Development of a rapid genetic system for linking traits to genes



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Abstract

Grapevine research conducted out throughout the world has identified approximately 30,000 genes in the grapevine genome. The challenge facing researchers is how to rapidly determine gene function and identify those genes that control key traits for vine performance, berry composition and wine attributes. This project describes a grapevine model system for rapidly linking genes to traits. The system is based on a new grapevine type known as a microvine that has a dwarf structure and fruits in less than one year. Advantages include reduced experimental time for studying fruit traits and small space requirements removing the need for field plantings.

Executive Summary

This report describes the successful development of a rapid method for linking traits to DNA markers and genes using a new grapevine type known as a microvine.

The grapevine plant has remained relatively unimproved for hundreds of years due in part to the difficulty of developing improved varieties. The plant is not conducive to genetic studies and as a result our knowledge of the inheritance and genetics of key traits responsible for vine performance, berry composition and wine attributes is very limited. Despite this there has been a rapid increase in grapevine genomic knowledge over the last 10 years, including the availability of a grapevine draft genome containing approximately 30,000 genes. However the grapevine plant still remains the limiting factor in applying this to grapevine improvement and the advancement of scientific knowledge. Grapevine is a difficult plant for genetic studies due to the size of the plant requiring plant populations to be maintained in the field, the long generation time (often three or more years), and the absence of genotypes for advanced strategies for linking genes to traits. Removal of these major limitations of long life cycle, need for field plantings and unique genotypes has now been achieved by the development of a unique genetic system based on a new grapevine form known as a microvine.

The microvine is a rapid flowering dwarf grapevine. The microvine was developed by CSIRO and the unique phenotype was found to be due to a DNA mutation in the *VvGAI1* gene (Boss & Thomas, 2002; Franks et al, 2002). In this project report we describe the creation of unique microvine phenotypes and genotypes suitable for rapid inheritance and genetic studies in grapevine and demonstrate by examples how the model microvine system can be used to rapidly study the inheritance and genetics of floral and berry traits. The model microvine system includes:

1. The development of microvine lines suitable for rapid evaluation of transgenes targeted to the berry to determine gene function. It has been shown that introduced genes expressed in the berry can now be evaluated in approximately one year which is 2-5 times quicker than the current situation with normal grapevines and avoids the need for GM field trials.
2. The development of microvine populations for inheritance studies and for linking DNA markers and genes to traits by genetic mapping. Rapid breeding methods have been developed and microvine populations produced that flower and fruit in less than one year for berry phenotyping and genetic mapping. The inheritance of the trait responsible for methoxypyrazines in aroma and flavour has been determined. The linking of DNA markers to traits and gene expression analysis has allowed the identification of candidate genes for berry size (fleshless berry mutant) and flower sex and the production of DNA SNP markers linked to the berry skin colour locus has resulted in the discovery of a novel lethal recessive locus (*Vvlrl1*) in the grapevine genome. This microvine system is a significant advance over existing field based systems that require 2-5 years to produce fruiting progeny populations suitable for inheritance and genetic mapping studies of floral and berry traits.

3. The rapid development of highly homozygous (>95%) inbred microvine lines suitable to link genes to traits by mutagenesis strategies. Induced mutagenesis has not been used for genetic analysis in grapevine due to the problems of managing highly heterozygous plants and the long generation time. The breeding process to obtain similar inbred lines would have taken 15-20 years using traditional breeding methods and varieties.

The above results illustrate the potential of the microvine system to provide an elegant solution in terms of small space requirements and short life cycle length for rapid grapevine genetic studies. The ability to investigate fruit traits and conduct multi-generational crosses over shortened time periods should see a rapid advancement in our knowledge of which genes control key fruit traits in grapevine, especially if the system is adopted by both Australian and international researchers.

For the Australian industry to remain competitive, it is likely that genetic solutions will become increasingly important through the use of improved planting material better suited to Australian conditions. The foundation of genetic improvement is an understanding of which genes control key traits and then applying this information to rapidly select improved varieties by advanced genetic methods. The ability of the microvine system to rapidly link genes and DNA markers to traits offers the real possibility to develop a smart Marker Assisted Breeding initiative to increase the efficiency of breeding improved rootstocks and scions for the Australian industry. Successful development could see a reduction from the traditional 25-30 year period required for breeding, evaluation and release to industry of a new variety to 10 years or less.

This project involved a number of international collaborations including; Prof. Laurent Torregrosa and Dr Alain Bouquet from UMR DIAPC, Montpellier, France; Dr. Anne-Françoise Adam-Blondon from URGV, INRA, Evry, France; Prof. Riccardo Velasco from IASMA Research Centre, San Michele a/Adige, Italy and Prof. José Miguel Martínez-Zapater from Departamento de Genética Molecular de Plantas, CSIC, Madrid, Spain.

Background

Since 2001 there had been a rapidly increasing amount of grape genomic information produced and placed into public databases in a large part due to the efforts of the International Grape Genome Program. Draft genetic and physical maps of the grapevine genome were developed and in 2007 two draft genome sequences of grapevine were published with estimates of approximately 30,000 genes present in the genome (Jaillon et al, 2007b; Velasco et al, 2007).

Grapevine had moved quickly from a situation where there was very little genomic information available to one of abundance. To take advantage of this genomic information for grapevine improvement required the development and use of new methods to determine the function of the 30,000 genes and the linking of genes to key traits.

Grapevine is a difficult plant for functional genomics due to:
1. The size of the plant requiring plant populations to be maintained in the field.
2. The long generation time, often three or more years, inhibiting genetic crosses proceeding beyond the first generation.
3. The cost and time (>5 years) involved in OGTR approved field trials of transgenic plants for fruit evaluation due to poor flowering under glasshouse conditions.
4. The absence of a large mutant population for linking genes to function.

These problems are especially relevant for research on fruitfulness, floral development, fruit development and metabolites responsible for grape composition and wine attributes. Knowledge of which genes control key traits is currently very poor for grapevine. Exploitation of grape genomic information by Australian grapevine researchers and industry will depend on the ability to quickly identify and confirm the function of genes that are involved in key traits, especially berry quality traits and an understanding of gene function is an important first step in applying this information for the benefit of the industry.

The ideal system for rapid genetic studies in grapevine would have many of the features that originally made Arabidopsis the model annual plant of choice for linking genes to traits by forward and reverse genetic approaches; small space requirements, small diploid genome, short generation time, homozygous lines for genetic mapping and mutagenesis and efficient transgenic methods.

We had previously reported the discovery of a L1 Pinot Meunier mutant plant (L1 mutant) of dwarf stature and precocious rapid flowering, producing bunches in place of tendrils (Boss & Thomas, 2002; Franks et al, 2002). A single mutated DNA base in the grapevine *GA insensitive* gene (*VvGAI1*) was found to be responsible for the phenotype and genetic studies showed that the mutated allele (*Vvgai1*) behaved in a semi-dominant manner (Boss & Thomas, 2002). This microvine plant had features suitable for developing a model grapevine system for forward and reverse genetic studies. It was proposed that a successful outcome of this project would remove the major impediments listed above for grape genetic studies.

Project Aims & Performance Targets

The aim of the project was to develop genetic systems to rapidly link DNA markers and genes to traits using the microvine plant as it had traits similar to an annual plant. It flowers and fruits within a year and due to its' small size is easily maintained under glasshouse conditions. As a step towards a fast grape genetic system this project investigated:

1. Development of microvine lines and progeny populations for inheritance studies and rapid genetic mapping of floral and berry traits for linking traits to DNA markers and genes.

2. Development of microvine lines amenable to transformation for rapid gene function studies in the berry by reducing the time from transformation to glasshouse fruit production to less than one year and removing the need for OGTR approved field trials.

3. Development of inbred microvine genotypes suitable for future experiments to create mutant populations for linking genes to traits.

Revised Outputs and Performance Targets approved by GWRDC on 11th September 2008. Submission of Final Report in September 2010 was approved by GWRDC on 30 June 2010.

Outputs and Performance Targets 2006-2007

OUTPUTS	PERFORMANCE TARGETS	TARGET DATE
Appoint staff	Postdoctoral Fellow appointed	June 07
Microvine crossed to a fleshless berry mutant	Crosses done, seed collected and sent to French collaborators.	June 07
Microvine crossed to genotypes that are more easily transformed	Crosses done, seed collected for germination.	June 07
Inbred microvine individuals assessed for homozygosity	DNA extracted from five inbred genotypes (S3) and the original microvine and evaluated for homozygosity using 100 DNA markers spread across the grapevine genome.	June 07

Outputs and Performance Targets 2007-2008

OUTPUTS	PERFORMANCE TARGETS	TARGET DATE
Additional key traits identified for genetic studies	Key traits identified by discussions with industry representatives, prioritised and feasibility of inheritance studies determined.	January 08
Berry flavour inheritance	Berry sample collection started and analysed by GC-MS to identify segregation and inheritance of flavour secondary metabolites	June 08
Two microvine populations established for flavour assessment	Two microvine populations (MVxR, MVxCS) of at least 96 individuals established in the glasshouse for DNA extraction and berry evaluation.	June 08
Genetic map construction	DNA extracted from populations and DNA marker research initiated to create genetic maps for linking DNA markers to berry traits	June 08
Transgenic microvine development	Embryogenic cultures established from anthers of selected microvines and evaluated for transformability by *Agrobacterium* and regeneration.	Jan 08
Inbred microvine lines for mutagenesis	Near homozygous lines identified by DNA markers selfed to create a new selfed population. Seed collected for germination.	Aug 07
Additional key traits identified for genetic studies	Crosses initiated, seed collected for germination.	June 08

Outputs and Performance Targets 2008-2009

OUTPUTS	PERFORMANCE TARGETS	TARGET DATE
Berry flavour	If trait shows segregation, fine scale genetic mapping with DNA markers and candidate genes initiated to localise the trait. If no segregation then backcrossing done to determine genetics.	June 2009
Berry size and flesh development	DNA obtained from French collaborators and fine scale genetic mapping with DNA markers and candidate genes done to localise the trait.	June 2009
Additional key traits identified for genetic studies	Seed germinated and populations established in glasshouse.	June 2009
Transgenic microvine development	Study continued into refining transformation protocols.	June 2009
Inbred microvine lines for mutagenesis	DNA extracted from five inbred genotypes and evaluated for homozygosity using 100 DNA markers spread across the grapevine genome. Decision taken on suitability of genotypes for future mutagenesis studies.	March 2009

Outputs and Performance Targets 2009-2010

OUTPUTS	PERFORMANCE TARGETS	TARGET DATE
Additional key traits identified for genetic studies	Progeny populations analysed to determine the segregation and inheritance of the selected traits	June 2010
Berry flavour	Study continued to fine scale genetic map with DNA markers to localise the trait.	June 2009
Berry size and flesh development	Study continued to fine scale genetic map with DNA markers and candidate genes to localise the trait.	June 2009
Extension and communication	Manuscript submitted to industry journal.	June 2010
Final report produced	All data analysed, summarised and communicated to industry in a Final GWRDC Report.	30 June 2010

Methods

Plant material

Fourteen cultivars were used for this study: Ugni Blanc fleshless berry mutant (UBM), Ugni Blanc (UB), Trebbiano (synonym of Ugni Blanc), 00C001V0008 (a female picovine), Chardonnay, Cabernet Sauvignon, Riesling, Pinot Noir, R23, Shiraz, Sultana, Richter 110, Grenache and the L1 Pinot Meunier microvine mutant (L1). Progeny from the cross Cabernet Sauvignon x Riesling were also used.

The following breeding was done to produce microvine progeny: Grenache x L1 microvine, L1 microvine x Riesling, L1 microvine x Cabernet Sauvignon and the picovine 00C001V0008 x UBM. The progeny from the L1 microvine x Cabernet Sauvignon and L1 x Riesling were produced to investigate berry flavour inheritance. Progeny from the 00C001V0008 x UBM cross were used to investigate the inheritance of the fleshless berry phenotype and 478 vines were generated and respectively named 06C020**V**0001-**V**0106 for the progeny established in Adelaide and 06C020**F**0001-**F**0372 for the progeny established with collaborators in Montpellier. Other microvine crosses and progeny were used to investigate the additional key traits of disease resistance, flower sex and berry colour.

For inbred lines, starting with the microvine L1, successive selfing was done to produce progeny with increasing homozygosity at each generation from the first generation S1 to the fifth inbred generation S5.

All the plants were grown in glasshouses or growth rooms, except Trebbiano that was maintained in the Waite vineyard. Growing conditions and protocols for breeding and seed germination were described in (Chaïb et al, 2010).

Genetic transformation

Somatic embryo cultures were initiated from anthers of immature flowers (Franks et al, 1998). Embryogenic callus initiation, maintenance media, culture conditions, *Agrobacterium*-mediated transformation and transgenic plant regeneration protocols were essentially as described previously (Bouquet et al, 2006; Iocco et al, 2001). After 2 weeks on C1 maintenance medium, embryogenic tissue was transferred to GS1CA medium for 2 weeks prior to transformation. Embryogenic callus was transformed with binary vectors (pBINmgfp5-ER, pCLB1301, pCLB2301) containing the green fluorescent protein (GFP) gene. The pBINm-gfp5-ER binary plasmid (Haseloff et al, 1997) contains *gfp-5-ER* under the control of CAMV35S and contains *nptII* for kanamycin selection of transgenic plants. The binary plasmids pCLB1301 and pCLB2301 were derived from pCAMBIA1301 (GB: AF234297) and pCAMBIA2301 (GB: AF234316) respectively (Hajdukiewicz et al, 1994). The vector pCLB1301 is a modified pCAMBIA1301 vector with the existing 35S promoter and *gusA* replaced by *gfp-5-ER* driven by the 35S promoter in the same orientation as the hygromycin (*htpII*) plant selectable marker. The vector pCLB2301 has the kanamycin (*nptII*) plant selectable marker and modified from pCAMBIA2301 as described for pCLB1301 to contain *gfp-5-ER*.

