. As physical and genetic maps become fully integrated, the densest molecular map will be the reference genome sequence, as well as the ultimate integrated and functional map. There is no requirement to generate additional molecular marker maps because sequence tags of any type can directly be mapped *in silico*. In addition, molecular markers for any genome position can be readily generated for positional identification of genes. However, genetic maps and rapid mapping technologies are still strongly required to understand the genetic structure of phenotypic traits and to associate specific genomic regions to trait values through quantitative trait locus (QTL) mapping as well as to identify the genes and gene variants underlying those QTLs. The demands of molecular markers in all these approaches will be efficiently covered by the sequence information and tools to identify and detect DNA sequence polymorphisms.

Genetic diversity analysis at the genome level. Publications of the grapevine genome sequences were almost coincident in time with the first commercial applications of highly efficient and low cost parallel pyrosequencing technologies (Margulies et al. 2005). Their applications are radically changing not only genome sequencing approaches (Wicker et al. 2006) but genetic diversity (Novaes et al. 2008) and gene expression studies (Weber et al. 2007) as well. In grapevine, the application of these new sequencing strategies to re-sequence additional genotypes for sequence comparison with the reference sequence (Jaillon et al. 2007) will provide a view for nucleotide variation within the species. The results of preliminary re-sequencing studies of specific expressed gene tags in selected sets of cultivars (Salmaso et al. 2004, Lijavetzky et al. 2007) and the information provided by the Pinot Noir sequence (Velasco et al. 2007) suggest that two randomly selected grapevine genomes could differ in 10–16 nucleotides per kb. These results represent 2–3 times higher nucleotide diversity than what has been reported in *Arabidopsis* (Clark et al. 2007). Furthermore, while two *Arabidopsis* genotypes can differ in up to 4% of their size (Clark et al. 2007), first estimations suggest that the two genomes present in the cultivar Pinot Noir could display up to 11% of size variation (Velasco et al. 2007). Further genome re-sequence analyses on a larger number of unrelated genotypes will provide more consistent and definitive values for grapevine to develop a view of the information content and genetic variation within the *pan-genome* of the species *V. vinifera*. This is a basic step in the search for genes and gene variants responsible for phenotypic variation. Furthermore, the analyses of the type of nucleotide variation encountered and its distribution along gene families can help identifying gene functions involved in adaptation or selected along the domestication process. Finally, the extent of linkage disequilibrium (LD) between nucleotide polymorphisms and its distribution along the genome will be relevant to the understanding of the evolutionary history of the species and to the evaluation of the possibilities of genome-wide association studies in grapevine. So far, the first analyses of LD in grapevine using either simple sequence repeat (SSR) markers on a core collection of 141 cultivars (Barnaud et al. 2006) or single nucleotide polymorphisms (SNPs) in a selected sample of 11 cultivated and wild grapevine genotypes (Lijavetzky et al. 2007) provided very divergent results for what could be related with the type of markers used in each case (Varilo et al. 2003).

Genetic diversity within the genus *Vitis*. *V. vinifera* is the only living species of the genus *Vitis* indigenous to Eurasia. The genus, almost exclusively present in the Northern Hemisphere, consists of ca 60 species mainly American and Asian. These species are of high interest because they show multiple resistance traits to pest and diseases that affect or can affect *V. vinifera* (Alleweldt and Possingham 1988, This et al. 2006). Furthermore, they are all inter-fertile, providing the possibility of genetic transmission of these traits in breeding programmes. Comparative genome sequence analyses within the genus

Vitis will generate a basic view of its information content and genetic variation. This will help the understanding of the evolutionary forces shaping speciation and adaptation processes in the genus and will contribute to the identification of relevant genes and gene variants to support future breeding programmes in *V. vinifera* as well as in other *Vitis* species and hybrids (e.g. rootstocks).

New genetic tools. The characterisation of DNA sequence polymorphisms is the basis to develop new molecular markers. SNP and insertions/deletions (INDELs) are the most abundant types of DNA sequence polymorphisms found in genomic sequences (Rafalski 2002). SNPs can be used as genetic markers for many genetic applications such as cultivar identification, construction of genetic maps, assessment of genetic diversity, detection of genotype/phenotype associations or marker-assisted breeding. Furthermore, the development of high throughput genotyping methods make SNPs highly attractive as genetic markers (De La Vega et al. 2005). SNPs generally display only two alleles per locus that reduces their polymorphism information content with respect to other molecular markers such as SSRs. However, multiplexing possibilities go from single SNP analysis to hundreds of thousands of SNP markers and largely overcome this limitation. In addition, independent of the detection method used, the two alleles can always be identified without confusion (Rafalski 2002). For example, when considering cultivar genetic identification, genotype information at 6–9 nuclear SSRs loci is currently the accepted strategy (This et al. 2004). However, this strategy still has problems arising from difficulties in multiplexing and allele identification among laboratories. Genetic identification can be easily solved with a set of 48 selected SNPs that for this purpose are as informative as 15 microsatellite loci (Lijavetzky et al. 2007) while being faster and cheaper to genotype (Cabezas et al., unpublished results). Millions of putative SNPs were detected between the two genome sequences of the heterozygous cultivar Pinot Noir (Velasco et al. 2007) and several hundreds have been mapped (Troggio et al. 2007). The generation of SNP chips containing 5–10 thousand SNPs could become a rapid tool for QTL and mutation mapping or a primary tool for genome-wide association mapping.

