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RESEARCH PAPER

Characterization of *Vitis vinifera* L. somatic variants exhibiting abnormal flower development patterns

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Abstract

Mutants have proven to be a key resource for functional genomic studies in model annual plant species. In perennial plant species where mutants are difficult to generate and to screen, spontaneous somatic variants represent a unique resource to understand the genetic control of complex developmental patterns. The morphological and histological characterization of six *Vitis vinifera* L. somatic variants that display four different abnormal phenotypes of flower development are described here. A phenotype of reiterated reproductive meristems (RRM), with both flower and petal reiteration, was observed in a somatic variant of the cultivar Carignan. An abnormal development of reproductive organs was displayed by the unfused carpels (UFC) somatic variant of cv. Bouchalès, while a somatic variant of cv. Mourvèdre named carpel-less (CLS) developed abnormal ovules in the absence of carpels. Finally, three independent somatic variants in cvs Gamay, Morrastel, and Pinot displayed a phenotype of multiple perianth whorls (MPW). Gene expression studies showed that the expression profiles of *VvMADS-box* 1, 2, and 3 (putative orthologues of *Arabidopsis* flowering genes *AG*, *SEP*, and *AGL13*), were altered during grapevine flower development in the somatic variants, whereas the corresponding original cultivars displayed similar *VvMADS-box* gene expression profiles. Phenotypic and molecular characterization of these variants allowed the development of hypotheses on genetic functions that might be altered in most of the variants in light of the current ABCDE flower model.

Key words: Flower development, grapevine, MADS-box, reproductive organs, somatic variants.

Introduction

Genes involved in flower development have been extensively studied in the Angiosperms using *Arabidopsis* and *Antirrhinum* as genetic models, leading to the identification of genes and gene networks controlling these processes (Theißen, 2001; Ausín *et al*., 2005). Among them, the MADS-box gene family has been shown to play a central role in the determination of flower meristem and flower organ identity. The current genetic model explaining the specification of the flower organ identifies five genetic functions (A-B-C-D-E) mainly specified by MADS-box genes with specific roles in the initiation and development of flower organs in the different flower whorls, from sepals to carpels (Ferrario *et al*., 2004). While spectacular progress has been made in the understanding of the molecular regulation of flower development in model herbaceous plants, an increasing number of reports show differences in the regulation and the function of homeotic genes in other plant species (Ferrario *et al*., 2004).

We are interested in the regulation of reproductive development in grapevine (*Vitis vinifera* L.), a woody perennial vine with a pattern of organ formation and

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Abbreviations: CLS, carpel-less; DF, double-flowered; MPW, multiple perianth whorls; RB, ramose bunch; RRM, reiterated reproductive meristems; UFC, unfused carpels.

development distinct from those previously described for other plants (Mullins *et al*., 1992; Boss *et al*., 2003; Carmona *et al*., 2007). Grapevine flowers, with four whorls from sepals to carpels, belong to the regular flower type of Eudicot plants and have been well described at the anatomical and morphological level (Pratt, 1971; Hardie *et al*., 1996). However, the underlying molecular genetic mechanisms regulating flower development are poorly understood. Some putative orthologues of genes controlling flower initiation and development in *Arabidopsis* have been isolated and characterized (Boss *et al*., 2001, 2002; Carmona *et al*., 2002, 2007; Calonje *et al*., 2004; Boss *et al*., 2006; Sreekantan *et al*., 2006), and their expression analyses have suggested some functional differences in this species compared with model plants (Boss and Thomas, 2002; Calonje *et al*., 2004; Sreekantan *et al*., 2006). Still, expression of some of these genes in *Arabidopsis* transgenic plants displayed phenotypes similar to those produced by overexpression of the endogenous *Arabidopsis* genes (Boss *et al*., 2006; Carmona *et al*., 2007). However, their role in grapevine flower development remains unknown since genetic transformation is still a lengthy and tedious process in this species (Bouquet *et al*., 2003).