Phenotyping

For berry flavour, GC-MS was used to determine secondary metabolite peak differences. Populations were visually phenotyped for plant stature (wild-type (wt), VvGAI1/VvGAI1; microvine, VvGAI1/Vvgai1; picovine, Vvgai1/Vvgai1), flower sex (hermaphrodite, female, male), berry colour (Black, white) and the fleshless berry (Flb) phenotype. For powdery mildew disease resistance, detached leaves from progeny were inoculated with powdery mildew and incubated on moist filter paper in large petri dishes at 22°C and examined 6 days later for an infection response and sporulation.

Genetic mapping

Young leaf tissue was collected and processed by The Australian Genome Research Facility (AGRF) to extract DNA.

DNA markers development

Single Nucleotide Polymorphism (SNP) markers were developed to cover the 19 chromosomes of the grapevine genome through a Spain-Australia collaboration and an Italy–France-Australia, collaboration (Lijavetzky et al, 2007).

To increase SNP marker density on chromosomes 2 and 18, useful markers from six published maps representing the major diversity of molecular markers available in grapevine (Adam-Blondon et al, 2004; Di Gaspero et al, 2007; Lamoureux et al, 2006; Riaz et al, 2004; Troggio et al, 2007) were tested. Thirteen cultivars and progeny from various microvine crosses were genotyped to identify markers that were polymorphic and followed a Mendelian segregation. Unique SNP markers were also developed from examination of DNA genomic sequences present at NCBI (National Center for Biotechnology Information).

Selected sequences featuring targeted SNP were submitted to the AGRF for primer design and genotyping. The MassARRAY® iPLEX Gold assay with matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) detection (Sequenom, http://www.sequenom.com/) was used for SNP analysis.

Genetic mapping & linkage group analysis

The construction of marker maps was performed using MapManager QTXb20 (Manly et al, 2001). Marker analysis was organized into three steps: (1) marker independent segregation was checked, (2) pair wise recombination fraction was estimated and (3) a recombination value cutoff was used to form linkage groups and compare marker order according to the rapid chain delineation method. MapChart v2.2 (Voorrips, 2002) was used for graphical presentation of genetic linkage groups.

Gene expression

RNA extraction and cDNA synthesis

Inflorescences or leaves were collected, frozen and finely ground in liquid nitrogen. About 150 mg of powder was used for RNA purification using a Sigma Spectrum Plant Total RNA Kit (Sigma-Aldrich, St Louis MO, USA). According to the manufacturer's instructions, protocol B was followed, including an on-column DNase digestion using the Qiagen RNase-free DNase I (Qiagen, Valencia CA, USA). Total RNA was recovered by two successive elutions using 30 µl of Elution Solution. Concentration and quality of total RNA was determined by spectrophotometric analysis and agarose gel electrophoresis.

First strand cDNA synthesis was performed from 1 µg of total RNA using 1 µl of 50 µM oligo(dT)20 and Invitrogen SuperScript^{TM} III RNase H^{-} Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen, Carlsbad CA, USA). After the reaction, the final volume of cDNA was adjusted to 200 µl with sterile nanopure water.

Real-time quantitative PCR was conducted as previously described in (Sreekantan et al, 2006) with some modifications as follow. Two µl of diluted cDNA and 2X Fast Start SYBR Green Master (Rox) (Roche Diagnostics, Mannheim, Germany) were used. Serial dilution standards of purified PCR product were included in each run, ranging from 10^{-3} to 10^{-6} for the genes of interest and from 10^{-4} to 10^{-6} for the housekeeping genes (Ubiquitine, EF1-α).

Results & Discussion

The original L1 microvine was heterozygous for the *Vvgai1* mutation and had the genotype (*VvGAI1/Vvgai1)*. Breeding with the microvine can produce 3 different genotypes and associated phenotypes and for reader clarity the naming convention adopted for describing phenotypes and genotypes is; wild-type vines with normal stature (*VvGAI1/VvGAI1)*, microvines with reduced stature (*VvGAI1/Vvgai1)* and picovines of very small stature (*Vvgai1/Vvgai1)*. All microvine and picovine plants containing the *Vvgai1* mutation are not only dwarf but are rapid flowering and have a short generation cycle.

Development of genetic lines and progeny populations for rapid genetic and phenotypic analysis of key traits

Grapevine is a perennial plant and plant improvement in grapevine has been considerably limited mainly due to the long life cycle (minimum 3 years from seed to seed) and the field requirement for cultivation. These two major biological constraints have represented serious obstacles in terms of development of crosses for genetic studies. Not only does it slow down the characterization of agronomic traits of interest, from linking traits to DNA markers and genes by genetic mapping but it also slows down the breeding of new elite varieties containing a combination of favourable traits.

As an example, in order to study key berry traits an F1 cross between Cabernet Sauvignon and Riesling was initially developed but trait characterization was slow as the progeny plants started to produce fruit 3-5 years after field planting. Moreover, the plants had a normal stature (*VvGAI1/VvGAI1)* and were grown in a vineyard. As a consequence, environmental conditions could not be controlled and environmental variables become problematic when studying traits associated with quantitative variations such as berry flavour. In addition, while an initial trait mapping study could be done using an F1 population, further crosses such as backcross or selfing are often required to fine map a trait and identify a candidate gene responsible for the trait. The delay in grapevine fruit production at each new generation considerably limits the linking of traits to DNA markers and genes. This example illustrates the constraints associated with genetic studies in grapevine and the potential of the *Vvgai1* mutated allele to provide an elegant solution in terms of space requirements and short life cycle length for studying fruit traits and conducting multi-generational crosses.

Consequently, the L1 mutant was respectively crossed to Cabernet Sauvignon and to Riesling to produce two new progeny populations. All parents being hermaphrodite, the L1 mutant was used as a female parent and flowers were emasculated to receive pollen from Cabernet Sauvignon or Riesling, as male parents. The segregation for the *Vvgai1* mutated allele resulted in 50% of the progeny possessing the dwarf stature and precocious flowering properties, as the wild-type cultivars were *VvGAI1/VvGAI1* and the L1 mutant *VvGAI1/Vvgai1*. Microvine individuals were isolated for each population and were grown in a glasshouse. The progeny began to flower and produce fruit a few months after seed germination.

Evaluation of the previously described *Vvgai1*-related crosses suggested that a more efficient system to investigate the inheritance and genetic mapping of a particular trait could be developed by the use of a picovine (*Vvgai1/Vvgai1*) female flower (*f/f*) plant as pollen acceptor crossed to a wild-type genotype containing a trait of interest. Thus no emasculation would be required prior to pollination. The *Vvgai1* mutated allele contributed by the picovine would result in 100% of progeny having the short stature and rapid flowering phenotype of the microvine genotype (*VvGAI1/Vvgai1*). All microvine progeny derived from a female picovine crossed to wild-type *V. vinifera* cultivars with hermaphroditic (*H/H* or *H/f*) flowers would also be capable of producing fruit.

As an example of this approach, picovine female plants were first bred by selfing of the L1 mutant and a highly fertile fruitful genotype, 00C001V0008, was selected, from the S1 generation, as a female parent for breeding. To study the genetics of both the fleshless berry mutation (*flb*) (Fernandez *et al.* 2006a, Fernandez *et al.* 2006b) and flower sex determination, plant 00C001V0008 was pollinated with pollen from the FLB mutant. Nearly 2000 seeds were harvested with the number of seeds per berry similar to wild-type vines but less on a bunch basis due to fewer flower numbers per bunch produced by picovine genotypes. Seed germination success rates were 24-59% which was comparable to that achieved in our hands with wild-type seeds. A subset of 103 plants was grown (Fig. 1) in a growth room at a density of 34 microvines per m^2 and scored for flower sex type, Flb^- phenotype and time to flowering.

Figure 1. Microvine progeny derived from the cross between a female picovine (*Vvgai1/Vvgai1*) and the Ugni Blanc mutant (*VvGAI1/VvGAI1*) maintained in a growth room for phenotyping flower sex and fleshless berry characters. Ribbons indicate phenotyped plants.



The rapid flowering phenotype conferred by the *Vvgai1* mutation was observed in the mutant microvine progeny from the 00C001V0008 x FLB mutant cross with 76% flowering and phenotyped within 3 months after potting. The remaining progeny flowered within one year after a pruning treatment. Genetic mapping was carried out with DNA markers and confirmed that the *Vvgai1* mutation had no effect on the segregation of other traits studied or on genetic mapping suggesting that a *Vvgai1*-based breeding system would have general application for many other traits.

As a result of the success of these crosses, a number of other crosses were carried out to produce microvine populations for studying the inheritance of additional key traits and mildew resistance.

Development of inbred lines suitable for future studies to create mutant populations for linking genes to traits and lines for improved genetic mapping.

We demonstrated above that the use of the L1 mutant or a female picovine line was a powerful way to create plant material with a short life cycle and decreased space requirements for grapevine inheritance and genetic studies. However, another constraint limiting grapevine genetic studies is the high level of heterozygosity of the grapevine genome. As an illustration, L1 mutant, Shiraz, Chardonnay and the Ugni Blanc mutant were genotyped with a set of 480 SNP markers and the results show that most of the molecular markers were heterozygous in all genotypes. As a result, only 7-11% of the markers were informative to map a trait of interest from Shiraz, Chardonnay or the Ugni Blanc mutant when crossed with the L1 mutant (Table 1).

Genetic dissection of traits based on *Vvgai1* would gain a significant advantage if homozygous lines were used to increase the informativeness of bi-allelic SNP markers used for genetic mapping. In addition, homozygous lines would be a valuable resource for reverse genetic studies employing mutagenesis and TILLING (McCallum *et al.* 2000). Breeding of homozygous lines was conducted with the objective of obtaining black (*B/B*) and white (*b/b*) berry grapevines with ≥ 95% homozygous genomes. Also desirable would be the production of homozygous lines differing in both stature and flower sex. To achieve this, successive inbreeding by self-fertilisation (selfing) was conducted while maintaining a heterozygous state at the two loci for stature (*VvGAI1/Vvgai1*) and flower sex (*H/f*).

The L1 mutant (*VvGAI1/Vvgai1; H/f; B/b)* was used as starting material to conduct successive self-fertilization inbreeding for 5 generations. The original genotype had a homozygosity level of 6.84% based on the analysis of 263 SNP and 51 SSR markers spread among the grapevine genome (Table 2; Fig. 2). After the first 3 generations of selfing (S1-3), families with a low fertility index or abnormal phenotypes were excluded from further breeding. Many mutant phenotypes were observed in these initial generations including sterility, seedlessness, floral and berry abnormalities. Abnormal flower phenotypes included multiple unfused carpels (Fig. 3a), multiple pistils (Fig. 3b) and petals

that abscise at the top and sides instead of calyptra dehiscence (Fig. 3c). In some genotypes, flowers with unfused carpels were capable of forming misshaped berries (Fig. 3d). The unexpected level of abnormal phenotypes in these early selfed generations suggested that the highly heterozygous genome of the L1 mutant was masking multiple mutations.

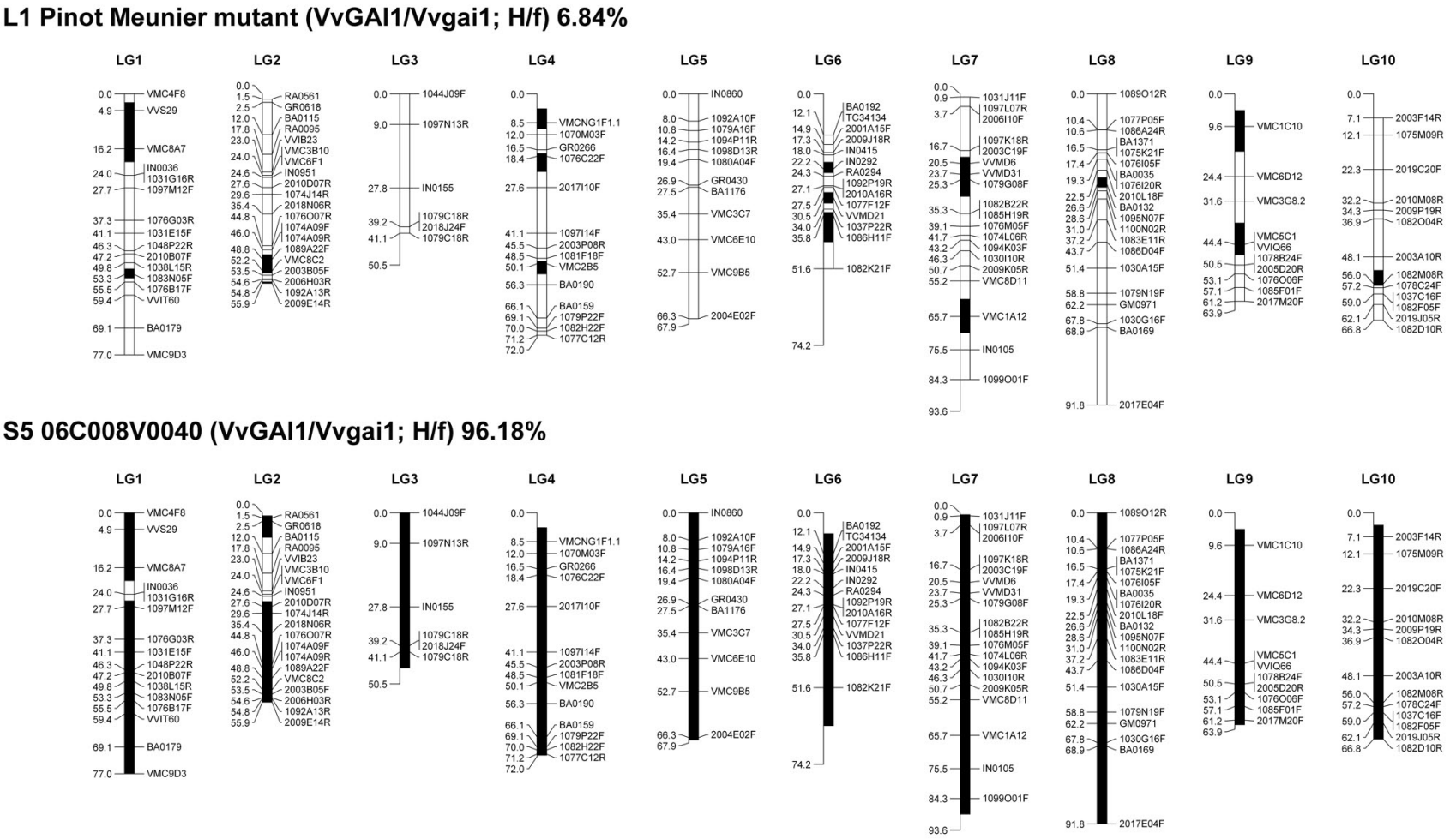
Table 1. Informativeness of grapevine bi-allelic SNP markers increases with increasing homozygosity of one parent. Percentages within the matrix correspond to the expected number of polymorphic markers from a set of 480 SNPs that can be mapped for the male parent of an F_1 progeny population and linked to a phenotype. Homozygosity levels of each genotype are also indicated in parenthesis for the same marker set.