Progressive identification and annotation of all the genes in the grapevine genome, first based on expressed sequence tags (ESTs; da Silva et al. 2005; Peng et al. 2007) and later on genome sequence (Jaillon et al. 2007, Velasco et al. 2007), has fostered the design and development of microarrays for transcriptome profiling. These tools have been useful in the characterisation of plant developmental processes (Deluc et al. 2007, Grimplet et al. 2007, Pilati et al. 2007) as well as in the study of plant responses to physical (Cramer et al. 2007, Tattersall et al. 2007) or biological agents (Espinoza et al. 2007). Together with strategies to characterise the grapevine proteome (Vincent et al. 2007, Lückner et al. 2009) and metabolome (Deluc et al. 2007), they represent high throughput phenotyping approaches that are useful in

developing candidate gene hypotheses when studying the effect of different gene variants in the same genetic background (Ageorges et al. 2006, Fernandez et al. 2007). Current microarrays already represent ca 50% of grapevine genes. As genome annotation and protein identification progresses, these tools will eventually cover all the annotated sequences and eventually, with the development of grapevine genome tiling arrays (Stolc et al. 2005), all the reference genome sequence. However, these technologies are so rapidly evolving that it is difficult to predict whether all of them will be developed for grapevine or new approaches will substitute for them in the future.

From molecular function to biological function. The grapevine genome sequence is now the framework for analysing gene sequence families such as transposable elements (Benjak et al. 2008, Moisy et al. 2008) or families of transcription factors (Matus et al. 2008, Díaz-Riquelme et al. 2009) and within those families the specific function of any sequence. Before, discussing how functional analyses could be approached in grapevine, it is of interest to briefly summarise what has been learnt from functional analyses in *Arabidopsis* and the evolution of function search goals.

The sequence of the *Arabidopsis* genome (*Arabidopsis* Genome Initiative 2000) generated a strong international commitment to identify the function of all the plant genes within the so called 2010 programme (Somerville and Dangl 2000). At that time, approximately 55% of *Arabidopsis* annotated genes could be assigned a putative function, but not a biological role, based on sequence comparison to genes in databases. Only about a thousand genes had been assigned a function by direct experimental evidence. Nowadays, many molecular and genomic tools and strategies have been developed in *Arabidopsis* helping to obtain functional information on the encoded products of annotated genes in a systematic way, such as expression profiling, promoter information, protein expression in heterologous systems, protein interaction data, subcellular localisation of protein products, etc. These approaches provide an image of what the molecular function of the gene product can be, e.g. if it has an enzymatic or regulatory activity, a structural function, a binding capacity, etc. However, understanding the biological role within the organism has required the development of genetic tools to analyse the phenotypic effect of different gene alleles either through the construction of transgenic plants or the use of mutants selected in forward or reverse screenings (Alonso and Ecker 2006). Fortunately, *Arabidopsis* genetics was already well developed when the genome sequence became available, and sequence information provided the required synergy to develop a model system in which both forward and reverse genetics are extremely efficient as tools in the search for sequences responsible for specific phenotypes or biological functions for annotated sequences. Current goals in the study of the biological roles of *Arabidopsis* genes surpass their function within the organism and question their roles within the species. In other words, the goal now is to identify those genes

contributing to species genetic variation and ultimately to understand their role in species adaptation and evolution (Koornneef et al. 2004, Tonsor et al. 2005, Mitchell-Olds and Schmitt 2006).

When considering a given phenotypic trait such as flowering time, every approach used to analyse the genetic and molecular basis of the trait will provide a slightly different answer. For example, when flowering transition was transcriptionally analysed, hundreds of genes showing significant expression changes upon flowering induction were identified (Schmid et al. 2003). On the other hand, a combination of forward and reverse genetics analyses provided direct experimental evidence of a role in flowering induction for about 120–140 genes (Boss et al. 2004). Finally, quantitative genetic analyses of the variation for flowering time in natural populations of *Arabidopsis* have so far identified over 20 QTLs (Alonso-Blanco et al. 2005). Seven of them are major effect QTLs and were molecularly characterised, permitting in some cases the identification of genes not uncovered by classical genetic analyses (Michaels and Amasino 1999, Johanson et al. 2000). In addition, the set of genes with a major contribution to natural genetic variation for flowering time in *Arabidopsis* is not necessarily the same, explaining natural variation for the same trait in rice or in other species belonging to unrelated taxonomic groups (Ausin et al. 2004). Focusing on crop species, natural genetic variation is the primary tool for genetic improvement and understanding its genetic and molecular basis can speed up the breeding process.

