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The microvine, a model for studies in grapevine physiology and genetics

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ABSTRACT

Context and challenges: Like most other perennial crops, the grapevine needs to undergo a juvenile period before fruiting. Thus, the development of reproductive organs from seedlings is possible only after the second or third vegetative cycle. Each proleptic axis then displays only one to three inflorescences per growing cycle. These biological features and the size of the adult vine are major hindrances to the design of experiments on fruit and plant physiology, and complicate and lengthen the time required for studies in grapevine breeding and genetics.
Significance of the review: The microvine is a dwarf phenotype resulting from a mutation in the *VviGAI1* gene, which induces miniaturization of all vegetative organs and conversion of tendrils into inflorescences without affecting berry development. The small size of the microvine allows tight control of environmental conditions. Spatial developmental gradients fit well with temporal series of each phytomer position. Thus, kinetic profiles can be inferred from spatial information. In the first part of the paper, we describe the molecular and genetic mechanisms determining microvine phenotypes, reviewing the main biological properties of the microvine model. Subsequently, the results of recent studies in which the model was used for research in grapevine physiology and genetics are summarized. The review focuses on experiments investigating the effects of temperature on vegetative and reproductive organogenesis, berry development, and biomass allocation at the whole-plant level. Furthermore, we discuss and illustrate how the model can be used to identify (QTL) quantitative trait loci in fruit development and adaptive traits that could be useful when selecting genotypes in anticipation of the effects of global warming.

KEYWORDS

genetics, grapevine, microvine, physiology, plant model, *VviGAI1*

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INTRODUCTION

As a perennial fruit crop, the grapevine (*Vitis vinifera*) needs to undergo a long juvenile period before the reproductive cycle can be induced. Seedlings and even vine cuttings sampled from adult plants produce fruit only after the second year. Moreover, common cultivars develop reproductive organs only once per growing cycle (generally yearly) and once per proleptic axis, under the usual culture conditions. These biological features, together with the large size of the adult vine, complicate the design of studies requiring specific conditions. Therefore, they represent major hindrances to conducting precisely controlled physiological, ecophysiological and omics experiments, and thus impede progress in grapevine breeding and genetics research.

The microvine ML1, an L1 Pinot Meunier mutant (Chaib *et al*., 2010), is a somatic variant obtained from Pinot Meunier through somatic embryogenesis. Its phenotype results from a somatic mutation in the *VviGAI1* gene, which is involved in gibberellin signalling. The mutation was originally found to be present heterozygously in the epidermal cells of Pinot Meunier, and is responsible for the variety's well-known hairy phenotype (Boss and Thomas, 2002). The presence of the mutation in all cell layers, in plants regenerated from Pinot Meunier epidermal cells, results in the miniaturization of all vegetative organs and conversion of tendrils into inflorescences, which leads to continuous flowering and fruiting along the vegetative axes.

The small size of the microvine makes this grapevine model very convenient for experiments carried out in growth chambers, where tight control of climatic factors (radiation; vapour pressure deficit, VPD; temperature; carbon dioxide concentration) is possible, thereby minimizing uncontrolled biases arising from environmental fluctuation (an avoidable feature of field studies on perennial vines). Indeed, it is possible to grow the vines at densities of up to 15–30 plants/m^2 and to limit their height to 1.2 m.

In microvine, the first fruits are mature 5–6 months after plantation of cuttings or seedlings. Because of the continuous initiation of reproductive organs, proleptic and sylleptic axes can display all developmental stages from young inflorescences (on distal phytomers) to flowering, berry green growth and ripening (on proximal phytomers). Under stable controlled conditions, the spatial gradients of vegetative and reproductive development mimic the temporal development of each phytomer, which allows inference of kinetic data from one-off spatial information along the proleptic axis. Additionally, synchronism between vegetative development and fruiting of the microvine simplifies the study of their interactions (compared with macrovines). The effects of contrasted source–sink balance on fruiting can be easily studied by manipulating shoot or fruit load.

Lastly,with greenhouse-grown microvines it is possible to study the relation between phenotype and berry development all year round, which greatly accelerates research on grapevine physiology and molecular biology. By reducing the time interval between successive generations, and by increasing the precision of phenotyping, genetic approaches become much more efficient than those usually applied to macrovines. In the first part of this paper, we describe the molecular and genetic mechanisms resulting in microvine and derived *Vvigai1*-bearing lines, reviewing the main biological properties of the model. Subsequently, we review recent projects in which this model has been used to study

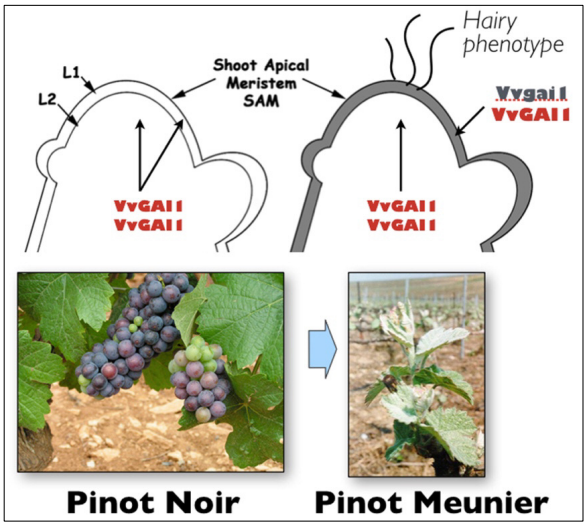


FIGURE 1. Genetic structures of Pinot noir and Pinot Meunier (also called Meunier) and their respective phenotypes.
Pinot Meunier is a somatic variant of Pinot Noir that carries the mutation in the VviGAI1 gene heterozygously. Localized in the epidermal cells (the L1 cell layer), the Vvigai1 mutation exacerbates the hairiness of vegetative organs, without any other significant phenotypic changes.

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grapevine development and fruit physiology under conditions of abiotic stress and to identify QTLs.

BIOLOGICAL ORIGIN OF THE MICROVINE

1. Tissue chimerism and phenotypic consequences

The meristem of higher plants is organized into several cell layers. The outermost layer, which comprises epidermal cells, results from anticlinal division (i.e. following a plane of division perpendicular to the surface). This L1 tissue develops as a single-cell layer that covers all the organs of the shoot system (Torregrosa, 2015). Below this is a multicellular zone called the L2 cell layer, from which all subepidermal tissues originate, the cells undergoing multidirectional division to form not only primary structures but also lateral meristems, vascular cambium, phellogen, and the tissues derived from them. In some species, a deeper cell layer called the L3 cell layer forms the core of shoot organs (pith), but it has yet to be clearly identified in the grapevine (Torregrosa *et al*., 2010).

Generally, cell lines that derive from cells initially located at the tip of the apical dome do not mix unless there is an accident during cell multiplication. The pattern of organization in the L1 and L2 cell layers is found in the various organs derived from the shoot apical meristem, particularly the axillary meristems at the origin of the caulinar organs. Because a somatic mutation is initiated by a single cellular event, it determines the nature of chimeric tissues or organs, so that they are composed of cells of different genotypes and potentially displaying some phenotypic diversity (Torregrosa *et al*., 2010). When a somatic mutation laterally arises lto a meristem, changes are localized to that sector of the mutated organ. If the mutation occurs in the initial cell of a meristem, it can spread to all tissues derived from the mutated cell. The resulting structure has a chimeric and periclinal genotype, and therefore includes cell layers that are not all genetically identical.

Periclinal chimeras can be stabilized by vegetative propagation, such as the use of cuttings or grafting. A somatic mutation can invade all the cell layers and spread uniformly to all derivative tissues, provided that three conditions are fulfilled: (i) the mutation is not lethal for the plant, (ii) the mutation appears in an initial cell within a meristem, and (iii) the mutation is established by cell substitution in both the L1 and L2 cell layers (Torregrosa *et al*., 2010). The probability of the simultaneous occurrence of these three conditions is very low, therefore most of the mutations develop sectorially or periclinally and give rise to chimeric tissues and organs.

In the 1990s, thanks to the use of codominant genetic markers (microsatellites, restriction fragment length polymorphisms), the existence of genetic chimerism was demonstrated in several vine varieties. Franks *et al*. (2002) showed that Pinot Meunier can have up to three alleles at some loci, whereas the grapevine, which has a diploid genome, can theoretically have two allelic forms for a heterozygous locus. Boss and Thomas (2002) succeeded in dechimerizing Pinot Meunier by somatic embryogenesis. They characterized the resulting L1 and L2 genotypes and studied the associated phenotypes. This work showed that in Pinot Meunier, cells of the L1 layer carry a mutation in the *VviGAI1* gene (Peng and Harberd, 1993; Peng *et al.*, 1997), and that this mutation confers the hairy phenotype to the variety (**Figure 1**).