	Female Parent	
Male Parent	**L1 mutant (34.45%)**	**06C008V0002 (98.94%)**
Shiraz (56.18%)	11.53%	42.77%
Chardonnay (59.16%)	7.37%	40.38%
Ugni Blanc mutant (66.31%)	11.37%	32.39%

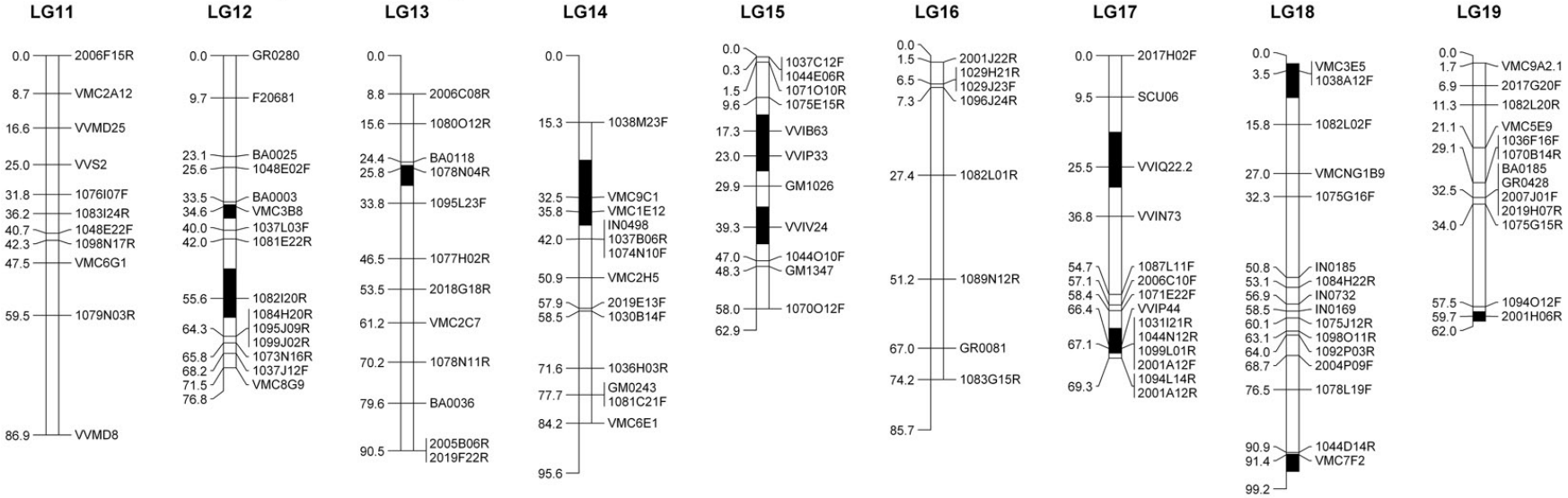
Table 2. Genome homozygosity of each inbred generation estimated using a set of 263 SNP markers. From a unique selfed third generation (S3) family, two S4 lines were selected to develop two highly homozygous S5 families.

L1 mutant	**S1**	**S2**	**S3**	**S4**	**S5**
6.84%	52.11%	73.37%	87.6%	93.51%	93.87%-98.47%
				94.27%	95.04%-98.09%

Figure 2. Graphic representation of the grape reference genetic map showing the repartition of homozygous and heterozygous genome portions for L1 microvine and 06C008V0040, an S5 inbreed line. For each chromosome, black and white areas respectively represent homozygous and heterozygous loci. Linkage groups are named LG1 to LG19. Cumulative genetic distances, in cM, are indicated on the left side of each linkage group and marker names on the right side. Only scored markers, located on the reference map, are shown which corresponds to 208 SNP of the 263 genotyped and 45 of the 51 SSR genotyped. A simple bar at linkage group extremities is used to represent the whole chromosome length when none of scored markers was present at these positions.



L1 Pinot Meunier mutant (VvGAI1/Vvgai1; H/f) 6.84%



S5 06C008V0040 (VvGAI1/Vvgai1; H/f) 96.18%

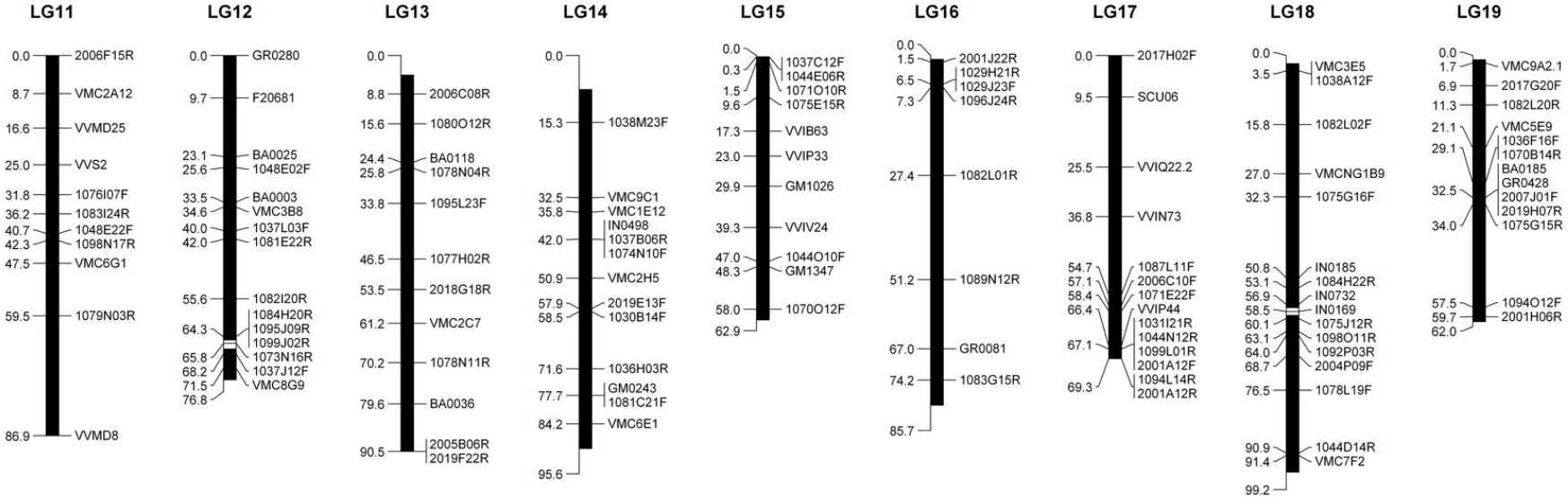
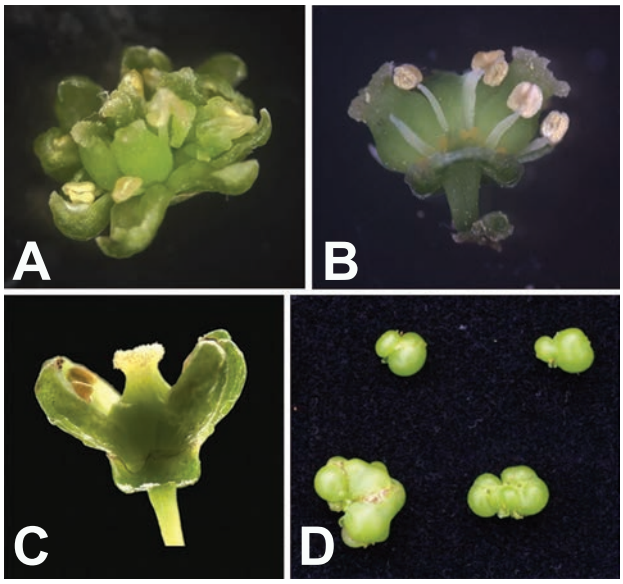


Figure 3. Abnormal floral and berry phenotypes observed in early generation inbred microvine lines. (A) Flower with multiple unfused carpels, (B) multiple pistils, (C) petals abscising apically and longitudinally preventing the formation of the cap, (D) deformed berries resulting from unfused carpels pre-anthesis.



At the S3 and S4 generations, marker-assisted selection was applied to identify inbred lines with the highest level of genome homozygosity. DNA screening at the S4 generation identified 8 plants with 88.85% to 94.55% homozygosity which were used for further inbreeding. The next generation of S5 individuals were found to have genome homozygosity levels between 93.87% and 98.47% (Table 2). Phenotyping and genotyping of individuals with >95% genome homozygosity within each of the two S5 families identified, for both families, all 9 possible combinations for flower sex (female, f/f; hermaphrodite, *H/H* or *H/f*) and stature; wild-type, microvine or picovine (Fig. 4). The linkage group map of the S5 individual in Figure 2 is representative of a highly homozygous microvine hermaphrodite where both loci (*VvGAI1/Vvgai1*; *H/f*) are still maintained in the heterozygous state.

These unique genotypes and phenotypes represent a biological solution for efficient inheritance analysis and genetic mapping of grapevine traits. For genetic mapping, bi-allelic markers like SNPs suffer from low informativeness especially in highly heterozygous species such as grapevine requiring the costly screening of a large number of markers to identify polymorphic markers. Informativeness of bi-allelic markers like SNPs increases in crosses involving homozygous lines. Typically, in a cross involving the L1 mutant, only 7-11% of SNP markers are potentially useful to link a marker to a trait of interest from the pollen donor parent (Table 1). Using an S5 homozygous female parent, such as 06C008V0002, most of the loci for the cross progeny will be aa♀ x ab♂. In this context, the probability of finding a marker linked to the trait of interest from the male parent has increased approximately 3-5 fold. The inbred line

06C008V0002 also displayed the same advantages as the heterozygous genotype 00C001V0008, it is a female picovine and all progeny would be rapid flowering microvines when crossed with a wild-type allowing rapid phenotyping of flower and fruit traits.

Figure 4. Highly homozygous S5 wild-type (right), microvine (centre) and picovine (left) lines.



These distinct but closely related highly homozygous lines are expected to be useful in future mutagenesis studies. Construction of mutagen-induced grapevine mutant populations would be a valuable resource for both forward and reverse genetic studies to link traits to genes (Henikoff *et al.* 2004, McCallum *et al.* 2000). The homozygous hermaphrodite (H/H) genotypes, either wild-type or picovine would be an obvious choice as starting material for mutagenesis as M2 progeny are usually required from a M1 population to identify recessive mutants. In addition, the homozygous background is highly advantageous when identifying mutations by TILLING to link the mutation responsible for the mutant phenotype (Comai and Henikoff 2006, Slade and Knauf 2005).

Grapevine breeding is slow and has been held back for many years partly due to the biological features of the plant (Alleweldt and Possingham 1988). Incorporation of the *Vvgai1* based system and associated homozygous lines into genetic improvement programs may result in significant benefits. In addition to the advantage of short generation time and small space requirements to screen large numbers of plants, the *Vvgai1* system also lends itself to efficient

Marker Assisted Selection (MAS) and gene pyramiding to identify and maintain elite genotypes, as demonstrated by our selection and maintenance of genotypes heterozygous at two loci (*VvGAI1/Vvgai1; H/f)* over five generations. The microvine genotype (*VvGAI1/Vvgai1*) would appear to be the preferred genotype for gene pyramiding. Once pyramiding is completed the desired genotype of interest can be recovered in a wild-type phenotype for cultivation in vineyards as demonstrated for the S5 progeny.

Inheritance studies and linking traits to DNA markers and candidate genes by genetic mapping

Molecular DNA markers and genetic maps

The choice of plant material is critical to conduct genetic studies, especially for perennial crops for which the production of new populations is time consuming and requires large field areas. Our solution, as described above, has been to use microvine populations to allow rapid phenotyping in both glasshouses and growth rooms. Moreover to efficiently link a gene to a phenotype, genetic maps sufficiently dense in DNA markers are required. Until recently, most of the genetic maps published in grapevine were based on the use of microsatellite (SSR) markers (Adam-Blondon et al, 2004; Di Gaspero et al, 2007; Riaz et al, 2004). These maps were informative enough to allocate a qualitative or a quantitative trait to one or more chromosome(s) but the region identified remained relatively large and rarely under 10 cM (Doligez et al, 2006; Marguerit et al, 2009). This coarse localisation is far too imprecise whether the objective is to link the trait to a marker for marker-assisted selection or to identify the gene(s) controlling the trait. To improve on this type of genetic mapping high numbers of molecular markers evenly spread across the genome are needed. Nowadays, bi-allelic markers such as SNPs are currently preferred, not only due to their suitability for use in high throughput platforms but also because they are highly abundant and usually evenly distributed along the genome with the frequency estimated to range from 4 to 16 SNPs per kb (Lijavetzky et al, 2007; Velasco et al, 2007). However, SNP markers are less informative than the multi-allelic microsatellite markers. Consequently, large numbers of SNP markers need to be tested to find polymorphic markers linked to a trait. In this context, the grapevine community has made a huge effort to develop SNP markers (Lijavetzky et al, 2007; Vezzulli et al, 2008b) and this was particularly facilitated by the availability of the two draft grapevine genome sequences (Jaillon et al, 2007b; Velasco et al, 2007).