The above developments described for *Arabidopsis* frame the challenge of undertaking functional analyses in grapevine and help to identify what can be considered as possible tools and reasonable goals. Regarding genetic tools, as we will see in the next section, grapevine genetics is in its infancy. Laboratory genetic systems as tools for genetic analyses need to be further developed from two promising starting systems: the near-homozygous line PN40024 used for the reference sequence (Jaillon et al. 2007) and the dwarf, short cycle plants, regenerated from somatic embryogenesis of Pinot Meunier (Franks et al. 2002). If efficient transformation procedures were developed for them, they could become basic tools for functional genomics strategies based on genetic transformation approaches (as presented in a different article of this special issue). Grapevine genetic resources have been the focus of renewed research interest in the last few years with an aim to catalogue and characterise existent germplasm collections at the genotypic and phenotypic level and to rescue minority cultivars and endangered wild populations before they disappear (This et al. 2006). These collections provide a representation of existing natural genetic variation in grapevine and are the basis for the development of useful genetic materials such as segregating populations or core collections (Le Cunff et al. 2008). Focusing functional analysis on the identification of those genes that have a major contribution to phenotypic variation of relevant traits within those collections will be of immense benefit, given their direct breeding implications. This objective is in fact better supported by

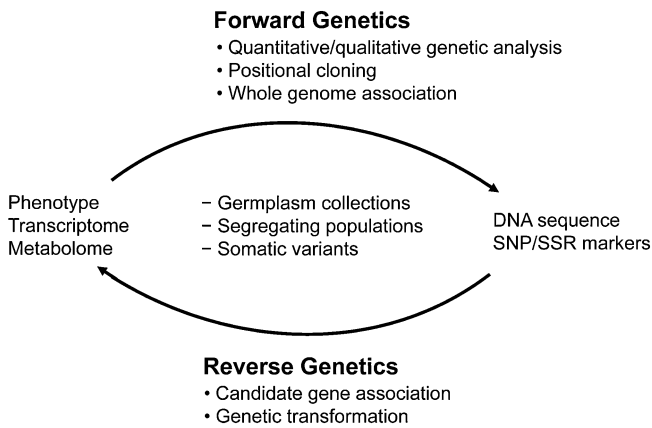


Figure 1. Forward and reverse genetic approaches to identify the molecular basis of natural genetic variation in grapevine. SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

the available genetic materials than a systematic search for the biological role of every annotated sequence, which was identified as a primary goal in the Arabidopsis model system. Figure 1 depicts how the phenotypic and genotypic characterisation of these genetic resources could allow forward and reverse genetic strategies to associate nucleotide variation with phenotypic variation. This priority does not exclude the need to generate additional genetic tools for future systematic approaches. However, as we will see, those can encounter further difficulties in the case of grapevine.

Natural genetic variation in grapevine

The establishment of biological function requires genetic analyses and these are based on genetic variation. In this section, we will consider the sources of natural genetic variation currently available in grapevine as well as artificial mutagenesis.

Genotypic variation. Cultivated grapevines are thought to have been domesticated from wild populations of *V. vinifera* (This et al. 2006) which is the only extant wild European taxon of the genus *Vitis*. Its distribution across Europe has been drastically reduced and it is legally protected as an endangered species in some European countries. This reduction has been due to two main factors: the pests and diseases affecting viticulture in Europe since their introduction in the second half of the 19th century, and the reduction of their habitats because human population expansion and current forest and river bank management (Arrigo and Arnold 2007). Consequently, populations are generally small and dispersed, and their genetic variation has been reduced. These small populations are being recorded from the near East to Central and Mediterranean European countries as well as in Northern African countries. However, considering the number of populations recorded, the low number of individuals per population and the identification of naturalised cultivars and rootstocks as well as hybrids in these populations (Arnold et al. 1998; Snoussi et al. 2004; Di Vecchi-Staraz et al. 2009), it is likely that only a few thousand wild

individuals exist. The number of cultivated genotypes belonging to the *V. vinifera* and maintained in germplasm collections were estimated to be about 10 000 (Alleweldt and Possingham 1988). However, modern viticulture, focused on a few cultivars for the production of single varietal wines, has dramatically reduced the genotypes in culture. Furthermore, complications arising from quarantine restrictions and costs of maintaining genetic materials in the field as living collections have caused, with some important exceptions, genetic resources to be fragmented into many small collections. In addition, the use of different names for the same material (synonyms) as well as the convergence of names for different genotypes (homonyms) has generated additional confusion that has slowly been resolved through international collaboration (This et al. 2004). Altogether, more accurate estimations of variety numbers might be closer to 5000 (This et al. 2006) with many of them being closely related. As a whole, between 10 000 to 20 000 wild and cultivated genotypes within the species *V. vinifera* may only exist today

The difficulty to manage large numbers of genetic materials has prompted the construction of core collections as a useful alternative. Nested core collections intended to maximise allelic variation at nuclear microsatellite loci have been generated (Le Cunff et al. 2008) and may be useful for LD-based association and identification of divergent progenitors to build mapping populations. Phenotypic characterisation of these collections is also being performed including morphological, phenological and agronomic traits.