Plants regenerated from L1 or L2 cells have very different phenotypes (**Figure 2**).

- Plants derived from cells of L2, the deepest cell layer, no longer have a mutation at the *VviGAI1* locus and are homozygous: *VviGAI1*/*VviGAI1*. The phenotype associated with this genotype is non-dwarf, similar to Pinot Noir and other grapevine cultivars (i.e. macrovines).

- Plants derived from L1 cells retain a mutated version of *VviGAI1* (i.e. *Vvigai1*), in association with the wild-type allele, *VviGAI1* (*VviGAI1*/*Vvgai*). These plants are dwarf, hairy, and display full conversion of all tendrils into inflorescences. This phenotype is called microvine because of the small size of the mutant.

Thus, the presence of the *Vvigai1* mutation in both cell layers (L1 and L2) confers to the microvine a very different phenotype from that of Pinot Meunier, which bears the mutation in the L1 cell layer alone. Although present in both cell layers of the microvine, the *VviGAI* locus is heterozygous, that is, each cell carries the mutated *Vvigai1* allele in association with the wild-type *VviGAI1* allele. *Vvigai1* is not a lethal mutation for the sporophyte or the gametophyte,

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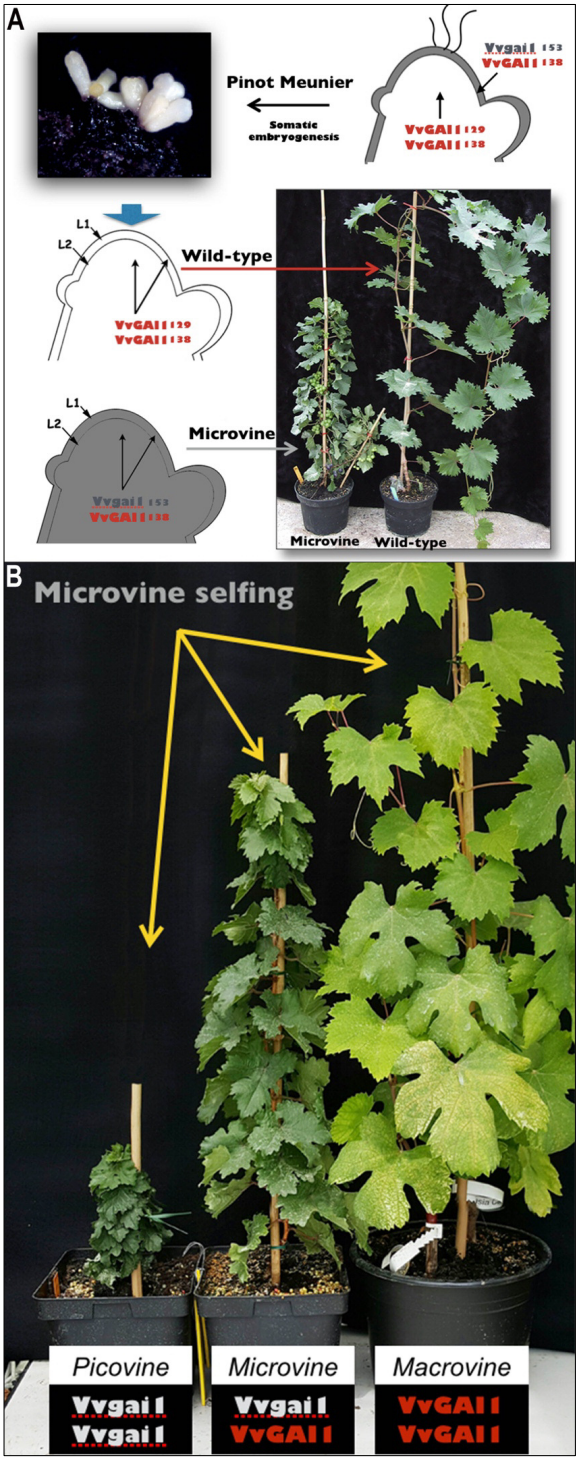


FIGURE 2. Variants of Pinot Meunier produced by somatic embryogenesis and microvine selfing. (A) Somatic embryogenesis from anthers of Pinot Meunier yields two types of plant. One type, called macrovine, no longer carries the Vvigai1 mutation in the L1 and L2 cell layers. It is homozygous for the non-mutated form of the gene (i.e. VviGAI1/VviGAI1) and exhibits the same phenotype as Pinot Noir (i.e. large size, juvenile period, production of clusters mainly from proleptic axes, low trichome density). The other type, called microvine, carries the Vvigai1 mutation in all its tissues. Microvine is heterozygous for the mutation (i.e. VviGAI1/Vvigai1) and displays a miniaturized phenotype and extreme hairiness; it produces inflorescences from both the winter buds and the conversion of tendrils into inflorescences. (B) Three genotypes and their corresponding phenotypes can be obtained from selfing of microvine: the microvine and the macrovine described here, in addition to picovine, an extremely miniaturized vine with the homozygous genotype (i.e. Vvigai1/Vvigai1). In this figure, the numbers associated with each VviGAI allele correspond to the number of base nucleotides in the VVS2 microsatellite marker (Boss and Thomas, 2002).

therefore this status can be altered by microvine selfing to produce the following phenotypes (and genotypes): (i) the macrovine (*VviGAI1*/*VviGAI1*), (ii) the microvine (*VviGAI1*/*Vvigai1*), and (iii) the picovine (*Vvigai1*/*Vvigai1*). The last of these is characterized by extreme dwarfism (Boss and Thomas, 2002; King *et al*., 2001).

2. Molecular mechanisms associated with the *Vvigai1* mutation

Comparison of the allelic forms of *VviGAI* in Pinot Meunier and the microvine (Boss and Thomas, 2002) showed that the *Vvigai1* mutation corresponds to a single-nucleotide change affecting the DELLA motif of the protein, which plays a key role in gibberellin signalling (**Figure 3**). After transient transformation of epidermal onion cells, green fluorescent protein (GFP) fusions to *VviGAI1* and *Vvigai1* sequences responded differently to the application of gibberellin: the fluorescent signal of the GAI1–GFP fusion disappeared rapidly from the nucleus after hormonal stimulus with a gibberellin A3, which indicates its degradation. Conversely, the gai1–GFP fusion remained insensitive to hormonal signalling, which indicates that the degradation of the protein triggered by gibberellins no longer occurs when DELLA is mutated to DELHA.

The GAI protein is an important regulator of vegetative growth and reproductive development in plants (Carmona *et al.*, 2008). In grapevine, gibberellins, whose synthesis is amplified under shade, stimulate growth and inhibit the formation of inflorescences (Coombe, 1967). This effect is mediated by the nuclear protein GAI1, which in its mutated form gai1 no longer transmits the hormonal signal. Thus, vegetative growth and inhibition of the conversion of tendrils into inflorescences are no longer maintained, resulting in the dwarf phenotype and continuous fructification along the stems. In fact, among the