Within the framework of this project, the development of molecular markers was performed for different purposes. Firstly, a set of 281 SNP markers, evenly distributed over the genome, and heterozygous within the L1 microvine were identified and used to select the near-homozygous lines within all five selfed generations. These markers were genotyped on the Cabernet Sauvignon x Riesling and on the L1 x Cabernet Sauvignon progenies to identify useful markers that followed a Mendelian segregation. From this analysis 263 SNP markers were validated and confirmed as useful. In addition, in order to have at least one marker every 10 cM on each chromosome, 53 SSR markers were used to complement the SNP markers.

In addition, another set of 217 SNP markers were developed to improve the coverage over the genome and to increase the proportion of polymorphic markers across genotypes. These markers were also tested for Mendelian inheritance as described previously and all were validated. Altogether, 480 SNP markers were developed for genetic analysis such as trait mapping, genetic diversity characterization or association genetic studies. In the case of genetic

mapping, Table 3 illustrates how the increased number of molecular markers increased the genome coverage and refined the precision and reliability of a trait location during fine mapping. Table 3 also shows that when both parents have a high level of heterozygosity, a high proportion of polymorphic markers will be common to both of them and therefore, less informative for genetic mapping purposes. And while the use of the L1 microvine to develop crosses is beneficial in term of biological properties, the heterozygous genome of the L1 microvine is not as useful as a more homozygous inbred microvine genotype. Nevertheless, the disadvantages of using SNP markers is becoming less of an issue, especially with the recent development of a *Vitis* 9K SNP genotyping array (Myles et al, 2010) and increasing availability of next-generation sequencing technologies for SNP discovery. The SNP markers developed in this project and the advances in technology for SNP discovery will benefit not only academic research but it will also provide the industry with major tools for high throughput marker screening for variety identification, certification and protection as well as applied marker-assisted selection for breeding purposes.

Table 3: Number of polymorphic markers in different SNP panels for each parent of the following crosses; Cabernet Sauvignon x Riesling, L1 microvine x Cabernet Sauvignon and 00C001V0008 x Ugni Blanc Mutant.

	281 SNP panel		480 SNP panel	
	Polymorphic markers	Average number of markers per chromosome	Polymorphic markers	Average number of markers per chromosome
Cabernet Sauvignon	62	3.3	121	6.4
Riesling	54	2.8	94	4.9
Common	91	4.8	165	8.7
L1 microvine	133	7	170	8.9
Cabernet Sauvignon	3	0.2	68	3.6
Common	14	0.7	96	5.1
00C001V0008	80	4.2	105	5.5
Ugni Blanc Mutant	26	1.4	101	5.3
Common	88	4.6	193	10.2

For candidate gene identification by fine genetic mapping and positional cloning approaches (see below for the fleshless berry locus and flower sex locus) the identification and development of SNP markers targeting specific regions was required. In this case, the chromosomal regions of interest were aligned with the corresponding zones from six published maps representing the major diversity of molecular markers available in grapevine at this time (Adam-Blondon et al, 2004; Di Gaspero et al, 2007; Lamoureux et al, 2006; Riaz et al, 2004; Troggio et al, 2007; Vezzulli et al, 2008a). The availability of the two draft genome

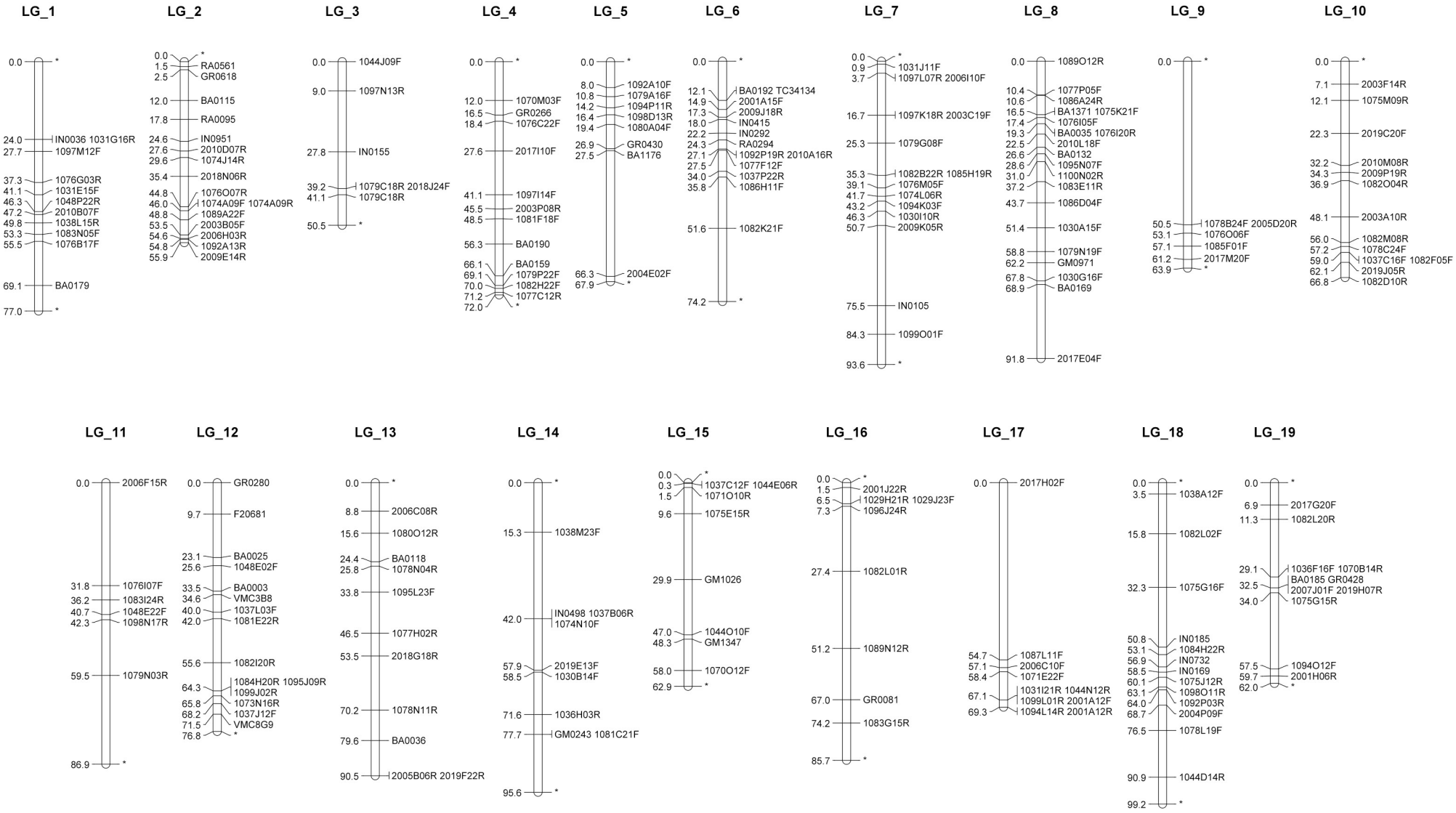
sequences was also utilized to detect SNPs preferentially located in predicted transcribed regions. As the two genome sequences were produced from the inbred genotype PN40024 (Jaillon et al, 2007a) and the heterozygous cultivar Pinot Noir (Velasco et al, 2007), the alignment of both sequences provided an additional approach to identify potential SNPs in a target region for fine genetic mapping.

Inheritance and genetics of berry aroma and flavour

Methoxypyrazines are important volatiles that contribute to wine aroma and flavour and are present in cultivars such as Sauvignon blanc and Cabernet Sauvignon. They are thought to contribute to the vegetative aroma intensity. Progeny from the L1 microvine x Cabernet Sauvignon cross were screened to determine the inheritance of the flavour compounds methoxypyrazines. Berries were collected from the progeny and analysed by GC-MS to determine secondary metabolite peak differences. The results showed that all progeny possessed methoxypyrazines. However only the Cabernet Sauvignon parent produced these compounds and not the L1 microvine. This indicated that this flavour trait was inherited in a dominant manner and that Cabernet Sauvignon was homozygous for the trait. As all progeny had the trait, genetic mapping could not be done on the F1 population, as there was no segregation of the trait. To produce a population that segregated for methoxypyrazines a backcross F2 microvine population was created taking advantage of the rapid flowering phenotype of the microvine. Analysis of fruit obtained from the F2 progeny showed that there was segregation of methoxypyrazines and that genetic mapping could be carried out to link the trait to DNA markers.

As an initial step for genetic mapping for flavour compounds the parents, L1 microvine, Riesling and Cabernet Sauvignon were genotyped with a set of 480 SNP markers and genetic maps created. The SNP genetic maps across all 19 chromosomes for Cabernet Sauvignon are shown in Figure 5. The results showed that most of the SNP markers were heterozygous for all of the parents and could be mapped to their genomes. The localisation of these markers will allow for the future fine scale mapping of the methoxypyrazine trait in the F2 progeny.

Figure 5. Genetic map of Cabernet Sauvignon created with 480 SNP markers

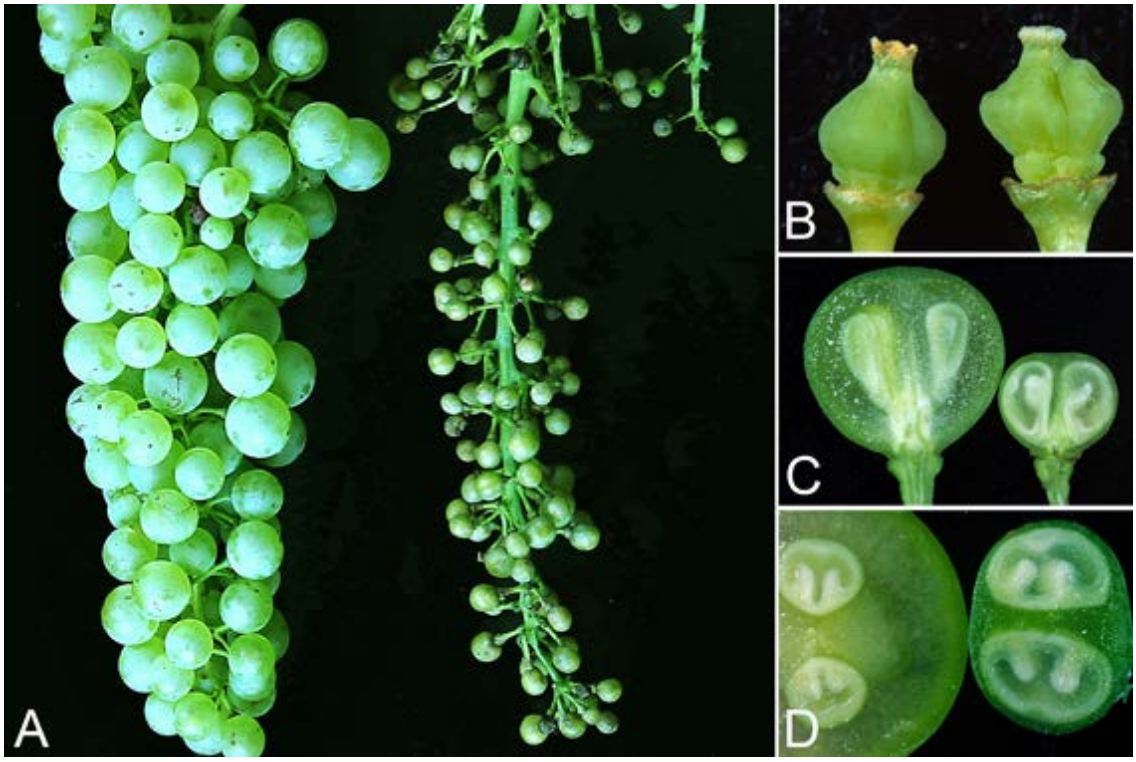


Inheritance and genetics of berry size, the Fleshless Berry Mutation (FLB)

In grapevine the genetic determinism of berry growth and size is unknown. This trait is very difficult to study for several reasons. Firstly, berry size variability exists within a cluster. Secondly, at the physiological level, three main stages occur during berry growth, an early cell division phase followed by two successive cell elongation phases, suggesting the existence of complex regulation mechanisms. Thirdly, berry size is also strongly influenced by environmental conditions such as water stress. In order to have a better understanding of the genetic control of berry growth in grapevine we investigated a natural mutant that produced berries without flesh.

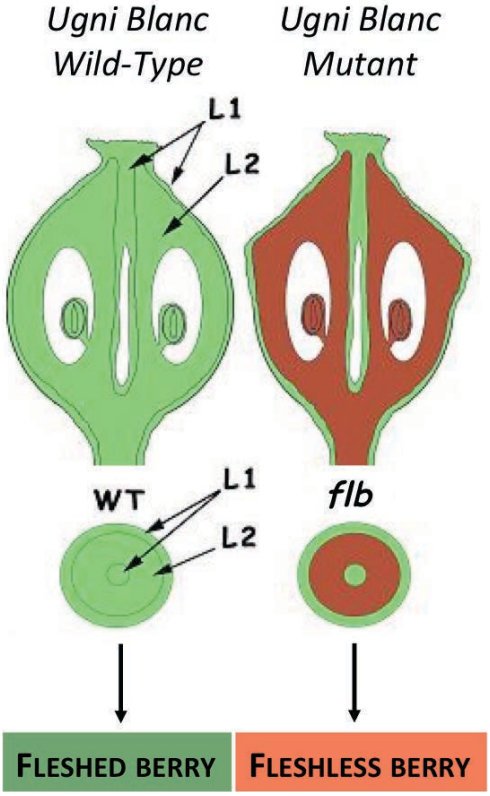
The grapevine fleshless berry phenotype (*flb*) is the result of a spontaneous somatic mutation event. The phenotype originally appeared on a single branch on a single plant of the *Vitis vinifera* cultivar Ugni Blanc and is characterized by major disturbances in ovary and mesocarp development (Fig. 6) (Fernandez et al, 2006b). At the flower level, a misshaped pistil shows a transversal wrinkle at the style base (Fig. 6B). At the berry level, an impaired growth of the pericarp results in the development of a fleshless berry that is typically reduced to an organ of seeds and skin (Fig. 6C and 6D).