Somatic variation. Somatic cell variation constitutes an additional source of phenotypic variation in grapevine. Somatic variation results from mutation or epimutation events taking place in single cells belonging to a specific meristem cell layer (L1 and L2 layers are distinguished in grapevine shoot apical meristems) (Thompson and Olmo 1963). These somatic events are in the heterozygous state and therefore, a derived phenotypic effect will only be produced by gain-of-function mutations or by loss-of-function mutations when affecting the only functional allele in a locus or resulting in haplo-insufficiency. Furthermore, for the mutant phenotypes to be observed, the mutant cell has to 'colonize' the corresponding cell layer (either L1 or L2) in at least one shoot apical meristem and derived organs. Once a bud meristem is colonised in either the L1, the L2 or both cell layers, the mutation with or without an associated phenotype could be transmitted to next plant generations by bud propagation and through sexual reproduction if present in the L2 (Franks et al. 2002).

Somatic variation has been the basis of the progressive improvement of specific genotypes through cycles of vegetative propagation, because farmers have generally selected those plants with the best productive features as the sources of buds. Somatic variation has also provided a way to improve some qualitative differences such as colour, aroma or seedlessness to given genotypes. Well-known somatic variants affect conspicuous quality traits.

As a rule of thumb, the more ancient and widely cultivated genotypes are, the higher the number of recognised somatic variants they generally display (This et al. 2006).

Somatic variation can also provide information on gene function, offering the opportunity to compare the original with the variant plant at morphological and molecular levels (e.g. transcriptional profiling) and to develop testable candidate gene hypotheses. Large collections of somatic variants are maintained in stock centres (This et al. 2006) that could be used for such a purpose. Recently, a few of them have been analysed at the genetic and molecular level (Boss and Thomas 2002, Kobayashi et al. 2004, Fernandez et al. 2006, Lijavetzky et al. 2006, Walker et al. 2006, Chatelet et al. 2007) (Table 1). The number of analysed variants is not large enough to make general conclusions, however several trends can be observed. First, all somatic variants except one show a dominant phenotype. The only exception is constituted by the loss of berry colour because of a loss-of-function mutation affecting the functional allele at a heterozygous *VvMybA1* locus (Walker et al. 2006). Second, in those few cases where the genes responsible for the variation and the functional polymorphisms were identified, altered genes generally encode proteins with regulatory function and the functional polymorphisms mostly result from genome reorganisations, either related to transposable elements or deletions. This conclusion is biased by the fact that only somatic variation conspicuously affecting the phenotype has been analysed. Although these genome reorganisations commonly affect all types of gene sequences, only those producing a major phenotypic change have been identified. Similarly, single nucleotide mutations like the one responsible for the dwarf phenotype of Pinot Meunier (Boss and Thomas 2002) or small INDELS such as those causing somatic variant alleles at microsatellite loci are also described (Franks et al. 2002). Studies investigating the nature and frequency of somatic changes are still missing in woody plants as this genetic variation could be useful in adaptation, in genetic improvement and functional analysis studies.

Cryptic genetic variation. The phenotype is in general a poor predictor of the underlying genetic potential or genetic diversity. One important part of grapevine genetic variation is not evident in the phenotype mostly because of dominance and epistasis. Grapevine plants are highly heterozygous and, with the exception of some recently bred table grape varieties, it is rare to find many homozygous microsatellite loci in a given cultivar (Cabezas et al. 2006). The average observed heterozygosity is around 0.8 for microsatellite loci and 0.65 for gene sequence haplotypes (Salmaso et al. 2004, Lijavetzky et al. 2007). These high heterozygosity values mean that almost any locus segregates in a selfed population giving the opportunity to analyse the effect of specific recessive alleles that are covered by dominance effects in the original genotypes. Every selfed progeny of a grapevine genotype segregates variant phenotypes in Mendelian and non-Mendelian ratios as a result of chimerism and epistatic interactions

Table 1. Genetic and molecular information available on grapevine somatic variants.

Cultivar	Phenotype	Locus	Molecular		Functional polymorphism		Functional		Effect	References
			Function	Position	Mutation	Alteration				
Pinot Noir	Dwarfism	<i>VvGAI</i>	DELLA Prot.	Exon	aa substitution	Modified protein			Dominant	Boss and Thomas (2002)
Red table grapes	Colour	<i>VvMYBA1</i>	MYB TF	Promoter	Retrotransposon	Expression			Dominant	Lijavetzky et al. (2006), Walker et al. (2006)
Cabernet Sauvignon white				Gene	Deletion	Lack of protein			Recessive	Lijavetzky et al. (2006), Walker et al. (2006)
Ugni Blanc	Berry development	<i>Fleshless</i>	-	-	-	-			Dominant	Fernandez et al. (2006)
Sultamina derived cultivars	Seedlessness	<i>SDI</i>	-	-	-	-			Dominant	Cabezas et al. (2006)
Carignan	Cluster size	<i>RRM</i>	TFL1	Promoter	Transposon	Overexpression			Dominant	Fernández et al. (2009)
Chasselas	Leaf shape	<i>CIOUTAT</i>	-	-	-	-			Dominant	Lijavetzky et al unpublished data

Prot., protein; aa, amino acid; TF, transcription factor.