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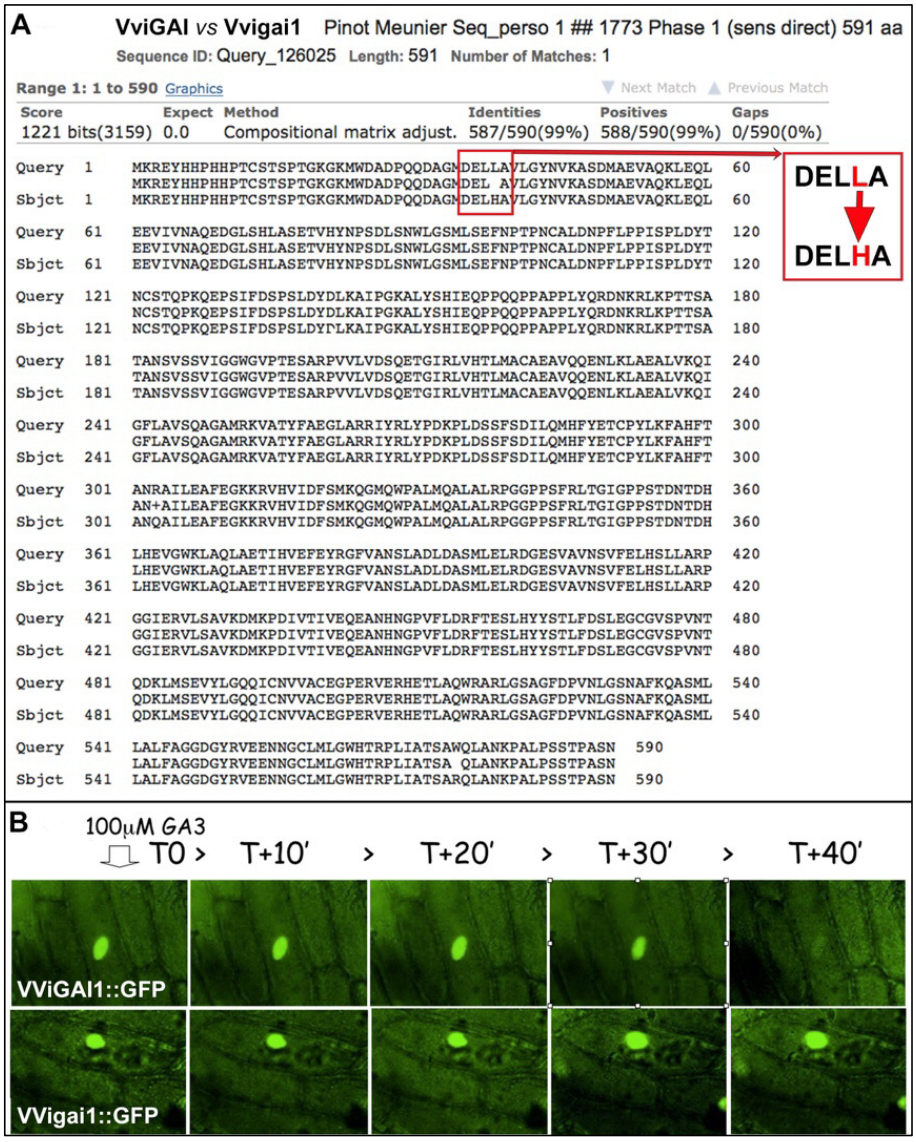


FIGURE 3. VviGAI1 and Vvigai1: protein alignment, protein localization, and response to gibberellins.
(A) Protein alignment (BlastP) of the VviGAI1 and Vvigai1 alleles present in the microvine. VviGAI1 corresponds to the functional allele and Vvigai1 to the mutated form. (B) Protein localization and response of both alleles to gibberellin. The construction and production of the fusion proteins, and the protocol to transiently express GAI–GFP in onion epidermal cells, are detailed in Feechan et al. (2013). After bombardment, gibberellin A3 (100 µM/L) was added directly onto onion strips. Confocal observations were at successive 10¢ intervals, starting from T0.

VviGAI isogenes present in the grapevine genome, *Vvigai1* is expressed mainly in vegetative organs such as buds and young leaves, whereas other forms, without any mutation in the DELLA protein motif, are expressed in reproductive organs; *Vvigai2* is an example of the latter (unpublished data, **Figure 4**). This explains why the *Vvigai1* mutation does not interfere directly with berry development, which is similar to macrovine varieties (Rienth *et al.*, 2016a).

PHYSIOLOGICAL AND GENETIC FEATURES OF THE MICROVINE

1. Spatiotemporal dynamics of development and growth

The vegetative and reproductive development of microvine are shown in **Figure 5**. The possibility of converting spatial observations (along the proleptic axis) into temporal dynamics at a given stage of vegetative or reproductive development was studied. Two

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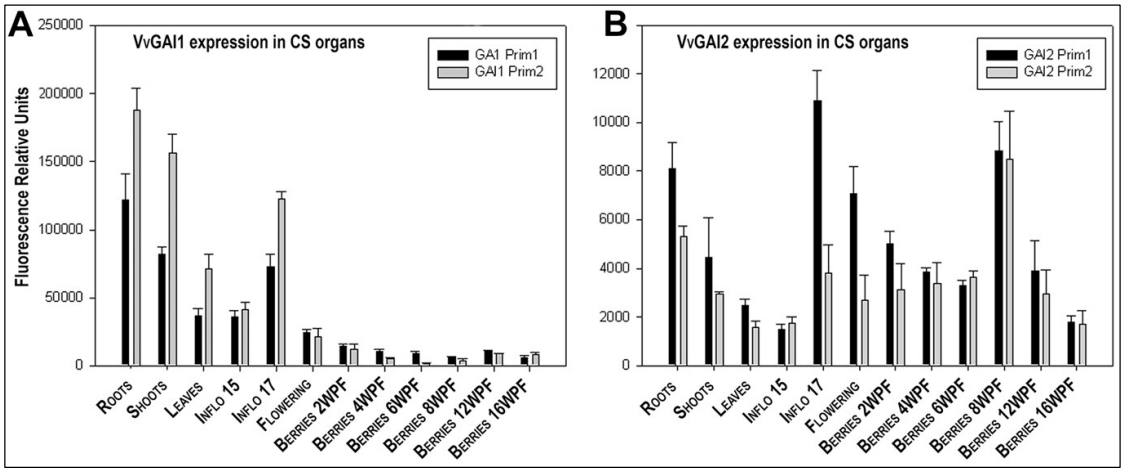


FIGURE 4. Differential expression of VviGAI isogenes, assessed in vegetative and reproductive organs of Cabernet-Sauvignon (CS).
(A) VviGAI1, the wild-type version of the gene, which is mutated in the microvine. (B) VviGAI2, an isogene of VviGAI1 with a similar genetic structure to that of VviGAI1 cloned from Pinot Meunier. VvGAI11 primers: Prim1 F, TGAGAGGGTGCTGTCG, and Prim1 R, CCCTTCCAATGAGTCAAACA (both before the stop codon); Prim2 F, CTCACCCTAACC CGCTTGTA, and Prim2 R, GGGAAACAAA AGGGTGACA (both after stop codon). VvGAI2 primers: Prim1 F, CGTTTGACAGGCATTGG, and Prim1 R, ATAGCTACGGCTTCCTTTGG (both before the stop codon); Prim2 F, AGCCGTAGCCGAGATTTACA (before the stop codon) and Prim2 R, TGCAACCAAAATCCAATTACA (after the stop codon). RNA extraction, cDNA synthesis and gene expression quantification as described in Fernandez *et al.* (2007).

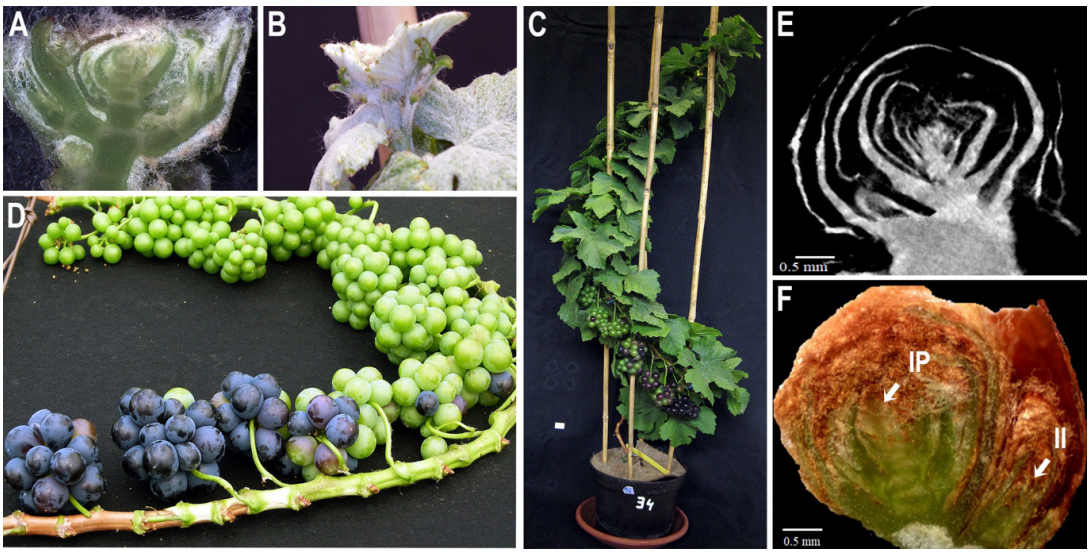


FIGURE 5. Vegetative and reproductive development of the ML1 somaclone n°7, a microvine line regenerated from Pinot Meunier cl. ENTAV 8 according to the method described by Torregrosa (1998).
(A) Longitudinal section of an apex, showing preformation of between seven and nine phytomers before the emergence of caulinar organs. (B) Emergence of young inflorescences just below the apex. (C) An 8-month-old ML1 microvine displaying all typical stages of the reproductive cycle. (D) Phytomers carrying bunches ranging from the green to ripening stages, with concomitant lignification of the shoot (leaves have been removed for clarity). (E) Longitudinal section of a winter bud analysed by tomography. (F) Longitudinal section of a winter bud exhibiting a lateral inflorescence primordium (IP) on the primary axis and secondary primordial shoots (II).

distinct reproductive development patterns occur simultaneously: (i) fructification of the primordial shoot within winter buds, and (ii) continuous fruiting of proleptic axes, resulting from the conversion of tendrils into inflorescences.