Figure 6. Major phenotypes associated with the *flb* mutation: Ugni Blanc Mutant on the right and Ugni Blanc Wild-Type on the left. (A) Bunches at the ripe stage. (B) Pistils from flowers at anthesis. (C) Berries at veraison, longitudinal section. (D) Berries at veraison, transversal section.



In a previous genetic study (Fernandez et al, 2006a), evidence suggested that the mutation was linked to a single dominant locus. In the different selfing or crossing progenies studied, the Flb$^-$ phenotype was conserved at the flower morphology level but fruit set did not occur so that no berries at all are produced. The hypothesis of a periclinal chimeric status of the mutation in the Ugni Blanc mutant suggested that the mutated gene might be restricted to the L2 cell layer (Fig. 7) (Franks et al, 2002), so that, when both L1 and L2 cell layers contain the mutation, fruit set does not occur.

Figure 7. Hypothesis on the chimeric distribution of the *flb* mutation, restricted to the L2 layer (adapted from (Fernandez et al, 2006a)). Longitudinal (top) and transversal (bottom) sections of ovaries are represented with wild-type and *flb* tissues in green and red respectively.



The *flb* mutation was located at the top end of chromosome 18 (LG18) and initially mapped above the SSR marker VMC2A3, without any other flanking markers available. According to the 8X genome sequence (Jaillon et al, 2007a), VMC2A3 is located at the bottom of the scaffold_137 (1 Mbp), which is followed by the scaffold_1 (12.68 Mbp) (Figure 9).

The fine mapping of the *flb* mutation was conducted using the 00C001V008 x Ugni Blanc mutant microvine progeny that flowered rapidly and could be scored in a growth room. At following loci, the Ugni Blanc mutant was heterozygous for the fleshless berry trait (*FLB/flb*) and flower sex (Hermaphrodite/female; *H/f*),

but homozygous (white/white; *b/b*) for white berries. The other parental line, an S1 picovine, had wild-type fleshy berries (*FLB/FLB*), female flowers (female/female; *f/f*) and black berries (black/white; *B/b*). All progeny from this cross were microvines and the segregation observed for the berry flesh phenotype, flower sex and berry colour is presented in Table 4. While the inheritance of all the traits is as expected, the Flb$^-$ phenotype, indicative of the *flb* mutation, had a skewed segregation from 1:1 with 39% of the progeny having the mutant phenotype which was similar to previous crosses involving the FLB mutant as the pollen donor and wild-type *V. vinifera* cultivars (Fernandez et al, 2006a). The segregation distortion reached a 1:3 ratio when a larger number of progeny (426) were screened. This result suggested that more than one locus could be involved, the presence of a lethal locus or instability at the *flb* locus. The Mendelian inheritance observed for the other traits, both at the phenotypic and at the molecular levels, rejected the hypothesis of a lethal locus. Inheritance studies of the phenotype over two years revealed that the Flb$^-$ phenotype was unstable in the progeny and that about 4% of the progeny presented a reversion from the mutant phenotype to the wild-type and/or to the original fleshless berry phenotype. Consequently, an over-estimation of the wild-type (WT) phenotype due to a frequency of reversion events higher than expected was conceivable and would explain the segregation distortion for the Flb$^-$ phenotype, in various proportions according to the progeny studied. Thus it appears that the segregation distortion is mostly likely due to genetic instability at the *flb* locus.

Table 4. Inheritance of the *flb* mutation, flower sex and berry colour traits at the phenotypic and molecular levels in F1 microvine progeny. The first value corresponds to 106 06C020V progeny scored in Australia and the value in brackets to all progeny scored in Australia and France (06C020V and 06C020F individuals). Chi-square values at *p=0.05* are indicated in the last column. ns = non significant.

flb	56 (316) WT	34 (110) *flb*	0.02*
Flower Sex	53 (232) female	50 (225) hermaphrodite	0.76 ns
SNP VVMT136	52 (235) G/G	52 A/G (223)	0.92 ns
Berry Colour	41 black	34 white	0.42 ns
SNP VVMT002	56 T/C	48 C/C	0.43 ns

The reversion phenotypes, of chimeric bunches, observed in the 00C001V008 x Ugni Blanc mutant progeny was also seen in the original Ugni Blanc Mutant with some clusters composed of fleshless berries but with the occasionally fleshy berry. In our case, the occurrence of offspring showing the original Flb$^-$ phenotype, i.e. at the berry level, was not observed previously. Phenotype

reversions occur in both directions from *flb* to wild-type and vice-versa and sometimes affect the whole cluster with the chimeric status being more frequent. Therefore, the hypothesis of the chimeric distribution of the *flb* mutation can be refined to include the genetic instability reversion theory, as described in Figure 8. This model suggests that the unstable nature of the *flb* mutation tends to return to the wild-type phenotype as shown in the original mutant genotype. The reversion can arise simultaneously in both cell layers or can happen in the L1 cell first and later in the L2 cell layer, explaining the occurrence of Flb^{-} phenotype in the progeny as an intermediate state.

At the molecular level, the 00C001V008 x Ugni Blanc mutant progeny were genotyped with 111 SNP markers heterozygous in the Ugni Blanc mutant spread along the genome. The results confirmed that the fleshless berry trait, *flb*, was located on LG18. For fine genetic mapping SNP markers located above VMC2A3 were tested and the sequence of Scaffold_137 was screened for SSR motifs in order to identify a flanking marker above the *flb* mutation. Surprisingly, none of the 9 SSR markers, or any of the 14 SNP markers tested were polymorphic in the Ugni Blanc mutant (Fig. 9). More generally, it appeared that most of the markers developed using the chromosome 18 sequence were monomorphic, which agreed with the higher level of homozygosity observed for the Ugni Blanc mutant compared to the other cultivars (Table 1). Testing of an additional thirteen SNP markers for fine genetic mapping of chromosome 18 did not allow tight linkage of a marker to a candidate gene due to the instability of the phenotype and the high level of homozygosity on chromosome 18 of the Ugni Blanc Mutant.

Figure 8. Representation of the *flb* linked phenotypes observed in microvine progeny in relation to the chimeric distribution of the mutation and the hypothesis explaining the reversion phenomena. Note that the flower visible with the fleshed black berries has the *flb* type (left) and the white fleshless berries are on a heterogeneous bunch mixed with fleshed berries (right).

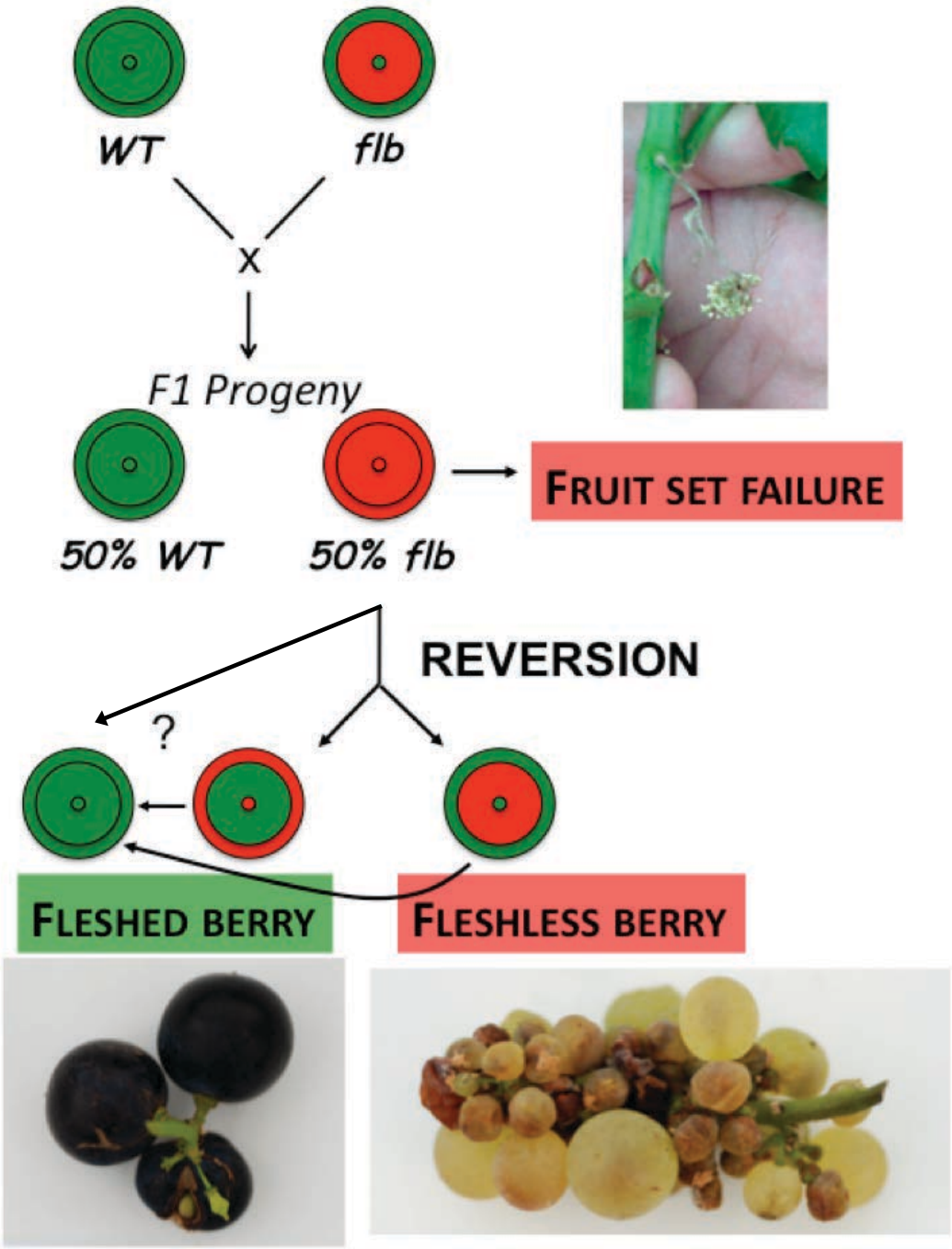
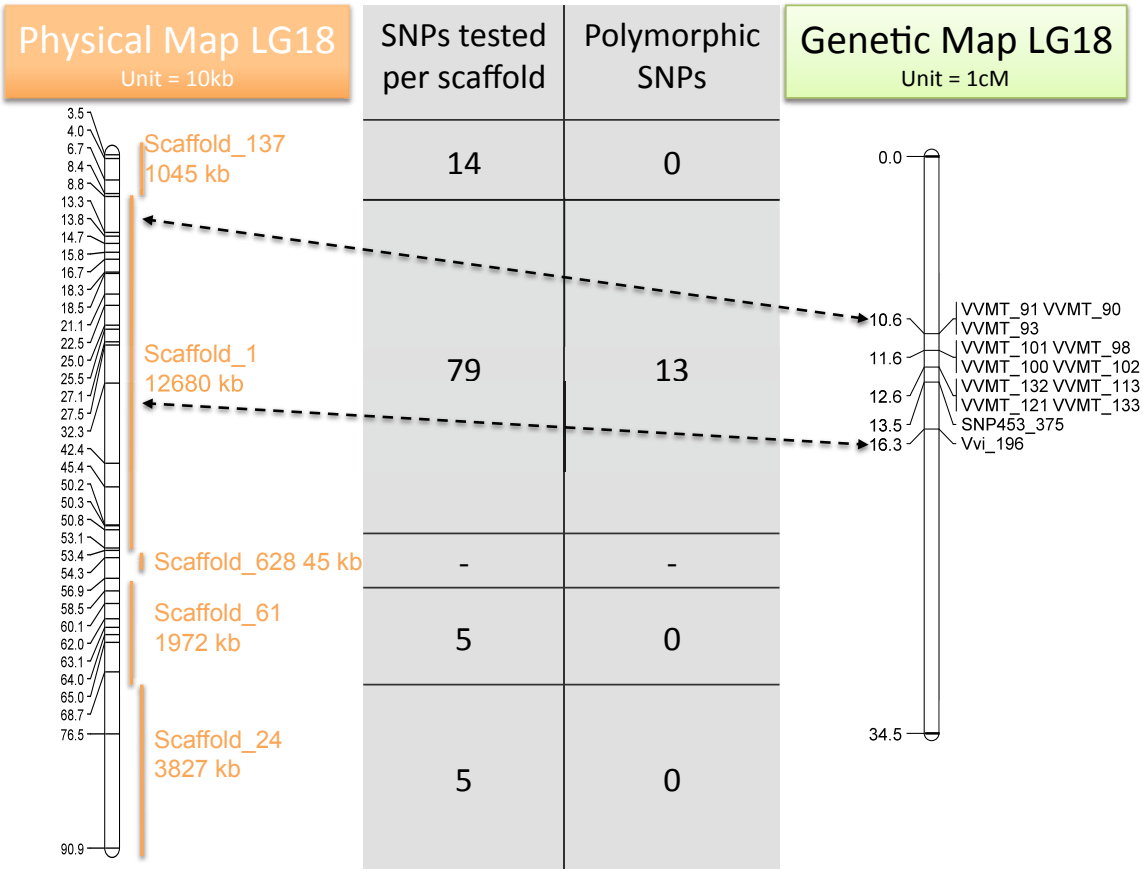


Figure 9. Alignment of the physical map, on the left, and the genetic map, on the right, for the *flb* locus on LG18.