Figure 2. Mutant phenotypes segregating in selfed progenies of grapevine cultivars. (a) Dwarf phenotype segregating in the progeny of the Spanish cultivar Callet. (b) Variegated phenotype segregating in the progeny of cultivar Aramón. Photos courtesy of Maria Napal.

(Figure 2). In any case, genetic analyses are facilitated in selfed progenies by the fact that only two alleles are generally segregating per locus.

Further relevant information on genetic variation comes from the analyses of genetic crosses. Given the high heterozygosity of grapevine genotypes, genetic analyses of phenotypic traits are performed on F_1 populations derived from crosses between different genotypes using a pseudo-testcross strategy (Grattapaglia and Sederoff 1994) and in many cases four alleles, are segregating for every locus. In these mapping populations, it is very common to observe transgressive segregations for most analysed traits indicating the generation of new genetic combinations with phenotypic effects (Doligez et al. 2002, 2006, Fischer et al. 2004, Cabezas et al. 2006, Costantini et al. 2008). In tomato, divergent accessions selected on the basis of genotype in place of phenotypic appearance, have shown to contain the greatest number of novel QTL alleles when analysed in advance backcross populations (Fulton et al. 1997a, 1997b) and this strategy could be useful in other species to uncover new and useful alleles (Tanksley and McCouch 1997). This systematic search could be useful in grapevine when considering not only divergent cultivars but wild *V. vinifera* genotypes as well as other *Vitis* inter-fertile species.

Induced genetic variation. Mutagenesis has not been extensively used in grapevine although physical (X-rays, gamma rays and ultraviolet light) and chemical (ethyl methanesulphonate (EMS) or ethidium bromide (EB)) mutagens have been investigated. Mutagenic bud treatments can result in plant chimeras (Alleweldt and Posingham 1988), and this complexity can be reduced when combined with somatic embryogenesis (Kuksova et al. 1997).

The main purpose of mutagenic treatments in grapevine has been to increase the level of somatic variation to select agronomically relevant phenotypes in specific

cultivar genetic backgrounds. For this reason, vegetative buds have been the subject of mutagenesis either in the field or in tissue culture. Among physical mutagens, X-rays (Pratt 1959) and gamma rays (Botta et al. 1987, Kuksova et al. 1997, Charbaji and Nabulsi 1999) have been used on buds of *V. vinifera* or *Vitis* hybrids including rootstocks (Charbaji and Nabulsi 1999). Botta et al. (1987) irradiated five different cultivars and described some interesting mutant phenotypes such as a low berry dropping-type in Dolcetto, a polyploid Barbera with large berries that were less susceptible to *Botrytis cinerea* infection, as well as somatic variants for large berries in the cultivar Delight and for seedlessness in Queen of Vineyards. The only published report on chemical mutagenesis of *in vitro* grown buds comes from experiments using EMS and EB on cultivar Pusa Seedless (Khawale et al. 2007).

Mutagenesis has not been used in grapevine as a source of variation for functional studies. Part of the problem for efficient production and use of mutant populations comes from the lack of near-homozygous lines and the reduced germination potential of many genotypes. The near-homozygous line PN40024 as well as potential lines that could be rapidly derived from the Pinot Meunier somatic dwarfs (Boss and Thomas 2002) could constitute potentially adequate genetic backgrounds for mutagenesis experiments. Given the lack of previous experience in large mutagenesis experiments in grapevine and considering the lessons learned from Arabidopsis, the use of several genetic backgrounds seems the most efficient strategy to increase the probability of hitting any gene in the genome.

Apart from buds, seeds and pollen grains could be interesting organs to mutagenise in grapevine. Where possible, pollen grain mutagenesis is preferred over seed mutagenesis because it targets a single germ cell, which guarantees that every mutation is a unique event. Seeds as buds and even pollen mutagenesis generate chimerical

mutant plants and it is important to fix the mutations either through a selfing generation or by bud grafting, in order to reduce redundancy of the same mutation events. In addition, it is very important to estimate the efficiency of the mutagenesis to ensure a balance between mutation load and fertility. In the case of pollen, these estimates are easily achieved using *in vitro* germination assays (Candela and Hake 2008).

Regarding mutagens, a broad spectrum of chemical and physical mutagens would be more interesting than restriction to a given type. EMS is the most widely used mutagen and has been the most efficiently used in the generation of Arabidopsis mutant populations (Page and Grossniklaus 2002). Chemical mutagens have been used to generate heavily mutagenised populations in which plants carry large numbers of mutations that can be identified via reverse genetic approaches such as targeting induced local lesions in genomes (TILLING) strategies, in which phenotype can be analysed in segregants generated by selfing (Gilchrist and Haughn 2005).