1.1. Temporal conversion of spatial profiles

Under controlled environments (25°C/15°C day/night; VPD, 1kPa; 12- to 14-h photoperiod), leaf, internode and berry organogenesis are constant at a given level of phytomer, regardless of bud break (Luchaire *et al.*, 2015). Leave and

internodes have a similar duration of growth in microvine and macrovine, namely *c.*220°C days (or 22 days) for leaves and *c.*150°C days (or 15 days) for internodes (Lebon *et al.*, 2004). Flowering (defined as 50% open flowers) occurs 320°C days (or 30 days) later than phytomer emission, which is similar to the interval between budburst and flowering in macrovine (Mullins *et al.*, 1992). Ripening (onset of sugar loading) starts *c.*500°C days (or 46 days) after flowering, and the physiologically ripe stage (arrest of sugar loading) is reached at *c.*900°C days (or 83 days) after flowering or 37 days after the start of sugar loading, similarly to in the macrovine (Romieu *et al.*, 2016; Mullins *et al.*, 1992). No effects of the *Vvigai1* mutation on final berry size was observed when compared with macrovine (see section 3.1.3); however, the leaf area of microvine was reduced by 50% and internodes were five times shorter.

The development of leaves and berries has proven spatially stable: the dynamics of leaf area and berry volume (at the herbaceous phase) of all phytomers are superimposed when expressed with respect to accumulated thermal time since the emission of the corresponding phytomer. Additionally, the phyllochron (i.e. the rate of leaf emergence) is constant on the main axis, reaching *c.*24°C days in the microvine, similar to in other varieties of *V. vinifera* such as Grenache (Lebon *et al.*, 2004), and it fluctuates only slightly with respect to light radiation (photosynthetically active radiation, PAR, between 19 and 25 mol/m^2 per day). Based on these outcomes, the conversion of spatial dynamics of leaf and berry development along the stems into time profiles was tested (**Figure 6**). For this purpose, the positions of the phytomers along the axis were converted into accumulated thermal time since their emission by multiplying their plastochron index (or phytomer position from the apex) by the phyllochron. The temporal profiles of leaf area and berry volume (green growth phase) resulting from this spatiotemporal conversion are similar to actual temporal profiles obtained at given levels of phytomer (Luchaire *et al.*, 2013; Luchaire *et al.*, 2015; Luchaire *et al.*, 2017). This property makes it possible to reconstruct temporal dynamics of development from a single spatial observation of the axis at a given stage.

1.2. Dynamics of inflorescence differentiation within winter buds

The level of differentiation of microvine winter buds (i.e. the number of preformed phytomers and inflorescence primordia) was analysed over an 80-day period of growth under controlled greenhouse conditions (25°C/15°C day/night;

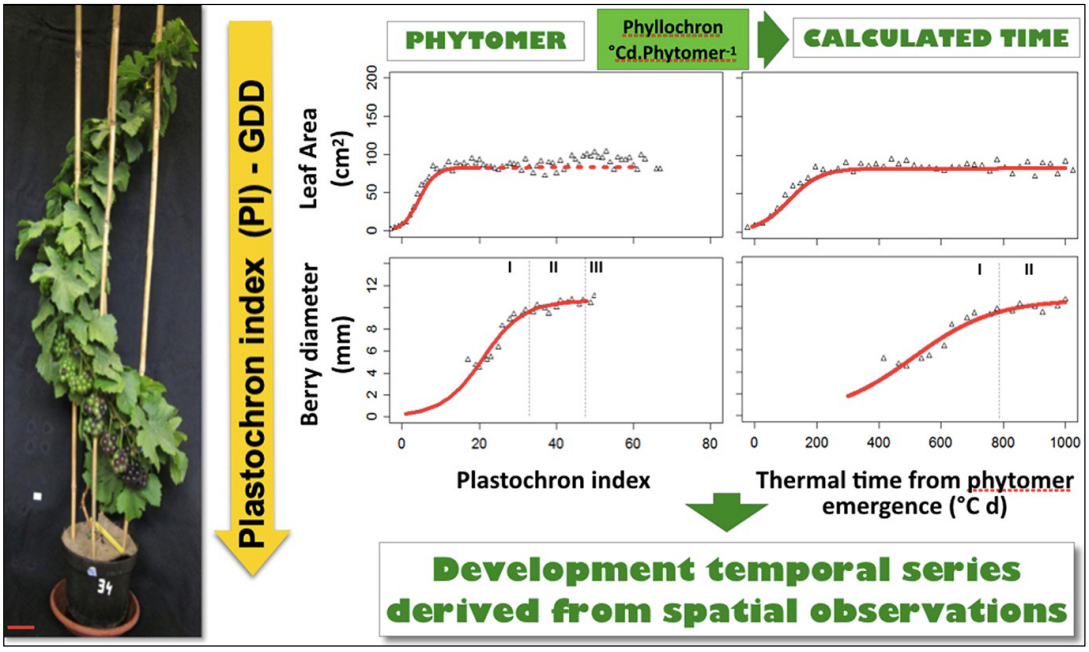


FIGURE 6. Conversion of leaf and green-stage berry growth data obtained at positions along the microvine main shoot (i.e. the plastochron index) into accumulated thermal time since phytomer emergence (Luchaire *et al.*, 2017).

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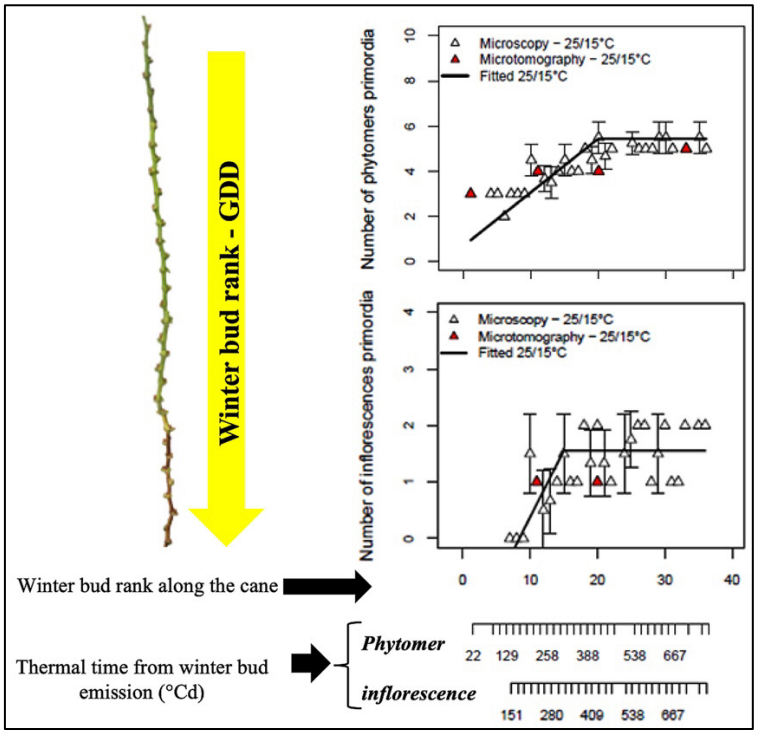


FIGURE 7. Number of preformed phytomers (top right) and inflorescences (bottom right) on the primordial shoot of primary winter buds, according to the plastochron index.
Scales below the graphs indicate the corresponding thermal time at each phytomer position.

VPD, 1 kPa; photoperiod, 12 h). Two imaging methods were compared: classic microscopy dissection and non-invasive X-ray microtomography with a resolution of 9 µm (Larabell and Nugent, 2010). The results showed that buds of the microvine have a complex structure of primary, secondary and tertiary buds of decreasing fertility, similar to that in non-dwarf vines (Alcântara Novelli Dias *et al.*, 2019). In the microvine, the maximum fertility is two inflorescences per primary bud. This compares with three or even four in some non-dwarf varieties.