Candidate genes linked to the fleshless berry mutation on chromosome 18 were investigated by transcriptional analysis of genes expressed in the Ugni Blanc mutant during reproductive development (Fernandez et al, 2006b). Results indicated that *VvMADS9*, a homologue of the PISTILLATA gene that controls stamens and petals organogenesis in Arabidopsis thaliana, could be a strong candidate gene responsible for the fleshless berry phenotype as the gene was expressed in both berries and inflorescences. We had previously shown that in Cabernet Sauvignon wild-type grapevines *VvMADS9* expression was restricted to a very specific stage of flower and inflorescence development and was not expressed in roots, leaves, tendrils, berries, buds or callus (Sreekantan et al, 2006). Therefore, we investigated if *VvMADS9* was up regulated in other tissues of the fleshless berry mutant by measuring its relative expression in young leaves. We confirmed that *VvMADS9* was expressed in leaves in the Ugni Blanc fleshless mutant but not in the wild-type Ugni Blanc, nor in the

picovine 00C001V0008. Consequently, *VvMADS9* expression in leaves was studied for the 106 microvine individuals of the 00C001V0008 x Ugni Blanc mutant cross segregating for *flb*. The results are summarized in Table 5 and show that *VvMADS9* is always expressed in the leaves of individuals with a mutant phenotype but also for half of the genotypes with a wild-type phenotype. This observation corroborates the hypothesis of the over-estimation of wild-type phenotypes described earlier, as a consequence of reversion events. At this stage, *VvMADS9* is the main candidate gene associated with the *flb* mutation. This possibility is supported by another example of an unstable mutation related to B-function MADS-box genes that has been reported in a recent study on a floral mutant in *Primula vulgaris* (Li et al, 2010). To confirm that *VvMADS9* is the mutated gene responsible for the fleshless berry phenotype DNA sequencing of the *VvMADS9* alleles, including the promoter region, from the Ugni Blanc and the Ugni Blanc fleshless mutant are required to identify DNA mutations that would explain the ectopic expression profile in the fleshless berry mutant. If proven then this would confirm a role for MADS-box genes in controlling berry growth and size.

Table 5. *VvMADS9* expression in leaves and the phenotype observed for 76 individuals of the 00C001V0008 x Ugni Blanc Mutant progeny.

VvMADS9 expression	Phenotype	Number of progeny
Detected	flb wt	28 20
Not detected	flb wt	0 28

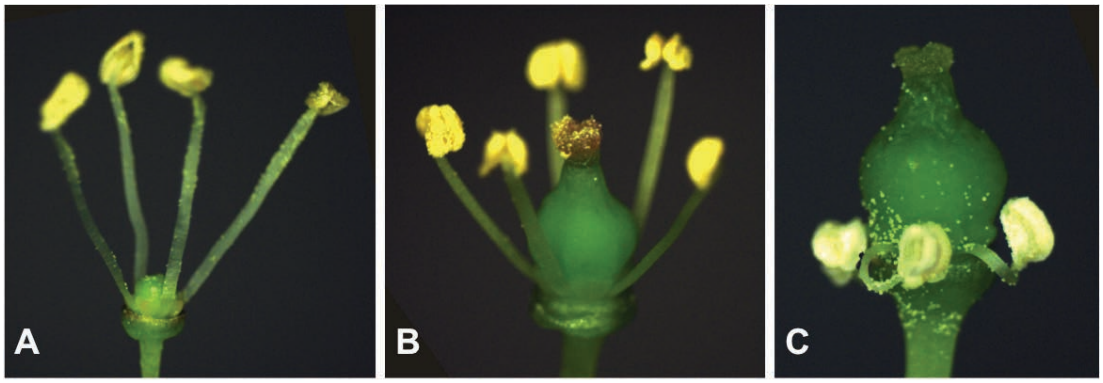
Inheritance and genetics of Flower Sex Determination (VvFSD)

Our own research and discussions with other grape breeders had identified flower sex as a serious impediment to efficient breeding and grapevine improvement with up to 50% of some progeny populations having individuals with female flowers that are of no commercial use. To improve future breeding efficiency a microvine cross was investigated to determine the genetics of flower sex and for the development of DNA markers to screen out female plants at the seedling stage.

Grapevine is dioecious and produces inflorescences composed of self-fertile hermahrodite flowers in the case of cultivated cultivars or unisexual flowers for various *Vitis* species (Carmona et al, 2008; Doazan & Rives, 1967). The genetics of flower sex determination has been previously described and is controlled by a single locus located on LG2 (Chromosome 2) with three alleles

being involved. The female flower allele (*f*) is recessive to both hermaphroditic (*H*) and male alleles (*M*) (Fig. 10) (Dalbo et al, 2000; Marguerit et al, 2009; Negi & Olmo, 1971).

Figure 10. Flower sex phenotypes. (A) Male, (B) hermaphrodite and (C) female flowers at anthesis.



The flower sex phenotype was previously linked to four SSR markers, UDV027, VVIB23, VMC3B10 and VMC6F1, covering a large region of about 12.8 cM (Di Gaspero et al, 2007). However, the occurrence of recombination events between these markers complicated the study, and accurate prediction was only reliable if female haplotypes or male haplotypes were observed for all the markers. Consequently, fine genetic mapping of the gene controlling flower sex was undertaken to identify a candidate gene and closely linked DNA markers following the same strategy presented for the study of the *flb* mutation using rapid flowering microvine populations.

The Ugni Blanc mutant was heterozygous for flower sex (*H/f*) and the fine mapping of the locus was done using the 00C001V0008 x Ugni Blanc mutant microvine progeny. The results previously presented in Table 4 shows that trait segregation followed the expected ratio of 1:1 with 232 individuals having female flowers (*f/f*) and 225 individuals with hermaphroditic flowers (*H/f*) both at the phenotypic and molecular levels.

Fine genetic mapping of chromosome 2 was performed by genotyping SNP markers identified by map alignment and using genome sequences. According to the 8X genome sequence, UDV027 is located on the scaffolds_112 (1,25 Mbp) and VVIB23, VMC3B10 and VMC6F1 are located on the scaffold_187 (0.61 Mbp). Estimated physical distance between UDV027 and VVIB23 was 1.31 Mbp, and this large region contained hundreds of putative candidate genes. To reduce the physical region associated with the flower sex trait, fine mapping of the chromosomal region was conducted and consisted of developing markers, mapping them and identifying a smaller target zone. Thus, respectively 21 and 38 SNP markers were developed for the scaffold_112 and

the scaffold_ 187. Of these SNPs, 46% were polymorphic in the Ugni Blanc mutant and informative for genetic mapping. The evolution of the genetic map of LG2 is presented in Figure 11. While many markers were available to cover the area, the precision of the LG2 map eventually reached a limit due to the low number of recombinant individuals identified amongst the 478 microvine progeny. As observed for the *flb* mutation mapping, not many recombinant events occurred in the area to allow fine localisation of the flower sex locus and massive markers co-localisation also observed. Consequently, the same sets of markers were genotyped on the L1 microvine selfed progenies that were developed to select homozygous lines and that also segregated for flower sex. In addition, a set of 29 cultivars and *Vitis* species, having hermaphrodite, male or female flowers, were also included in the analysis. As a result, the flower sex locus was successfully defined to a region of only 120 kb using the L1 microvine related individuals. This small region contained 8 potential candidate genes that was reduced to 5 genes when data from the *Vitis* diversity study was included.

An expression analysis during flower development was performed for the five candidate genes. Five stages of development were studied as described by (Chatelet et al, 2007): stage 1, inflorescence primordium still enclosed within the bud; stage 2, inflorescence clearly separated from the vegetative axis; stage 3, first flower buds visible at the top of the inflorescence; stage 4, initiation of new inflorescence primordial completed, all flowers well separated; stage 5, all flowers formed on fully expanded pedicels. Sampling was done on microvine plants with stages 1-2 combined and defined as the inflorescence just visible in the shoot tip but not clearly separated from the vegetative axis (Fig. 12).

Figure 11. Evolution of the LG2 genetic map using 106 individuals from the 00C001V0008 x Ugni Blanc mutant cross, from the beginning (on the left) to the end (on the right) of the fine mapping process for the flower sex locus.

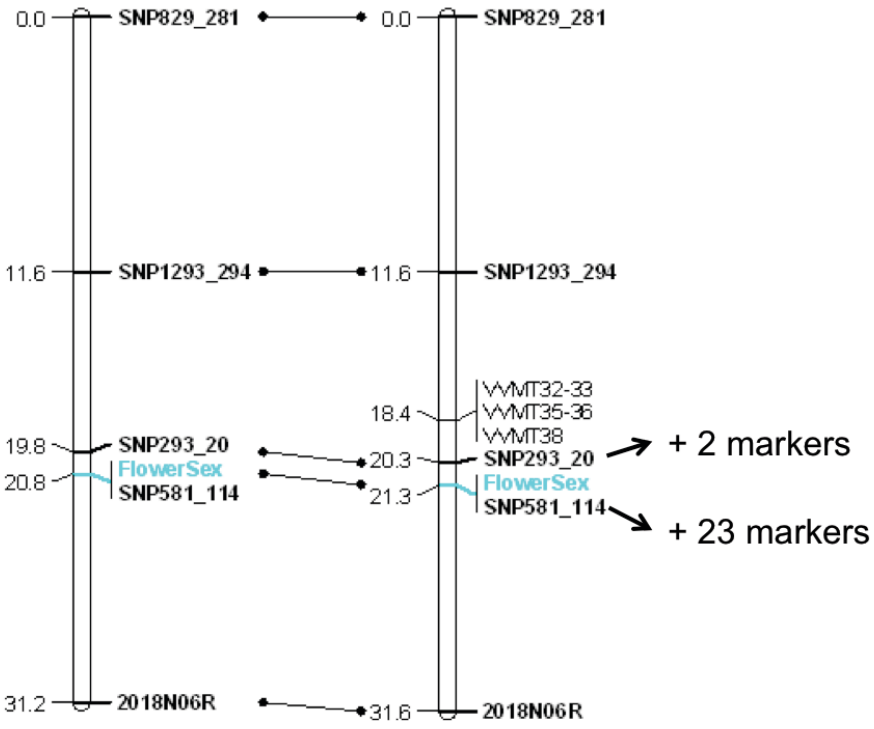


Figure 12. Inflorescence and flower phenotypes in microvine lines at various stages of reproductive development.

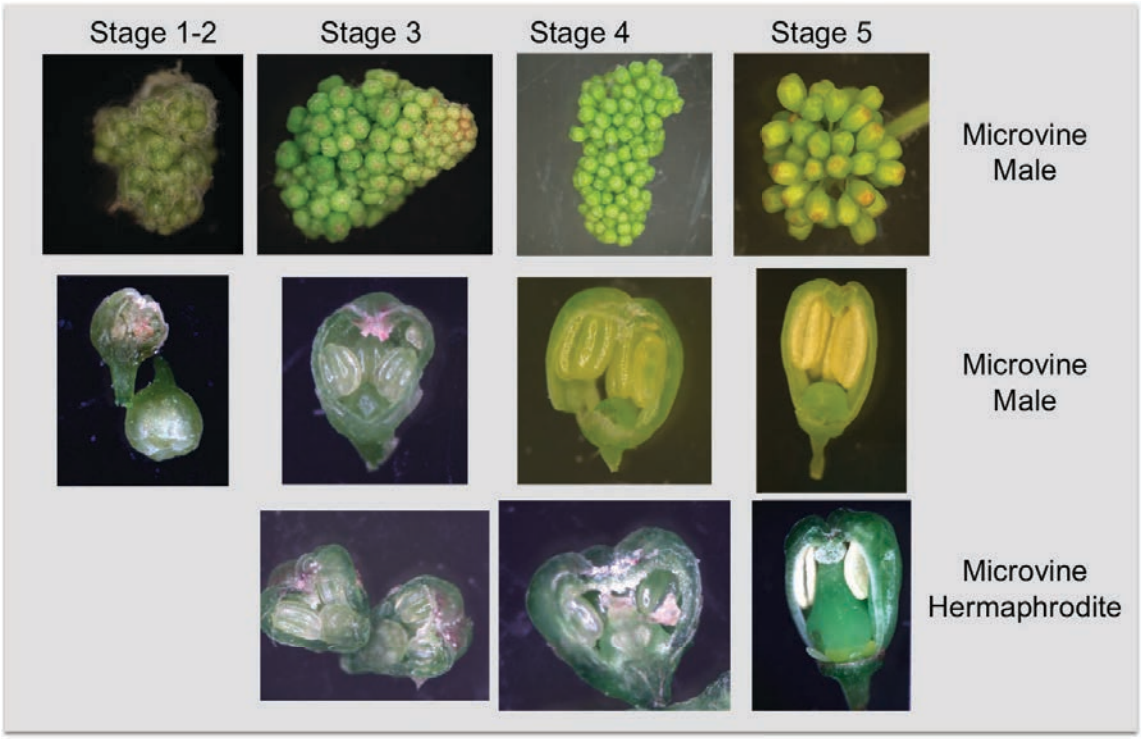


Table 6 shows the expression results obtained with only 3 genes being expressed during inflorescence development. Among them, only one gene showed an expression profile correlated with the flower sex phenotype. Expression and sequence analysis of the candidate gene supported the function as controlling flower sex. Further experiments are needed to understand the underlying molecular and biological mechanisms linking a single locus to three phenotypes. As a result of this work a unique ***V**itis **v**inifera* candidate gene controlling **F**lower **S**ex **D**etermination, *VvFSD*, has been identified and three SNP markers linked to the trait for marker assisted breeding applications.

Table 6. List of positional candidate genes located at the flower sex locus (ND = Non Determined).

Candidate gene	Expression analysis
1	ND
2	ND
3	detected
4	detected
5	not detected
6	detected
7	not detected
8	ND

Inheritance and genetics of berry colour

For efficient breeding and selection of new varieties with either black or white berries, SNP DNA markers linked to berry colour were investigated. Application of these markers would allow the selection of plants with only white or black berries at a young seedling stage without the need to wait 3 or more years for the plants to fruit in the field.

Grapevine berry skin colour is determined by the presence or absence of anthocyanin. This phenotype is controlled by a single locus localized on LG2 (Chromosome 2) and is characterized by a dominant black allele (*B*) and a recessive white allele (*b*). The locus consists of a complex *VvMYBA* gene family (Walker et al, 2007). SNP markers were designed from flanking non-coding regions near the *VvMYBA* gene family.

Inheritance studies of berry colour in microvine populations showed the expected segregation of 1:1 (black : white) when a heterozygous (*B/b*) black berry parent was crossed with a white berry parent (*b/b*). A number of SNP markers were tested across the progeny to identify a marker that was tightly linked to the berry colour trait. The SNP marker (VVMT2) was identified as

being able to distinguish the white and the black allele as follow: *b* = DNA nucleotide C and *B* = DNA nucleotide T (Fig. 13).