The use of natural genetic variation and induced artificial mutations can be very informative in establishing gene function in the absence of genetic transformation (Emmanuel and Levy, 2002; Jack, 2004; Koornneef *et al*., 2004). This genetic variation can be used in forward and reverse genetic approaches to support causal relationships between gene sequences and phenotypes. Natural genetic variation is starting to be exploited in the identification of genomic regions responsible for specific quality traits in grape (Cabezas *et al*., 2003; Doligez *et al*., 2006) and could also be used in both forward and reverse approaches. However, phenotypic variations for early development of reproductive organs have not been described in grapevine and although induced mutagenesis could be an alternative approach to generate phenotypic variation for this trait, mutagenesis studies are scarce in grapevine (Kuksova *et al*., 1997) and have not been reported to induce flowers with altered formation patterns. One interesting alternative to be considered in grapevine and other woody perennial species is somatic genetic variation, which can be maintained through vegetative propagation. In grapevine, somatic genetic variation has been successfully used so far in three cases to identify genes involved in gibberellic acid signalling (Boss and Thomas, 2002), anthocyanin production (Kobayashi *et al*., 2004; Walker *et al*., 2006), and berry early morphogenesis (Fernandez *et al*., 2006).

The goal in this work was to characterize grapevine somatic variants altered in flower development that could serve as genetic tools to identify the roles of genes involved in this developmental process. The phenotypic description of six somatic variants is presented here, as well as the corresponding RNA expression analysis of three MADS-box genes, previously reported to be related to the formation of the innermost floral whorls throughout flower development (Boss *et al*., 2001, 2002). The results allowed the development of working hypotheses on the role of genes that could be altered in each case.

Materials and methods

Plant material

The collection of grapevines at the INRA Domaine de Vassal (Marseillan, France, http://www.montpellier.inra.fr/vassal) was examined and found to include six accessions with developmentally aberrant flowers, with or without cluster architecture modifications. These were studied in comparison with the original variety grown under the same field conditions. Abnormal flowers were found in clonal mutants derived from the international cultivars Pinot and Gamay, and from the Southern Europe traditional cultivars Bouchalès, Carignan (known as Mazuelo in its country of origin Spain), Mourvèdre, and Morrastel (similarly known as Monastrel and Graciano, respectively). Early descriptions led to the introduction of these variants in the collection as double-flowered (DF) Gamay, Morrastel, and Pinot, ramose bunch (RB) Carignan, female Bouchalès, and female Mourvèdre. The genetic identity of cultivars and somatic variants was confirmed through genotyping the plants with 20 unlinked microsatellite loci; VVMD5, VVMD7 (Bowers *et al*., 1996), VVMD21, VVMD24, VVMD25, VVMD27, VVMD28, VVMD32 (Bowers *et al*., 1999), VVS2 (Thomas and Scott, 1993), VVIB01, VVIH54, VVIN16, VVIN73, VVIN73, VVIP31, VVIP60, VVIQ52, VVIV37, VVIV67 (Merdinoglu *et al*., 2005), VMC1b4 (unpublished), and VMC4f3 (Di Gaspero *et al*., 2000). PCR conditions were as described in Lacombe *et al*. (2003).

Microscopy analyses

Inflorescence samples were taken at anthesis from the original clones and simultaneously from the corresponding somatic variant of at least two of the five representatives established in the field. Alternatively, sampling used greenhouse-grown fertile cuttings according to Mullins *et al*. (1966). Samples were preserved in a 0.2 M phosphate buffer (pH 7.2), with 10% paraformaldehyde and 2.5% glutaraldehyde for a minimum of 48 h at 4 °C, and stored at the same temperature in 70% ethanol until further processing. For scanning electronic microscopy, after dehydration through a graded ethanol series, explants were critically-point dried with CO_2, sputtered with platinum, and observed with a JEOL JSM 6300F microscope.

Histological observations were performed on flowers embedded in Technovit Historesin® after dehydration as described above and following the manufacturer's specifications. For improved sample embedding, an extra step was introduced after ethanol dehydration using three successive (4, 24, and 24 h) 1-butanol baths before impregnation with resin. Semi-thin sections (3 μm) were stained with periodic acid–Schiff to reveal polysaccharide-rich structures and further stained with Naphthol Blue Black to reveal protein-containing bodies.

RNA extraction for gene expression studies

Sampling was done for several stages of inflorescence development defined as follows: stage 1, inflorescence primordium still enclosed within the bud; stage 2, inflorescence clearly separated from the vegetative axis; stage 3, first flower buds visible in the top of the

inflorescence; stage 4, initiation of new inflorescence primordia finished, all flowers well separated; stage 5, all flowers formed on fully expanded pedicels; stages 5+, cap drop starting. Flowers and inflorescences were randomly sampled for each clone from the five representatives cultivated in the field.