Biological mutagens such as the *Agrobacterium* T-DNA or different families of transposable elements have successfully been used in species such as Arabidopsis (Page and Grossniklaus 2002) or maize (Candela and Hake 2008) to generate mutant phenotypes while tagging the affected gene sequences. With these strategies, the number of mutations that can be obtained per plant is low and requires work with very large mutagenised populations to obtain enough coverage of the genome (Alonso and Ecker 2006). Given the size of the plant, these strategies are more difficult to implement in grapevine unless near-homozygous lines and seed stocks are developed. Furthermore, if these strategies are based on transgenic lines, their cultivation would generate additional regulatory conditions for plant growth in certain countries. Finally, as more information becomes available on active 'cut and paste' transposable elements (Benjak et al. 2008) and their requirements to transpose, it might be possible to devise reverse genetic strategies to exploit their natural transposition events in grapevine gene functional analyses.

Identification of QTLs and genes responsible for natural genetic variation

A major challenge in grapevine is the identification of those loci and underlying genes that contribute to the natural genetic variation for specific traits as well as understanding the nature and effects of their allelic differences. This information would be useful for cultivar improvement. The frequent quantitative nature of genetic variation requires the use of QTL mapping approaches to understand the genetic structure of traits; however, qualitative scoring has also been used in the identification of major loci. These approaches have already been used in grapevines to identify genomic regions responsible for the phenotypic variation at different traits either in F₁ progenies derived from cultivar hybridisations (see Costantini et al. 2009 for a recent review) or in selfed progenies (Duchene et al. 2009). In both types of crosses, only QTL that are in the heterozygous state in any of the parental cultivars will be identi-

fied. Given the perennial habit of grapevine and the possibilities of vegetative propagation, mapping populations can become permanent populations and can be established in different environments in order to analyse genotype \times environment interactions. However, a drawback is that maintenance of these progenies is expensive and requires experimental fields, and quarantine regulations limit the exchange of these populations between different continents.

As shown in Table 2, genetic mapping has been mostly used to identify genomic regions responsible for resistance traits in crosses that involved different *Vitis* species, rootstocks or breeding lines in which resistance traits have been introgressed from *Muscadinia rotundifolia* and other resistant species. For many of those traits, qualitative and quantitative genetic analyses have identified major loci explaining large proportions of phenotypic variation (Table 2). This is the case of the loci *Run1* (Bouquet 1986) and *Ren1* (Hofmann et al. 2008) responsible for dominant resistance to powdery mildew (previously known as *Uncinula necator* and later as *Erisyphle necator*). Similarly, resistance to downy mildew (*Plasmopara viticola*) is controlled by major loci like *Rpv1* (Merdinoglu et al. 2003) in *M. rotundifolia* as well as major QTLs in complex *V. vinifera* hybrids carrying introgressions from other *Vitis* species (Fischer et al. 2004, Welter et al. 2007). Interestingly, major loci are also identified for resistance to other diseases, such as the bacterial pathogen *Xylophora fastidiosa* causing Pierce's disease (Krivanek et al. 2006, Riaz et al. 2006, 2008), or pests like *Phylloxera* (Roush et al. 2007) or the nematode *Xiphinema index* (Xu et al. 2008). This situation can become more complex as different sources of genetic resistance are considered.

Regarding plant growth and physiology, only a few traits have been analysed till now and their genetic structure seems to be complex with multiple QTLs of small effects identified for many of the traits investigated (Table 2). One exception is the control of plant sex that seems to be regulated by a single locus in linkage group 2 (LG2) (Dalbo et al. 2000). A single locus on LG2 has also been identified as the main genetic determinant of berry colour (Doligez et al. 2002, Fischer et al. 2004, Lijavetzky et al. 2006, Salmaso et al. 2008). For other berry traits, most genetic analyses have been focused on table grapes (Table 2) providing evidence on the genetic control of traits such as seedlessness and berry size (Doligez et al. 2002, Cabezas et al. 2006, Mejia et al. 2007, Costantini et al. 2008). All studies identified the presence of a major seed development inhibitor (*SDI*) locus on LG18 with a dominant effect on seedlessness. This effect results from a somatic variation appearing in the cultivar Sultanina (Thompson Seedless, Sultana) that has constituted the basis for seedlessness breeding in table grapes. The pleiotropic effects of the *SDI* locus on berry size, and likely on additional phenological traits, may influence the phenotype of seedless table grape cultivars. Additional complexity on berry size QTLs has been described when analysing winegrape progenies (Fischer et al. 2004). Finally, location of major QTLs for Muscat flavour appears to be coincident in both table and winegrape cultivars (Doligez

Table 2. Genetic control of grapevine traits.