The spatiotemporal conversion approach described earlier in this review has been used to characterize the evolution of primary winter bud development along the proleptic axis of the microvine (Alcântara Novelli Dias *et al.*, 2019) (**Figure 7**). The number of preformed phytomers on the primordial shoot increases linearly with respect to the plastochron index (PI) in the non-lignified zone (PI < 25) of the proleptic axis. The temporal dynamics of bud development were calculated from the spatial profiles by using the equation proleptic axis PI ´ phyllochron (phyllochron of 24°C). The bud primordial shoot displayed a maximum of six phytomers from PI 20 (the lignified zone), that is, 430°C days (or 43 days) after its initiation. A maximum of two inflorescence primordia were observed in this zone.

The primordia of the first and second inflorescences were found between numbers 4 and 5 of the preformed phytomers of the primary axis, similarly to in the macrovine (Carolus, 1971). They were initiated from PI 14 and 18 of the proleptic axis, respectively, corresponding to 301°C days (or 30 days) and 392°C days (or 39 days) since bud emergence. The development of inflorescence primordia followed the same timing in buds of the macrovine (Vasconcelos *et al.*, 2009) as observed in those of microvines. Periderm formation at PI 25, corresponding to 516°C days (or 52 days) after bud emergence, was shown to be concomitant with the slowing down of bud development and probably their entrance into endodormancy, as reported in macrovines (Bernard, 1980). This pattern of bud development parameterized for the microvine can be used to evaluate and potentially predict how the effects of environmental stress in season 1 might affect fructification in season 2.

1.3. Dynamics of fruit development deriving from newly formed inflorescences

Newly formed inflorescences are smaller in the microvine (10–50 berries per cluster) than those initiated in the previous year in macrovine (Pratt, 1971; Mullins *et al*. 1992, Chaib *et al*., 2010). However, the flowers and young fruits of microvine do not display the extensive abscission found in macrovine. Spatial fruit development along the microvine axis occurs in the two classic growth phases, as observed for berries of macrovine (Conde *et al*., 2007; Vasconcelos *et al*., 2009).

Individual berries of the ML1 microvine reach a final weight of 1.2 g, similar to that of the berries of Pinot Meunier, from which the line derives. At the stage of physiological ripeness, when phloem unloading ceases, berries contain about 0.8 mmol of soluble sugars per berry, under non-limiting water supply; this is similar to equivalent values in macrovine varieties (**Figure 8**). The main microvine berry solutes (malic acid, tartaric acid, glucose, fructose and proline) have been analysed in detail (Rienth *et al.,* 2014a; Rienth *et al.*, 2016b). Microvine fruit accumulates malic acid during the green growth stage for about 40 days after fruit set. At the end of the herbaceous phase and throughout the second growth period or ripening phase, degradation of malic acid is triggered simultaneously with accumulation of sugars and proline, which is often used as an indicator of ripening. Tartaric acid is accumulated during the first growth phase only and levels remain stable afterwards. The slight decrease in tartaric acid observed during ripening may be attributable to enhanced tartaric precipitation, as shown by Rösti *et al.* (2018). At the end of the green growth stage, the two major organic acids represent approximately 500 mEq, which is similar to the acidity of the fruit of macrovines (Bigard *et al.*, 2017). The accumulation of sugars, triggered at *veraison*, continues until phloem unloading slows and fruit reaches its maximum volume. From this point, the amount of sugars per berry remains constant, but the concentration continues to increase due to the decrease in berry volume (i.e. over-ripening).

2. Genetics and genomics

2.1. Genetic mapping and pre-breeding

The *VviGAI1* gene and the flower sex type QTL are located on chromosomes 1 and 2, respectively. Thus, it is possible to use female

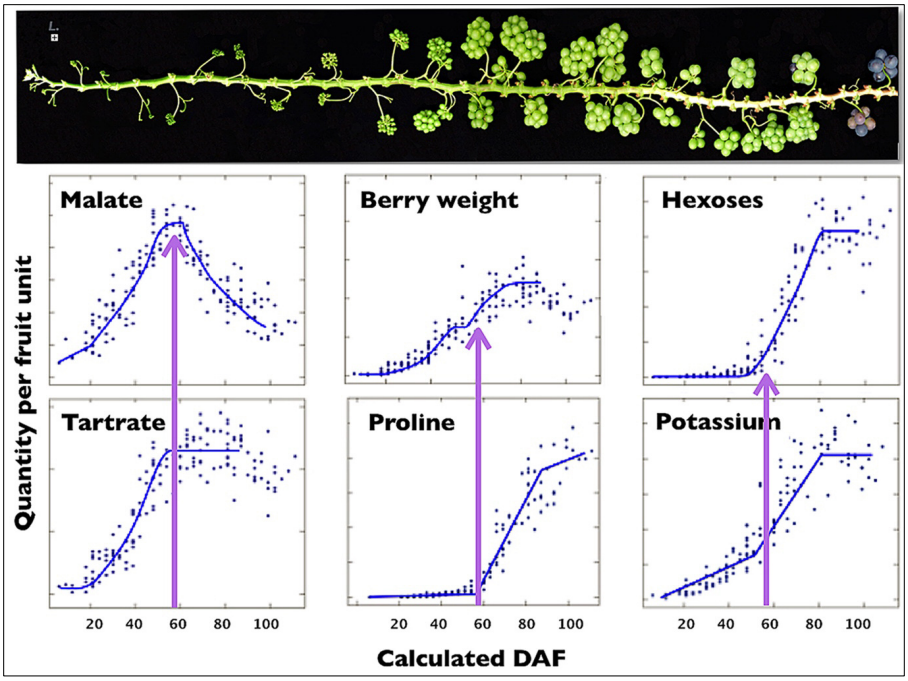


FIGURE 8. Spatiotemporal distribution of reproductive developmental stages from flowering to ripening. Days after flowering (DAF) on the abscissa was recalculated for each phytomer, converting each plastochron index value to thermal and calendar time, as described by Luchaire *et al.* (2017). Kinetics of fresh fruit weight, and the contents of major primary metabolites and potassium, are expressed as quantity per fruit, as described by Rienth *et al.* (2014a) and Rienth *et al.* (2016a).

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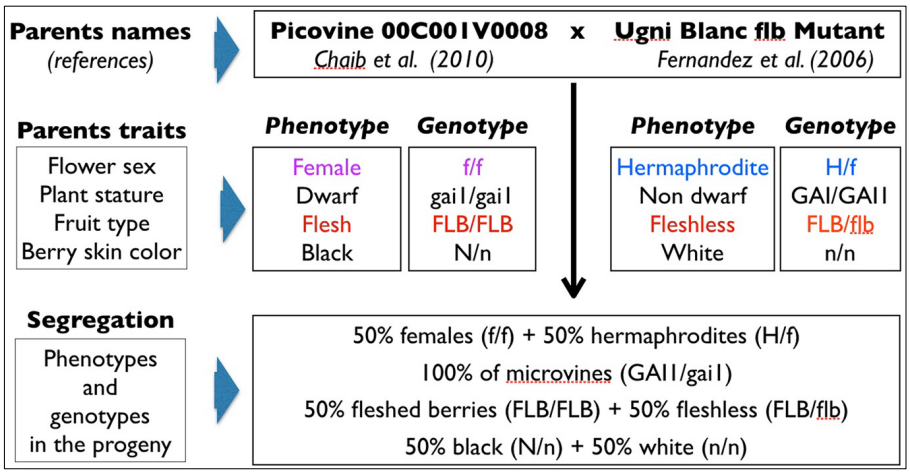


FIGURE 9. Distribution of genotypes and phenotypes within offspring derived from the cross between the picovine 00C001V0008 and the Ugni blanc Fleshless berry mutant.
The picovine 00C001V0008 has female flowers, dwarf stature, and black-skinned berries, and the Ugni lanc Fleshless berry mutant has hermaphrodite flowers, non-dwarf vine stature, and fleshless non-pigmented berries.

microvines or picovines, which facilitates crosses by avoiding the time-consuming emasculation of vines and reducing the risk of selfing (Chaib *et al.*, 2010). When a female microvine (f/f) is crossed with a hermaphrodite genotype (H/f, the most common genotype in *V. vinifera* varieties), the population is composed of female and hermaphroditic plants in a 50:50 ratio. When a picovine (*Vvigai1*/*Vvigai1*) is used for crossing, 100% of the progeny exhibit a microvine phenotype.