Figure 13. SNP DNA markers VVMT2 and VVMT4.

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gatgaccaaactgccactgattgaatgttgttggccctggttatggtttcctccttgaaacttg
aattgtccaaascttgatttttctcatagttggttctttgtgattgcagagaagctacacttg
tctgaagaagatttgcctcatcaatggcattctgttgctccaagcaatgctcataagtgagcaa
catgctatgaatctcttcaaactcaRggcttcatgcctagtcaagtcctaatcctctaagtaag
gaacgtgatcttgtttagaaattaattctctaattgcaacaagattatccacaatgaatttctC
CTTAAGAAGATATATACTCcatcat[C/A]ctcaaggagaagttgcataattctagcctttgat
gcRgcagagaagaatgWctttaagagaatttcaaa[T/C]gtcagaagtMaaagWgagaccgat
gatttgagaMatcacatGATTTTCAGTGAGAGATGAataaatccaacacatgactagatgatta
cgtagttgcttgagatgaaaatttggatttgcaatctcaaagttctcaagaaattttggtggga
caaggtcat

Both SNP markers have the same amplification primers:
PCR Primer A: acgttggatgCCTTAAGAAGATATATACTC
PCR Primer B: acgttggatgTCATCTCTCACTGAAAATC

VVMT2 [T/C]  SNP Extension Primer: TCTCICTTTIACTTCTGAC

VVMT4 [C/A]  SNP Extension Primer: ATGCAACTTCTCCTTGAG
```

The VVMT2 marker was also tested against the microvine inbred lines. The L1 microvine is heterozygous for the berry colour locus (*B/b*) and a segregation of 1:2:1 for *b/b*:*B/b*:*B/B* was expected. However, no homozygous (*B/B*) black berry lines were recovered. All black berry phenotypes were heterozygous (*B/b*) for berry colour according to the segregation of the linked VVMT2 marker. A screening of 104 S1 progeny from the L1 mutant with VVMT2 showed a skewed segregation ratio of 1:2.6:0 for [C/C]:[C/T]:[T/T]. This suggested that a lethal recessive locus was located close to VVMT2 and the berry colour locus.

To determine if the **l**ethal **r**ecessive **l**ocus (*Vvlrl1*) was specific to the L1 cell layer of Pinot Meunier from which the L1 microvine was derived, the VVMT2 marker was used to screen progeny obtained from selfing a wild-type L2 plant of Pinot Meunier that was isogenic to the L1 mutant but lacking the *Vvgai* mutation (Boss & Thomas, 2002; Franks et al, 1998). The segregation ratio for L2 progeny was close to the expected 1:2:1 for [C/C]:[C/T]:[T/T] indicating that *Vvlrl1* was not present in the L2 cell layer of the original Pinot Meunier plant but only present in the L1 layer. A similar study was conducted for Pinot Noir, the original cultivar from which Pinot Meunier arose as a somatic variant, and the segregation ratio of the progeny was again found to be 1:2:1 for [C/C]:[C/T]:[T/T] suggesting *Vvlrl1* was absent from the L2 cell layer.

To further our understanding into why no homozygous black berry genotypes (*B/B*) could be obtained from the selfed L1 microvine the genetics of *Vvlrl1* was investigated. Microvine progeny (F1) from two black berry parents, Grenache (B_G/b) x L1 microvine (B_{L1}/b), were screened with VVMT2 and black-fruited microvine individuals were identified that were homozygous [T/T] with the black berry genotype (B_G/B_{L1}). This showed that *Vvlrl1* was not present in the black berry wine grape cultivar Grenache and microvine progeny homozygous for black berry colour could exist when *Vvlrl1* was in a heterozygous state

(*Vvlrl1:VvLRL1*). An S1 progeny population was produced from one of these homozygous black berry colour F1 individuals (04C023V0004) that contained one black berry allele from each F1 parent (B_G/B_{L1}) and then screened with VVMT2 and VVMT4. The closely linked SNP marker VVMT4 (Fig. 13) is 70 bp from VVMT2 and distinguishes the black berry allele of Grenache (B_G) from the black berry allele of the L1 mutant (B_{L1}). All 93 progeny tested had the expected homozygous VVMT2 genotype [T/T] but an abnormal segregation for VVMT4 alleles. No S1 progeny were homozygous [C/C] at VVMT4 (B_{L1}/B_{L1}) confirming that lethality is specific to the homozygous state of *Vvlrl1* and very closely linked to the black berry allele of the L1 microvine on LG2.

An analysis of the segregation of the VVMT4 genotypes in the L1 mutant and 04C023V0004 S1 progeny populations indicated the biological basis of the lethal effect observed for the *Vvlrl1* locus. With both S1 parents heterozygous [A/C] at VVMT4, the expected segregation ratio if the *Vvlrl1* was affecting gametophyte development would be 1:≤1:0 and 1:2:0 if the lethality had a zygotic origin. For both progeny populations, the respective VVMT4 segregation ratios observed were 1:2.6:0 and 1:1.6:0 for [A/A]:[A/C]:[C/C]. This indicated that the recessive homozygous *Vvlrl1* locus had a lethal action at the zygotic level.

The new discovery that there are lethal genes in the grapevine genome that can cause unexpected segregation distortion is an important observation for genetic future studies.

Inheritance and genetics of mildew resistance

Powdery and downy resistance is an important trait for industry and SNP markers linked to a mildew resistance locus would allow marker-assisted-selection of resistant plants at a young seedling stage without the need for mildew infection trials.

The *Run1* locus has previously been characterized in detail and is located on chromosome 12 (LG12). It is responsible for both downy and powdery mildew resistance but is a complex locus with multiple RGA (Resistance Gene Analog) genes (Barker et al, 2005). As a first step to develop SNP DNA markers linked to the *Run1* locus a cross was made between the L1 microvine and a plant containing the *Run1* locus to determine inheritance. Progeny were then screened for resistance to powdery mildew and the expected ratio was observed with a 1:1 segregation for resistance:susceptible. This demonstrated that the *Vvgai1* mutation responsible for the microvine phenotype had no effect on the segregation of the *Run1* locus and that it would be possible in the future to develop SNP markers linked to trait for powdery and downy mildew resistance.

Development of microvine lines amenable to transformation for rapid gene function studies in the berry.

Grapevine transformation relies on the initiation and growth of embryogenic cultures initiated from immature anther filaments (Fig. 14). Once established these embryogenic cultures are treated with a disarmed *Agrobacterium* strain containing a disarmed binary vector that has the gene of interest and selectable marker for transfer into the grapevine embryogenic cells. To test the ability of different microvine genotypes to be transformed by Agrobacterium a binary vector containing the visual fluorescent green fluorescent protein (GFP) gene was used. Two different plant selectable markers, hygromycin (*htpII*) and kanamycin (*nptII*) were evaluated for selecting transformed cells and transgenic plant regeneration.

Embryogenic cultures of the original L1 mutant microvine were tested for *Agrobacterium*-mediated transformation and it was observed that the embryogenic tissue browned and no transgenic plants could be regenerated. To overcome this problem, new microvine genotypes were bred to obtain highly transformable microvine lines that produced either black or white berries. Over 32 genotypes derived from crosses between microvines and other cultivars were evaluated for (a) the ability to form embryogenic callus, (b) *Agrobacterium*-mediated transformation responsiveness and (c) transgenic plant regeneration potential. It was found that some microvine progeny did not produce embryogenic callus and of those that did many showed browning after Agrobacterium inoculation or poor transformation ability as judged by GFP positive cells. Other genotypes did not perform well after transformation on selection media containing either hygromycin or kanamycin and no transgenic plants were regenerated. The microvine genotypes most amenable to *Agrobacterium*-mediated transformation were derived from a Grenache x L1 mutant cross.

The high transformation efficient wild-type genotypes, Chardonnay (Iocco et al, 2001) and Portan (Torregrosa et al, 2002), were compared to the best microvine genotypes (Table 7). Two black berry and two white berry microvine lines were identified that performed as well or better than Chardonnay and Portan with high initial transformation efficiency and low browning (Table 7). For two microvine genotypes, transgenic plants could be selected with both antibiotics (Table 7) suggesting that these genotypes would be suitable for complex genetic studies requiring two independent transformation events with different selectable markers.

Table 7. Microvine genotypic variation in response to *Agrobacterium*-mediated transformation.

Genotype	Transformation efficiency[1]	Tissue browning[1]	Berry colour[2]	Transgenic plants[2]
04C023V0019	*****	**	Black	Yes (K)
04C023V0004	***	**	Black	Yes (K, H)
04C023V0006	***	**	White	Yes (K, H)
04C023C0013	*****	**	White	Yes (H)
L1 mutant	*	*****	Black	No
Portan	****	*	Black	Yes (K, H)
Chardonnay	**	***	White	Yes (K)

[1]GFP expression and tissue browning three days post *Agrobacterium* inoculation. Scoring was based on an arbitrary scale of 1-5 with 5 stars representing high transformation efficiency and high browning.
[2] Kanamycin selection (K), hygromycin selection (H).

The initial transformation efficiency of the selected microvine genotypes was observed to be high as judged by GFP expression of transformed cells (Fig. 15) and selection during culture resulted in the development of transgenic GFP positive embryos (Fig. 16). These GFP positive embryos germinated to produce GFP positive plantlets (Fig. 17). Transgenic plants were regenerated from all 4 microvine genotypes using either hygromycin or kanamycin selection and transferred to a PC2 glasshouse for plant growth.

Figure 14. Embryogenic tissue derived from a microvine plant.



Figure 15. GFP positive cells fluorescing green after *Agrobacterium*-mediated transformation.

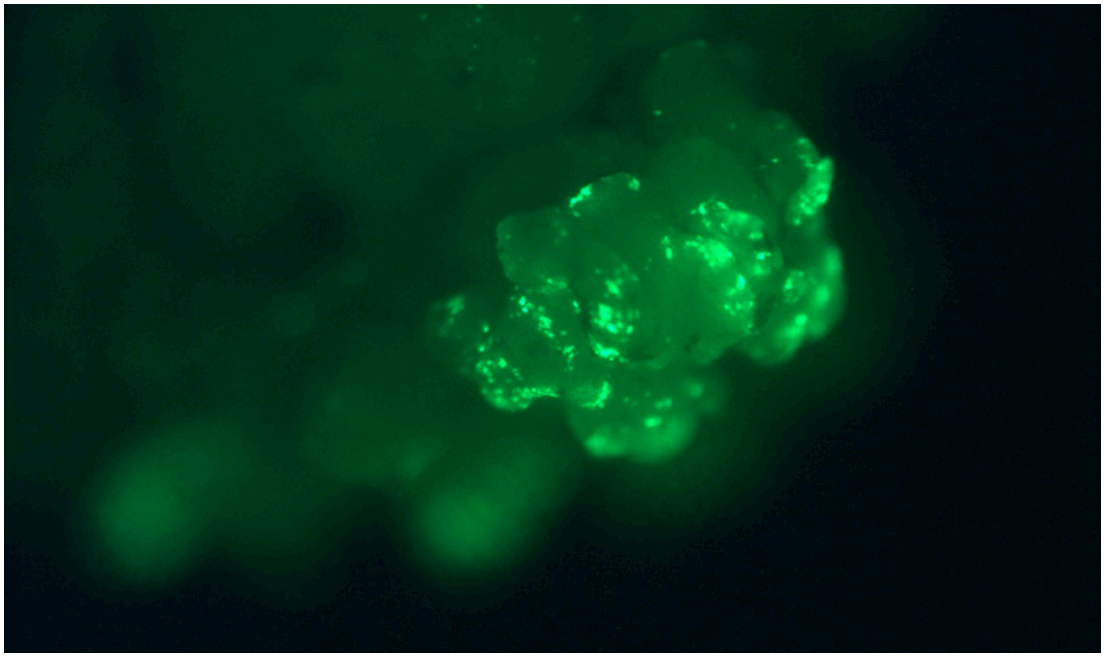


Figure 16. GFP positive microvine embryos fluorescing green after selection on medium containing the selection agent hygromycin.

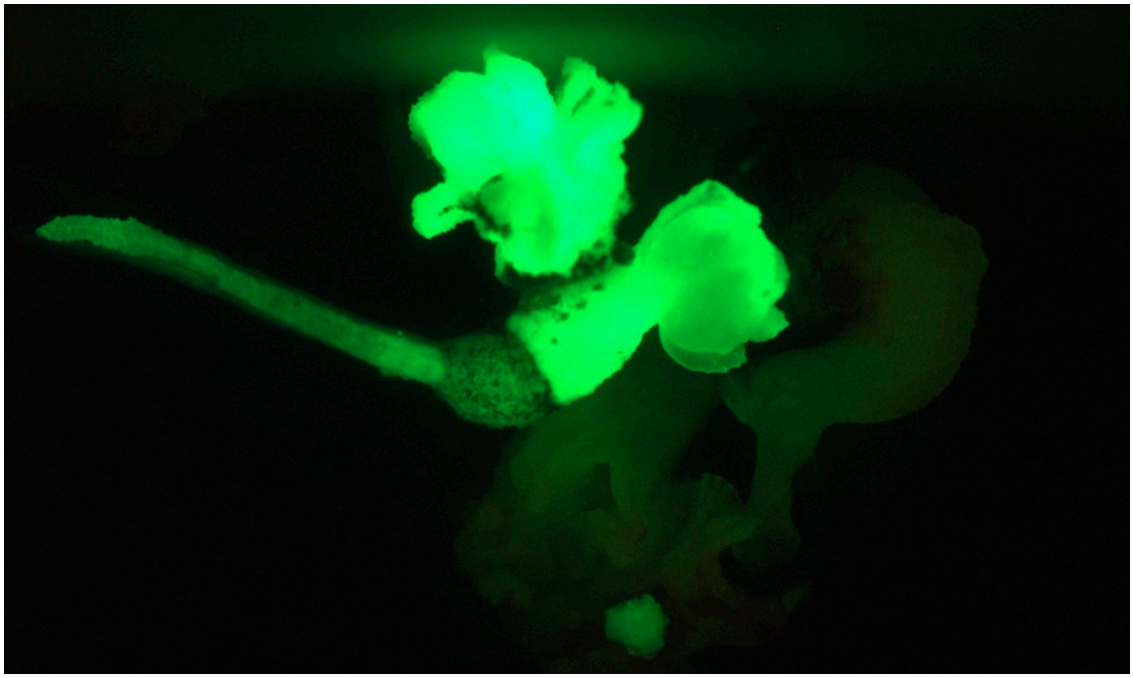


Figure 17. A transgenic microvine plantlet in culture prior to potting.

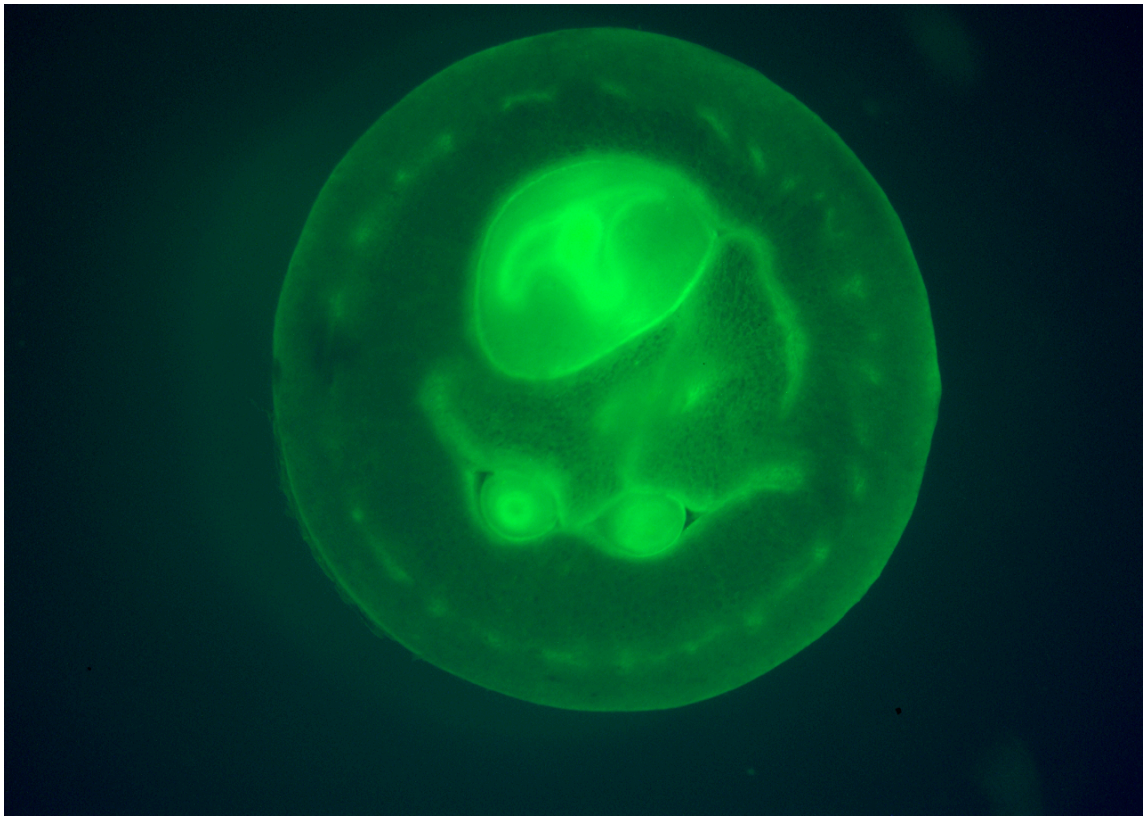


Compared to Chardonnay, the microvine genotypes had a similar average period of 287 days from *Agrobacterium* inoculation to transgenic plant regeneration. After transfer to the glasshouse the microvines grew rapidly and the rapid flowering phenotype conferred by the *Vvgai1* mutation was observed for the transgenic microvine plants with flowering and anthesis occurring on average 54 days after transferring plants to soil. Berry development was normal from berry set to harvest with berry ripening starting approximately 55 days after anthesis and complete fruit maturity (berry total soluble solids values of >22 ^{o}Brix) occurring 14 weeks post-anthesis (Fig. 18). Not only does the microvine flower rapidly but the multiple inflorescences at different developmental stages along a microvine shoot (Fig. 18) also allows a longer period of study and multiple sampling opportunities compared to the 1-2 inflorescences produced by a wild-type shoot each vegetative cycle (season). GFP expression was observed in the berry (Fig. 19) indicating that the microvine transgenic system is suitable for rapid gene function studies targeting the berry and floral organs.

Figure 18. Rapid flowering transgenic microvine plants with fruit.



Figure 19. A section through a berry from a transgenic microvine plant showing green GFP expression.