Trait	Genetic backgrounds analysed	Genetic structure	References
Resistance			
Powdery mildew	<i>V. vinifera</i> complex hybrids, <i>V. cinerea</i> , <i>V. rupestris</i>	Major loci or QTLs	Dalbo et al. (2001), Fischer et al. (2004), Welter et al. (2007)
Downy mildew	<i>V. vinifera</i> complex hybrids	Major and minor QTLs	Fischer et al. (2004), Welter et al. (2007)
Pierce's disease	<i>V. rupestris</i> , <i>V. arizonica</i>	Major QTL (71%)	Krivanek et al. (2006), Riaz et al. (2006, 2008)
Phylloxera	<i>V. vinifera</i> , <i>V. rupestris</i>	1–2 loci	Roush et al. (2007)
Xiphinema index	<i>V. arizonica</i>	Major QTL (70%)	Xu et al. (2008)
Plant physiology and growth			
Magnesium deficiency	<i>V. vinifera</i> complex hybrids	One QTL	Mandl et al. (2006)
Axillary growth	<i>V. vinifera</i> complex hybrids	Multiple QTLs	Fischer et al. (2004)
Leaf morphology	<i>V. vinifera</i> complex hybrids	Multiple QTL	Welter et al. (2007)
Plant sex	<i>V. vinifera</i> , <i>V. cinerea</i> , <i>V. rupestris</i> , <i>V. arizonica</i>	Single gene	Dalbo et al. (2000), Marguerit et al. (2009)
Flowering date	<i>V. vinifera</i> (SDL germplasm)	Multiple QTLs	Costantini et al. (2008)
Veraison date	<i>V. vinifera</i> complex hybrids and SDL germplasm	Multiple QTLs	Costantini et al. (2008), Fischer et al. (2004)
Ripening date	<i>V. vinifera</i> (SDL germplasm)	Major and minor QTLs	Costantini et al. (2008), Mejia et al. (2007)
Berry traits			
Colour	<i>V. vinifera</i> (winegrapes, complex breeds SDL germplasm)	Single gene	Doligez et al. (2002), Fischer et al. (2004), Lijavetzky et al. (2006), Salmaso et al. (2008)
Muscat flavour	<i>V. vinifera</i>	Major and minor QTLs	Battilana et al. (2008), Doligez et al. (2006), Duchene et al. (2009)
Berry size	<i>V. vinifera</i> (SDL germplasm)	Major and minor QTLs	Cabezas et al. (2006), Costantini et al. (2008), Doligez et al. (2002), Mejia et al. (2007)
Seed weight	<i>V. vinifera</i> complex hybrids	Multiple QTL	Fischer et al. (2004)
	<i>V. vinifera</i> (SDL germplasm)	Major and minor QTLs	Cabezas et al. (2006), Costantini et al. (2008), Doligez et al. (2002), Mejia et al. (2007)
seed number	<i>V. vinifera</i> (SDL germplasm)	Major and minor QTLs	Cabezas et al. (2006), Costantini et al. (2008), Doligez et al. (2002), Mejia et al. (2007)

SDL, seedless; QTL, quantitative trait locus.

et al. 2006, Battilana et al. 2008, Duchene et al. 2009). As these studies are expanded to further progenies, it will be possible to unravel additional complexity in the genetic structure of these traits.

Positional information on the location of major loci and QTLs facilitates the development of candidate gene hypotheses on the nature of the putative underlying genes (Costantini et al. 2008). However, final demonstration of the role of a specific gene in the determination of a given phenotypic trait requires clear positional identification through fine mapping or additional genetic and molecular proofs. For most QTLs, positional mapping requires Mendelisation through the generation of segregating populations where the QTL of interest segregates as a monogenic Mendelian trait (Alonso-Blanco and

Koornneef 2000). This is difficult to generate in grapevine because of their high heterozygosity and life cycle length. In fact, a positional cloning approach has only been tried for the *Run1* locus (Barker et al. 2005). Apart from *Run1*, associated with the presence of resistant gene analogues, the major berry colour locus mapped on LG2 has also been associated to the presence of a co-locating *MybA1* gene (Lijavetzky et al. 2006). The *MybA1* gene had been previously identified as responsible for berry colour based on the detection of colour somatic variants caused by the insertion and recombination of a *Gret1* retrotransposon in its promoter region (Kobayashi et al. 2004). Still, additional molecular analysis of this locus identified the occurrence of a closely linked *MybA2* gene that also contributes to variation in berry colour (Walker et al. 2007).

Loci, *Run1* and berry colour, exemplify the molecular complexity underlying natural genetic variation in grapevine. Consequently, apart from the positional identification of the genomic region where the gene or genes underlying the QTL are located, demonstration of the identity of the causative gene(s) and the functional polymorphism(s) represents one added difficulty when the available recombinants do not allow narrowing down the gene and gene variant. Several alternative experimental approaches can be combined in such cases to provide evidence of the molecular identification of a gene responsible for a QTL. Among them the Members of the Complex Trait Consortium (Members of the Complex Trait Consortium 2003) suggest at least two of the following strategies: the identification of DNA polymorphisms distinguishing alleles with different phenotypic effects; the existence of a mechanistic link between function of the gene and the trait of interest; functional studies on allele biochemical properties; mutational analysis showing that a loss-of-function mutation affects the trait of interest; the existence of natural genetic variation at a homologous locus affecting the same trait in another species; and transgenic complementation in different genetic backgrounds as described in an accompanying article (Vidal et al. 2010).