Examples of the genotypes and phenotypes obtained from the cross between the picovine 00C001V0008 (Chaib *et al.*, 2010) and the fleshless berry mutant (Fernandez *et al.*, 2006) are shown in **Figure 9**. The progeny obtained from this cross comprise 100% microvines (because the female parent has a *Vvigai1*/*Vvigai1* genotype) and a very small proportion of individuals with both hermaphrodite flowers and pigmented berries. Indeed, these two characters are present in the homozygous recessive state in one parent (f/f and n/n) and in the heterozygous dominant state in the other (H/f and N/n). It should be noted that because Ugni Blanc is heterozygous at the sex locus (H/f) and the picovine is f/f, selection of hermaphrodite individuals leads to segregation distortion in the progeny in terms of traits determined by the genes on chromosome 2.

Under standard thermal and photoperiod conditions (25°C/15°C day/night; VPD, 1kPa; photoperiod, 12h), the microvine produces two or three new inflorescences per week, making possible to repeat crosses on the same plants (**Figure 10**). Because of the small number of plants required for crossing, less experimental space is required and the hybridization can be spread over selected and potentially long periods. Less than 2 months after pollination, seeds can be harvested and embryos rescued, as described by Chatbanyong and Torregrosa (2015). Before the seedlings are acclimated, individuals can be multiplied by micropropagation (Bouquet and Torregrosa, 2003). The first grapes of the next generation can then be obtained only 12 months after the cross. Thus, linking a genotype to a phenotype in either F1 or F2 progenies is much quicker than in macrovine, for which several years are generally needed.

Moreover, if a trait of interest can be inherited through such crosses, it is possible to return to the non-dwarf phenotype (*GAI1*/*GAI1*) for breeding purposes. Indeed, 50% of the individuals from a cross between a microvine (*VviGAI1*/*Vvigai1*) and a macrovine (*VviGAI1*/*VviGAI1*) exhibit the same biological properties as conventional non-dwarf varieties. Thus, the microvine can be used both for the identification of QTLs of interest and to rapidly pyramid characters of interest from a pre-breeding perspective (Bigard *et al.*, 2017).

2.2. Functional genomics

The biological properties of the microvine are also of great interest for functional genomics (Vidal *et al.*, 2010). With genetic transformation of classic varieties, it takes several years to

obtain adult plants so that the fruit phenotypes linked to the ectopic expression of candidate genes can be studied (Vidal *et al.*, 2010; Dalla Costa *et al*., 2019). As in macrovines (Torregrosa *et al*., 2002), not all microvine genotypes proved suitable for transformation experiments (Chaib *et al.*, 2010). For instance, the L1 mutant microvine lines regenerated from Pinot Meunier proved recalcitrant to somatic embryogenesis regeneration (Chaib *et al.*, 2010). This is due to excessive browning reactions during *in vitro* culture (Torregrosa *et al.*, 2002; Torregrosa *et al.*, 2015).

For these reasons, Chaib *et al.* (2010) developed a set of microvine lines with improved capacity to be transformed by *Agrobacterium*. With such microvine lines, starting from embryogenic tissues compatible with *Agrobacterium tumefaciens*-mediated transformation (**Figure 11**), it is possible to recover transgenic fruiting plants in less than 1 year. As in classic genetics, it is then easy to derive F2 lines to establish transgenic loci in the homozygous state for further studies. Additionally, the microvines have a good aptitude for transformation by *Agrobacterium rhizogenes*, which means that transgenic roots can be obtained for axenic culture in a few weeks. These hairy roots can be either established in axenic culture (Cutanda-Perez *et al*., 2009; Huang *et al*., 2013) or grafted to establish semitransgenic plants (Torregrosa and Bouquet, 1997; Torregrosa *et al*., 2015).

We have used the microvine to ectopically express several genes (e.g. *VviHB13*, as shown in **Figure 11**; *VviSPEC2*, *VviHT6*, *VviTFL1*, etc.) in stably transformed plants (unpublished data). Several other groups (personal communications) have also used the microvine as an alternative to the macrovine for either classic genetic transformation or genomic edition studies. However, grapevine-stable transformation is not much easier with the microvine than with the macrovine, and today only a limited number of laboratories have mastered these transformation technologies. Thus, routine use of microvine for genetic transformation has yet to be established, due to the recalcitrance of the grapevine towards *in vitro* regeneration techniques using either embryogenic tissue/culture or protoplasts. This does not detract from the interest of this model, which makes it possible to obtain transgenic fruits in a few months, and it remains promising for all groups interested in regulation of grapevine fruit development.



FIGURE 10. The white tags of this microvine plant correspond to emasculated and pollinated inflorescences.
During the season, by keeping the female plants in a greenhouse, it is possible to pollinate dozens of inflorescences with the same plant to obtain hundreds of seeds per pollinated microvine plant.

MICROVINE RESPONSES TO ABIOTIC STRESS AND QTL MAPPING

1. Effects of temperature on carbon fluxes and fruiting

The effects of elevated temperature on growth and carbon distribution between vegetative and reproductive organs has been investigated on microvines grown in precisely controlled conditions. Two contrasting thermal regimens with a difference of 8°C (30°C/20°C versus 22°C/12°C day/night) were imposed in growth chambers for a period of 450°C d. For both regimens, VPD was 1 kPa and PAR was 19 mol/m^{-2} per day (photoperiod, 14 h). The phyllochron was *c*.24°C days for both treatments, similar to the results under controlled climatic conditions in a greenhouse (Luchaire *et al*., 2017).

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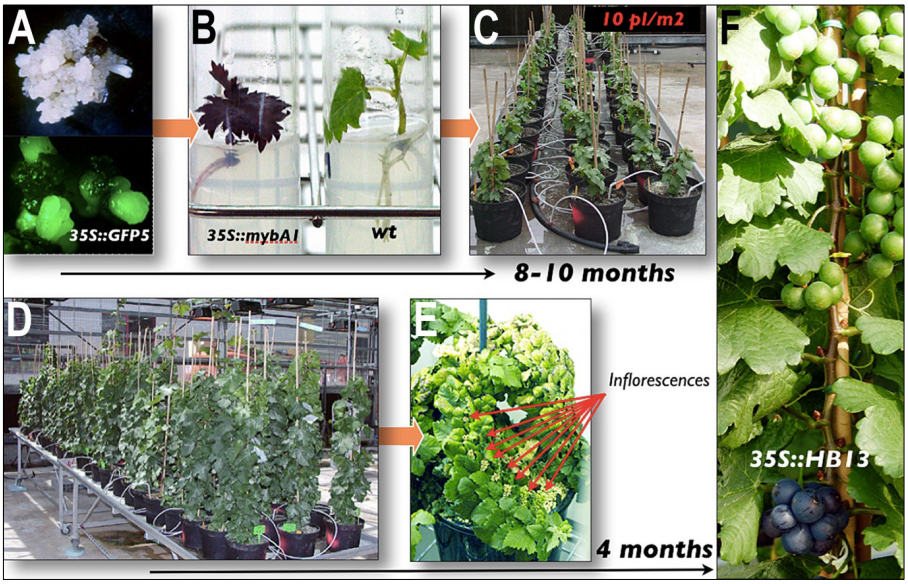


FIGURE 11. The recovery of transgenic microvines via Agrobacterium-mediated transformation of embryogenic calli

(A) Stable transgenic tissues expressing green fluorescent protein after selection with hygromycin. (B) Stable transformed grapevine plantlets expressing VlmybA1, micropropagated in vitro. (C and D) Acclimation of grapevine plants. (E) Development of inflorescences a few weeks after acclimation. (F) A transgenic microvine line overexpressing VviHB13, a major regulator of grape flesh development (Fernandez *et al.*, 2007).

Spatial observations of biomass, leaf size and carbon contents at harvest were converted into temporal profiles according to the method described in section 3.1. Luchaire *et al.* (2014; 2017) showed that high temperatures accelerate growth and biomass accumulation in vegetative organs (leaves and internodes) on a thermal time basis, at the expense of sugar accumulation in the internodes and the ratio of leaf area to leaf mass (thus resulting in thinner leaves). Additionally, fruit growth slows and sugar loading of proximal phytomers (from the post-flowering stage to onset of heat treatment) is delayed by *c.*400°C days at high temperatures. The inflorescence abortion rate is enhanced by elevated temperatures (+20%) at preflowering stages, concomitantly with the beginning of sugar loading in the proximal clusters (Luchaire *et al.*, 2014; Luchaire, 2015; Muller et al., 2016). These results suggest that high temperature decouples vegetative and reproductive development, thus increasing the total biomass of vegetative organs while reducing accumulation of carbon reserves and making continuous fruiting more difficult.