For model plant species, genetic transformation is a routine technology to confirm gene function and interactions. After nearly 20 years of intensive effort to improve transformation methods, this technology is still restricted to a limited number of grapevine genotypes and research groups (Bouquet et al, 2008). It is known that grapevine fruit best under field conditions but long timeframes (≥ 5-6 years) are required to obtain replicated fruit data over several seasons from transgenic plants. In addition, field trials usually need to be carried out under country specific GMO regulations and the OGTR (Office of the Gene Technology Regulator) regulates GM field trials in Australia. The requirement for regulated long-term field trials have reduced the attractiveness of such trials for scientific experiments. The containment of GM microvines to growth rooms or glasshouses appears to be a suitable replacement for grapevine GM field trials in many countries and will make it easier to carry out scientific studies investigating key genes regulating berry development and berry metabolism.

The black and white berry transformable microvine genotypes identified in this report are suitable for transgenic studies and represent ideal material to conduct transgenic forward and reverse genetic studies. Experiments investigating floral and berry traits can be carried out in a much shorter timeframe compared to using conventional wild-type grapevine genotypes due to the rapid flowering phenotype. Transgenic berries from microvine plants were observed to have

typical development and maturity stages. This may be due to the *VvGAI1* gene not being highly expressed in berry tissues as previously determined by Northern blot analysis (Boss & Thomas, 2002). It is also feasible to rapidly produce second generation T2 families to establish the transgene in the heterozygous or homozygous state to better study the phenotype especially for recessive trait investigations.

Outcome & Conclusion

This project has demonstrated that a new model grapevine system based on the *Vvgai1* mutation can be successfully used for rapid plant transgenic studies and for rapid genetic mapping and trait dissection beyond an initial F1 generation. The new picovine and microvine phenotypes and genotypes developed are expected to prove very useful for a variety of forward and reverse genetic studies not previously possible in grapevine.

The microvine system largely solves the problems previously identified for conducting rapid functional genome studies in grapevine which were:

1. The size of the plant requiring plant populations to be maintained in the field.
2. The long generation time, often three or more years, inhibiting genetic crosses proceeding beyond the first F1 generation.
3. The cost and time (>5 years) involved in OGTR approved field trials of transgenic plants for fruit evaluation due to poor flowering under glasshouse conditions.
4. The absence of a large mutant population for linking genes to function.

These problems are especially relevant for research on fruitfulness, floral development, fruit development and metabolites responsible for grape composition and wine attributes.

We have demonstrated that the *Vvgai1* mutation present in the original L1 microvine is transferred to progeny and that microvine progeny have the dwarf stature removing the need to maintain plants in the field while the rapid flowering phenotype reduces generation time to less than a year. We have shown that microvine populations can be maintained and fruit in glasshouses and growth rooms.

The constraints for carrying out inheritance and genetic mapping studies for linking traits to genes and DNA markers in grapevine have now been removed with the microvine system. Genetic mapping in grapevine has previously been limited to small F1 progeny populations (100-150 plants) maintained in vineyards (Doligez et al, 2006; Fernandez et al, 2006a; Vezzulli et al, 2008b). Reasons include breeding constraints (once a year flowering, emasculation, timing of anthesis of male and female parents), field space required (1-5 plants per m^2) and the 2-5 year delay before the appearance of flowers and fruit. The physical limitations and the genetic limitations of this F1 pseudo-backcross approach have restricted fine genetic mapping efforts and candidate gene identification of specific traits. We have demonstrated using the microvine system that it is possible to develop mapping populations beyond the F1 and F2 stages to study the inheritance of berry traits such as the flavour compounds (methoxypyrazines), berry size (fleshless berry mutant), berry colour as well as flower sex. Genetic mapping studies on the microvine populations allowed the linking of DNA SNP markers to traits and with this approach it was possible to identify candidate genes responsible for the fleshless berry mutant and flower sex. DNA markers developed during this study that are linked to traits such as

flower sex and berry colour are expected to be important in the future application of DNA marker assisted breeding strategies to produce improved grapevine varieties. Producing microvine populations beyond an F1 generation was shown to be important for studying the methoxypyrazine trait that showed no segregation in the F1 generation requiring the production of an F2 generation to produce a segregating population. Producing an F2 progeny population is also essential when studying recessive traits and the microvine system is a rapid means for obtaining an F2 population due to the short generation cycle.

The development in this study of microvine genotypes suitable for genetic transformation has solved the problem of how to conduct gene function studies in grapevine in a short time frame using a transgenic approach. It was demonstrated that transgenic microvines can be quickly produced and flower and fruit in less than one year. With this system it is now feasible to investigate the function of a gene targeted to the berry in less than one year and keep the plants in an Australian OGTR approved PC2 glasshouse without the need to conduct long-term field trials of genetically modified grapevines.

We have also shown in this study that it is possible to produce inbred microvine plants by selfing for 5 generations. With a normal grapevine plant under field conditions it would 15 - 20 years or more to produce 5^{th} generation plants. Use of DNA markers allowed the selection of individuals with highly homozygous genomes (>95%). It is expected that these near-homozygous individuals will provide the basis for the production of a mutagenised microvine population suited to linking genes to traits by using the TILLING method (Henikoff *et al.* 2004, McCallum *et al.* 2000) to link the mutation responsible for a mutant phenotype. The homozygous genotype and rapid fruit production of the inbred microvine lines is expected to represent significant advantages for identifying mutated genes responsible for key fruit traits in a mutagenised grapevine population.

In conclusion, we suggest that the grapevine *Vvgai1* based system described here has most, if not all, of the features and advantages that previously made Arabidopsis an attractive model plant system for studying gene function and identifying genes responsible for specific traits. We expect that the advantages of the system will encourage national and international scientists to adopt the microvine system for their own research studies investigating grapevine genes and traits.

Recommendations

The successful development of a new grapevine microvine system for rapidly linking genes to traits has identified a number of potential future research directions.

It is recommended that the microvine system continue to be used as a research tool to rapidly link key traits to DNA markers and genes. Adoption of this new microvine system by Australian and international grape researchers investigating genes controlling a variety of grapevine physiology and biochemical processes will lead to rapid advancement in our knowledge of genetic inheritance and the genes responsible for key berry traits.

It is recommended that further development of the microvine system be undertaken to produce a mutagenised population for linking traits to genes through a TILLING approach as this method has been successfully used in other crops.

At a broader industry R&D priority level the ability of the microvine system to rapidly link genes and DNA markers to traits offers the real possibility to develop a smart Marker Assisted Breeding initiative to increase the efficiency of breeding improved rootstocks and scions for the Australian industry. To date, marker assisted selection programs have been very difficult to develop for grapevine as the first requirement is DNA markers linked to traits of interest. It is recommended that an Australian Marker Assisted Breeding initiative be developed to produce improved scion and rootstock planting material better suited to Australian conditions through the production of new genotypes incorporating superior gene allele combinations. Successful development would see a reduction from the traditional 25-30 year period required for breeding, evaluation and release to industry of a new variety to 10 years or less.

Appendix 1: Communications

Communication activities have been through oral presentations at industry and scientific conferences, publications in scientific and industry journals and poster presentations at conferences.

Science & Industry Publications

Chaïb J, Torregrosa L, Mackenzie D, Corena P, Bouquet A, Thomas MR (2010) The grape microvine - a model system for rapid forward and reverse genetics of grapevines. Plant Journal **62:** 1083-1092

Vidal JR, Gomez C, Cutanda MC, Shrestha BR, Bouquet A, Thomas MR, Torregrosa L (2010) Use of gene transfer technology for functional studies in grapevine. Australian Journal of Grape and Wine Research **16:** 138-151

Walker R, Thomas MR (2009) Evolution of grapevine variety collections in Australia. Grapegrower & Winemaker 2009 Annual Technical Issue. **2009:** 78-80

Vezzulli S, Troggio M, Coppola G, Jermakow A, Cartwright D, Zharkikh A, Stefanini M, Grando MS, Viola R, Adam-Blondon AF, Thomas M, This P, Velasco R (2008) A reference integrated map for cultivated grapevine (*Vitis vinifera* L.) from three crosses, based on 283 SSR and 501 SNP-based markers. Theoretical and Applied Genetics **117:** 499-511

Carmona MJ, Chaïb J, Martinez-Zapater JM, Thomas MR (2008) A molecular genetic perspective of reproductive development in grapevine. J. Exp. Bot. **59:** 2579-2596

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Chatelet P, Laucou V, Fernandez L, Sreekantan L, Lacombe T, Martinez-Zapater JM, Thomas MR, Torregrosa L (2007) Characterization of Vitis vinifera L. somatic variants exhibiting abnormal flower development patterns. Journal of Experimental Botany 58: 4107-4118

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Conference Presentations

Chaïb, J., N'Diaye, A., Bouquet, A., Torregrossa, L., Mackenzie, D., Thomas, M. R. (2009) The grape fleshless berry mutation and the challenge of cloning a trait with an unstable phenotype. Plant Genomics European Meeting VIII., Lisbon Portugal

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Appendix 2: Intellectual Property

DNA markers identified as important for grapevine marker-assisted breeding and identified candidate genes for key traits remain confidential and have not been made public. Unique germplasm and genotypes produced during this project are only available under a Material Transfer Agreement.

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Appendix 5: Supplementary Data

None.

Appendix 6: Budget reconciliation

An End of Project Financial statement is submitted as a separate document.