Alternatives to QTL analyses

The limitations and time consuming nature of QTL mapping and positional identification has promoted the search for alternatives. One of them is the exploitation of LD to identify sequence polymorphisms associated with a particular phenotype, also known as LD- or association-mapping. This strategy can be directly applied to collections of genotypes without limits on the genetic diversity used. Furthermore, phenotypic information from the grapevine collections can directly be used in association-mapping avoiding the time required to construct and evaluate additional progenies. The resolution achieved for association-mapping will be related to the extent of LD in the collections used. It is not clear yet how fast LD decays in grapevine, however, even in autogamous species like *Arabidopsis*, LD seems to rapidly decay over 10 kb (Kim et al. 2007). Thus, when using a high number of markers, polymorphisms identified through this approach would be expected to be closely linked to the functional locus. The feasibility of LD mapping has been shown in a pilot experiment in *Arabidopsis* on previously known genes (Hagenblad et al. 2004). In grapevine, re-sequencing experiments could provide information on nucleotide diversity to generate the SNP panels required for genome-wide association scans. Even if LD mapping can help identify specific linked markers explaining part of the trait variation, it does not provide evidence of causal relationship between sequence variation and phenotypic variation. In fact, marker–trait associations may not be due to linkage or causal relationships, but instead may be related to unexpected statistical association. For example, when cultivars showing a given phenotype are more likely to be related to each other than what would be expected by chance. This is the result of population

structure and may occur because cultivars from a specific area are more related than those coming from geographically distant regions (Sefc et al. 2000). There are statistical methods that can help to correct for this problem (McCarthy et al. 2008), but further experimentation is required.

Perhaps, the most efficient solution in grapevine to get the maximum information from association studies will come from their combination with the use of segregating populations to test the predictions of association studies on allele effects. Correspondingly, at specific genomic regions, LD could also be used to reduce the size of QTL confidence intervals (Kruglyak 2008) or to perform candidate gene association studies in reverse genetics approaches as has been shown in maize (Thornsberry et al. 2001).

Conclusions

The last few years have seen the generation of increasing amounts of molecular information on *V. vinifera* culminating with the first genome sequences. Considering the rapid development of new technologies, no limitations are foreseen concerning the acquisition of molecular information from additional genome sequences within the genus *Vitis* or on nucleotide variation within any of the *Vitis* species. However, for this information to have an impact on genome-assisted breeding and viticultural production systems, further research is required to identify the genes and gene variants responsible for important agronomic traits and to assign biological function to annotated sequences.

Demonstration of biological function requires genetic approaches which work on genetic variation. The development of grapevine laboratory systems such as the Pinot Meunier dwarf plants or near-homozygous lines like PN40024 can provide basic tools to undertake the generation of mutagenised populations or other functional genetic tools. Of special interest, given its direct breeding applications, is the use of natural genetic variation as the basis of forward genetic analyses. This approach also requires the construction of appropriate genetic tools such as collections and core collections, mapping populations, introgression lines, etc. Quantitative genetic analyses have proven useful in the identification of major and medium effect QTLs, and LD-based mapping strategies could be more efficient alternatives once further molecular tools like SNP panels are developed. In any case, fine mapping and final identification of genes and functional polymorphisms can be a time consuming process for some traits, because of the size and the life cycle length of the plants.

Genetic resources in *V. vinifera* are likely limited to only several thousand genotypes in germplasm stock centres or in endangered wild populations. Inter-fertility between species of the genus *Vitis* opens the genetic variation available for breeding across the whole genus. Considering the relevance of genetic resources for the future of the crop and their current scarcity, major efforts should be dedicated to the collection and characterisation of the existing resources in the species and the genus. Genomic

tools and information can help to rapidly generate genotypic information; however, collection of phenotypic data requires more careful characterisation at morphological, biochemical, physiological or pathological and environmental response levels. Open databases with these phenotypic and genotypic data are required as well as more efficient ways to store and exchange biological materials representing all the available genetic diversity.

Understanding the genetic structure of major grapevine agricultural and quality traits in terms of their genetic components and the relationship between sequence variation and phenotypic variation could provide ways to improve current classical cultivars either through directed selection of somatic variants or through gene transfer technologies. In addition, this information will allow the efficient breeding of a new generation of cultivars with berry composition similar to existing ones but incorporating disease resistances and traits that are well adapted to the required environmental and growing conditions.

Acknowledgements

Research activity in the author's laboratories is funded by grants from the Spanish Ministry of Science and innovation BIO2008-03892 and GEN2006-27782-C2-1-E/VEG.

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Manuscript received: 5 March 2009

Revised manuscript received: 20 July 2009

Accepted: 21 September 2009