2. Circadian changes in gene expression during fruit development

Transcriptomics is now a very common approach in research to understand the genetic mechanisms regulating grape development. However, before the study reported by Carbonell-Bejerano *et al.* (2014), no work had attempted to investigate the circadian evolution of the grape transcriptome, due to the difficulties in conducting the necessary experiments outdoors with macrovines. The results published by Rienth *et al.* (2014b, 2014c, 2016a) were the first for a perennial fleshly fruit that addressed the circadian effects of elevated temperatures on grape transcriptional programme over several developmental stages.

Rienth *et al.* (2012; 2014b) initially conducted an experiment with microvines grown in climatic chambers (30°C/20°C day/night temperatures; VPD, 1 kPa; photoperiod, 14 h) for a 3-month period covering a complete reproductive cycle from flowering to ripening (Rienth *et al.*, 2014c). When proximal grapes reached physiological maturity, berries from two green and two ripening stages were sampled at different times of the day and night for a whole transcriptome analysis with NimbleGen Vitis 12′ microarrays (NimbleGen, 090818 Vitis 30 K). All genes developmentally modulated during the day also showed some variation of expression at night, but 1843 additional genes were regulated only at night. These results emphasize the importance of regulatory mechanisms associated with nocturnal fruit development. Circadian regulation is highly

developmental stage–specific, with only nine commonly deregulated genes between day and night at all stages. Genes related to photosynthesis appear strongly repressed at night, particularly in young green berries, and several functional categories related to secondary metabolism and abiotic stress show strong overexpression at night at all developmental stages (discussed later).

Based on these findings, Rienth *et al*. (2014c, 2016a) carried out two further experiments on the effects of elevated temperatures on microvine berry development. Temperature treatments were imposed at different stages between green growth and ripening over short periods (2 h, 35°C) or long periods (> 30 days, 20°C/15°C versus 30°C/15°C for berry green growth; 25°C/15°C versus 30°C/25°C for ripening stages). Berries were then sampled during day and night. The results of NimbleGen Vitis 12′ microarray assays showed that a large number of genes (5653) respond to increased temperature in the short term, at all stages of development. However, the nature of this temperature effect depends on the developmental stage. Berries close to *veraison* are the most sensitive to temperature elevation. Various genes of secondary metabolism (e.g. those encoding phenylalanine and anthocyanins) are suppressed by high temperature at *veraison*, particularly during the nocturnal phase.

To investigate the effects of elevated temperatures in the long term, we used high-throughput transcriptomic analysis through RNA-Seq (Illumina Technology, San Diego, CA, USA). A total of 10,788 genes were detected, depending on the stage, temperature regimen and photoperiod. Notably, the importance of 'heat shock'–type genes was highlighted. Temperature rises lead to acceleration of fruit growth during the green growth phase. At the onset of ripening, high temperatures increase malic acid respiration, delay accumulation of sugars, and down-regulate key genes of the flavonoid pathway. For the first time, decoupling of sugar accumulation and malic acid respiration during ripening was observed and related to changes in the carbohydrate status of the plant as a function of temperature (Romieu *et al*., 2016).

Expression patterns of genes putatively involved in sugar and malic acid accumulation and degradation confirmed physiological observations of sugar–acid decoupling. Together, these observations suggest that under cool conditions, malic acid respiration may not be necessary to provide supplementary energy in the fruit, because the energetic status of the plant is more stable due to lower vegetative growth and rates of cellular respiration. In cool climates, the allocation of carbon to fruit can support glycolysis, malate synthesis, and sugar accumulation in the vacuole. Conversely, under warm conditions, cytoplasmic sugars could be limiting at the onset of ripening, when cells start to accumulate sugars in the vacuole. Thus, malate would be drained from the vacuole to supply energy through respiration and/or acid–sugar exchange at the tonoplast.

3. Identification of genetic traits relating to the aromatic character of Cabernet-Sauvignon

The methoxypyrazines are a family of volatile compounds found in many fruits and vegetables and especially in grapes; they provide herbaceous flavours (green capsicum aroma) to the wines of some varieties, such as Cabernet Sauvignon and Sauvignon Blanc. Although several methoxypyrazine biosynthetic pathways have been proposed, the genes encoding the corresponding enzymes remain to be elucidated.

Dunlevy *et al*. (2013) have produced an F2 population derived from an F1 microvine obtained in 2005 by crossing Cabernet Sauvignon with a picovine. The Cabernet Sauvignon variety is capable of producing the molecule 3-isobutyl-2-methoxypyrazine (IBMP), the major compound associated with capsicum flavours. In contrast, the microvine derived from Pinot Meunier produces very little IBMP. In the F1 offspring, all individuals produced IBMP, suggesting that this trait is controlled by a homozygous dominant gene in Cabernet Sauvignon. In the F2 progeny, 43 lines accumulated IBMP and 21 individuals lacked this compound, confirming the dominant homozygous genotype for Cabernet Sauvignon and the homozygous recessive genotype for the picovine progenitor.

After genotyping and phenotyping the entire F2 progeny, the IBMP accumulation locus was localized to a 2.3-Mb region of the linkage group no. 3. This QTL included 261 genes, from which two candidate methyltransferase genes were identified: *VviOMT3* and *VviOMT4*. Screening of a collection of 91 grapevine

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genotypes differentially accumulating IBMP into the grapes identified *VviOMT3* as the most likely regulator of IBMP accumulation in grapevine fruits. Moreover, the data suggested that the low level of methoxypyrazines found in most cultivated grape varieties is a consequence of human selection for mutations in methyltransferase. The markers identifying this locus are valuable tools for the selection of grape varieties that are aromatically typified by IBMP and recall Cabernet wines.

Interestingly, Guillaumie *et al.* (2013) simultaneously reported a genetic analysis of the biosynthesis of 2-methoxy-3-isobutylpyrazine, in which they used a classic macrovine segregating progeny of 130 individuals, obtained in 1995 by crossing Cabernet Sauvignon with the *Vitis riparia* Gloire de Montpellier (Marguerit *et al.*, 2009). Owing to the genotypes of Cabernet Sauvignon (H/f) and *V. riparia* (M/f), only 25% of the individuals of this progeny displayed hermaphroditic flowers, whereas 50% of the individuals were male, predicting to phenotype fruits with this material. Fortunately, IBMP is also present in leaves, which allowed mapping of the corresponding QTL. From the studies of Dunlevy *et al.* (2013), Guillaumie *et al.* (2013), Houel *et al.* (2015) and Bigard *et al.* (2017), two major advantages of using microvine compared with macrovine can be highlighted: (i) because not all berry traits are present in vegetative organs, the microvine can be much more useful to study and segregate reproductive traits; and (ii) the genetic mapping of reproductive traits can be quicker and much easier with microvine.

4. Identification of stable QTLs for development under fluctuating environments

Houel *et al*. (2015) reported the first genetic study on microvines in which the aim was to test the stability of QTLs of vegetative and reproductive development under fluctuating environments. A set of 129 hermaphroditic microvines, derived from picovine 00C001V0008 ´ Ugni Blanc Fleshless berry mutant (Fernandez *et al.*, 2007) (see section 3.2.1), was genotyped with the Illumina 18 K SNP (single nucleotide polymorphism) chip. Forty-three vegetative and reproductive traits were phenotyped over four vegetative cycles in the field, and a subset of 22 traits were additionally characterized over two cycles in climatic chambers under two contrasting temperature regimens. Ten stable QTLs were identified on the parental genetic maps for development and composition of the berry and the leaf area.

A new major QTL accounting for up to 44% of variance in berry weight was identified on chromosome 7 of the Ugni Blanc parent. This QTL colocates with QTLs for number of seeds per berry (accounting for up to 76% of total variance), fruit acidity before maturation (up to 35% of explained variance), and yield components such as number of clusters and berries per cluster (up to 25% of explained variance). Additionally, a minor leaf surface QTL was found on chromosome 4 of the same parent. This study, which combined use of a microvine population to boost and facilitate phenotyping with high-throughput genotyping technologies, was innovative in grapevine genetics and also genetic research on perennial fruit crops. It allowed identification of 10 stable QTLs, including the first QTLs of berry acidity in a *V. vinifera* intraspecific cross.

This progeny was also included in a study addressing diversity in fruit volume, main sugars and amounts of organic acids in *V. vinifera* (Bigard *et al.*, 2018). A panel of 33 genotypes (12 macrovine cultivars and 21 microvine offspring) were phenotyped at two stages of fruit development: the end of the green growth phase, when organic acidity reaches a maximum, and the physiologically ripe stage, when sugar unloading and water uptake stop. To determine the date of sampling at each critical stage, fruit texture and growth were carefully monitored. Analyses at both stages showed large phenotypic variation in malic and tartaric acids, as well as in sugars and berry size. At the ripe stage, fruit fresh weight ranged from 1.04 to 5.25 g and sugar concentration from 751 to 1353 mmol/L. Organic acid content varied in both quantity (from 80 to 361 mEq/L) and composition, with the ratio of malic to tartaric acid ranging from 0.13 to 3.62. At the intergenotypic level, the data showed no link between berry growth and osmoticum accumulation per fruit unit, suggesting that berry water uptake does not depend on fruit osmotic potential alone.

The findings of these studies suggest interesting possibilities for breeding to mitigate the effects of climate warming on viticulture through (i) identification of contrasted genotypes for fruit primary metabolites that are affected by temperature, and (ii) mapping of QTLs of

development, including the regions regulating berry composition.

5. Effect of application of exogenous stimulants of fruit metabolism

Two studies using the ML1 microvine were recently performed to analyse the effects of exogenous compounds on aroma accumulation during grape ripening. The first study dealt with the effect of vine-shoot aqueous extracts, which have been proposed as biostimulants to improve wine aromatic profile. Sánchez-Gómez *et al*. (2018) and (2019), respectively, studied the effect of vine-shoot extract and guaiacol solutions applied at 21 stages of fruit development on the final composition of the microvine grapes. The results confirmed that these extracts and solutions can modify the content of glycosylated aromatic compounds at physiological ripening, especially aglycones such as alcohols, terpenes and C13-norisoprenoids.

OPTIMAL GROWING CONDITIONS AND BIOLOGICAL LIMITATIONS

Although the microvine represents a unique and very versatile biological model, some specific issues should be considered for optimizing its use. Over the past 15 years, we have acquired a wide empirical knowledge regarding the optimal cultivation of microvine under different growing conditions in greenhouses, fields and climatic chambers.

Microvine development is optimal under well-controlled environmental conditions (day/night temperature, 25°C/15°C; air VPD, 1–1.5 kPa; daily PAR, 20–25 mol/m^2/s; photoperiod, 12 h) and when the ratio of leaf area to fruit fresh weight is less than 1 m^2/kg. Even short abiotic constraints, such as high temperature (> 33°C), low radiation (PAR, < 15 mol/m^2 per day) and high VPD (> 3 kPa), block fructification and trigger inflorescence abortion. Rates of inflorescence abortion are higher when carbon reserves (i.e. starch) are reduced, particularly in young plants. Thus, although it is possible to obtain fruiting organs from 5-month-old microvine cuttings, for some studies including abiotic constraints, for example, it is better to use plants aged at least 1 year (Luchaire *et al*., 2016).

Under standard conditions of management, either outdoors or in a greenhouse, we obtained successive cycles of fruiting for at least 5 years, without repotting. For the most fertile lines, it should be noted that the number of ripening berries needs to be controlled to avoid source–sink imbalance, so as not to hamper development of new inflorescences and accumulation of metabolites in the fruits. Because the microvine can carry several levels of cluster at the ripening stages, a good balance is achieved by limiting the number of ripening berries to 8–15 per cluster. After each cycle of growing, we recommend pruning the plants to two to four buds and then exposing them to temperatures ranging from 0 to 10°C for 1 week to stop growth, break bud dormancy, and homogenize subsequent budburst and growth.

Finally, the short internodes in microvine increase leaf shading, which can promote development of fungal diseases. Powdery mildew (*Erysiphe necator*) on leaves and green berries, and grey mold (*Botrytis cinerea*) on ripening fruits, should be carefully controlled. Regular fungicides can be applied to control these diseases, but prophylactic methods are also efficient. Microclimate can be improved. Each microvine can be limited to a single proleptic axis by removing all lateral branches and two out of three leaves (e.g. the leaves of P0 phytomers that do not display inflorescences). In the greenhouse, powdery mildew can be efficiently controlled by the use of sulphur-burning vaporizers; their effects are more gentle for the plants than direct application of sulphur.

Under some conditions, yellow spider mites (e.g. *Eotetranychus carpini*, *Tetranychus urticae*) can also be an issue. A range of acaricides can be used to limit the proliferation of these pests, but biological control with predators (e.g. *Phytoseiulus persimilis combined with Amblyseius californicus* systems from Biobest) have proved very efficient.

We now have several microvine lines that have been pyramided for genes of fungus tolerance. The use of this material, combined with targeted biological control of arthropods, allows microvine plants and populations to be grown without any need for chemical spraying. This is safer for staff and students carrying out management and phenotyping of these plants.

CONCLUSIONS

In the years since the microvine phenotype was initially reported by Boss and Thomas (2002) and proposed as a model for genetics and

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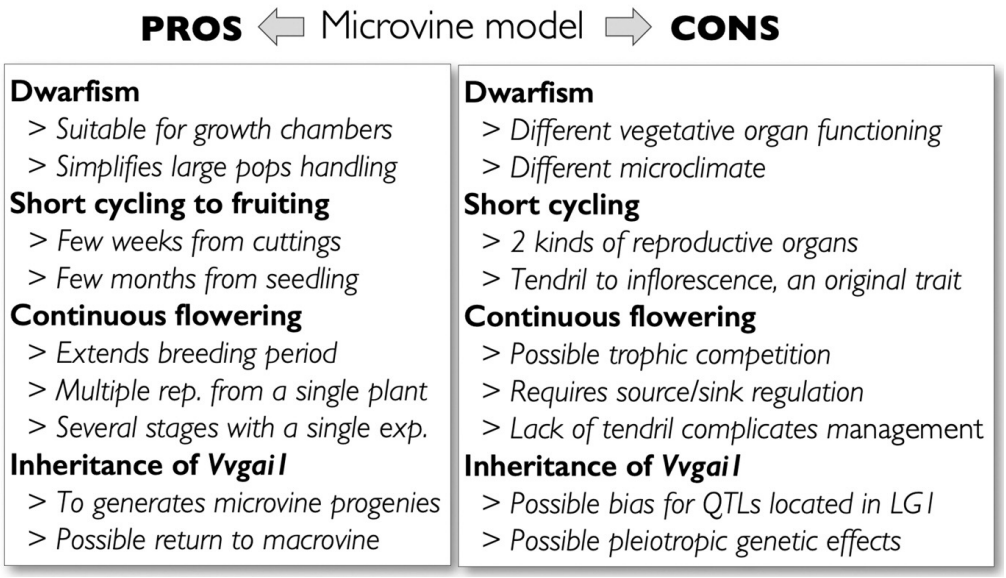


FIGURE 12. Pros and cons of the microvine model

genomic studies by Chaib *et al.* (2010), it has been used in various physiological studies. Given its original biological properties (e.g. small size, continuous fructification) and the possibility of inferring temporal observations from spatial data, this model can be used in fundamental studies on vine response to abiotic constraints or on fruit physiology under well-controlled environments. This model has pros, enabling new experimental opportunities, but also, as with other models, some cons (**Figure 12**). Of the latter, compatibility with the research purpose need to be considered.

The microvine has been used in several studies to address the effect of elevated temperatures over short or long periods on vegetative and reproductive development and on gene expression and phenotypic plasticity in grapes. Microvine has also proved to be a convenient system for accelerating conventional and reverse-genetics approaches, including identification of genetic determinants of stable developmental traits under fluctuating thermal conditions or major loci controlling grape composition. The model is being used to study the effects of physical factors (drought, carbon dioxide concentration, temperature, etc.) on development of the vine and quality of the grapes. It is also being used to develop genetic tools (markers of QTLs, prebreeding lines pyramiding several agronomic traits of interest) for selection of new varieties displaying original properties such as traits for adaptation to climatic changes (Escudier *et al*., 2017; Ojeda *et al*., 2017; Ollat *et al*., 2018; Torregrosa *et al*., 2017).

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