Total RNA was extracted from 1–2 g of inflorescence tissue as described by Tesnière and Vayda (1991). To get sufficient material, stage 1 and 2 samples were combined before extraction processing. A 100 μg aliquot of crude RNA was further purified using the Qiagen RNeasy cleanup mini-protocol including a DNase treatment, resulting in 10–30 μg of clean RNA. Quality and quantity were evaluated by both electrophoresis and spectrofluorometry.

Real-time PCR analysis

Real-time PCR analyses were performed as described in Fernandez *et al.* (2006). Using Primer3 software, specific primers (T_m=58–60 °C) were designed (Rozen and Skaletsky, 2000) to amplify 100–200 bp in the 3′ untranslated region of the *VvMADS1* (GenBank ID: AF265562), *VvMADS2* (GenBank ID: AF373601), *VvMADS3* (GenBank ID: AF373602), and ubiquitin extension protein *VvUBI* (GenBank ID: CF406001) genes (see Table 1). The expression levels of the MADS-box genes were determined for three technical replicates and corrected using the corresponding expression level of ubiquitin, *VvUBI*.

Results

Origin of the somatic variants

The somatic variants analysed were collected from a wide range of geographical origins and over a long period of time. DF Gamay was introduced from the Ravaz collection (France) in 1949, DF Pinot from the Champagne area in 1951, and DF Morrastel from a French grower in 1956. RB Carignan was introduced from Perpignan Research Station (France) in 1955. Female Bouchalès and female Mourvèdre were introduced more recently from SEPPIC Montauban (France) in 1973 and from the Magaratsh collection (Crimea) via the Oberlin Institute (Colmar, France) in 1979, respectively. The somatic variants, established as five plants for each accession (as were their putative original clones), were recorded as displaying stable phenotypes after repeated vegetative propagation and over successive reproductive cycles. The genetic identity of cultivars and somatic variants was confirmed by genotyping 20 independent microsatellite loci. All the somatic variants displayed genotypes identical to those of the plants from which they were derived (data not shown).

Grapevine flower development

The reproductive development of *V. vinifera* has been extensively described (Srinivasan and Mullins, 1981). While most of the *Vitis* species are dioecious, in almost all *V. vinifera* cultivars, the flower assumes a classical hermaphrodite floral organization with a whorl of five fused sepals followed by a five-petal whorl, five stamens, and the gynaecia being composed of an ovary with two carpels each containing two ovules (Fig. 1A–D).

Flower development in somatic variants

The vegetative development of the somatic variants was similar to that of the corresponding original plants in each case. No differences in inflorescence number or position were observed between the cultivars and the respective variants. Based on initial morphological examinations, the variants were classified in three major phenotypic groups: (i) reiteration of reproductive meristems (RRM; Carignan variant); (ii) reiteration of perianth whorls (Gamay, Morrastel, and Pinot variants), and (iii) abnormal development of the reproductive whorl (Bouchalès and Mourvèdre variants). Scanning electronic microscopy and histological observations further confirmed this preliminary phenotypic classification and led to the suggestion of specific denominations better suited to the actual flower defects extensively explained below. The Carignan variant was described by the name RRM and all the variants showing reiteration of perianth whorls were described as 'multiple perianth whorls' (MPW). Somatic variants displaying abnormal development of the reproductive whorls and initially known as female variants were more specifically described as 'unfused carpels' (UFC) for the Bouchalès and 'carpel-less' (CLS) for the Mourvèdre variants. Flower development within each one of these phenotypic groups is described below.

Reiterated reproductive meristems (RRM)—Carignan variant

This variant was characterized by an alteration of early inflorescence architecture. After the early stages of inflorescence development, the differentiation of flower meristems (stages 1–2) was impaired and primordia reiterated the development of inflorescence ramifications (Fig. 2A). Histological observations showed reiteration of adjacent bract-floral meristems in tandem, with at least two floral

Table 1. *Primers used for real-time PCR*

Target gene	Primer A sequence	Position relative to stop	Primer B sequence	Position relative to stop	Product length
VvMADS1	TGTGGGTCTCTTCGTGGAGT	+16	TGTGGCAGGCAACAGAGTTA	+200	184
VvMADS2	ATGCCCTTGTATGGTGATGA	+55	GAAAGCAAGTATCATAGGTTTCCA	+216	161
VvMADS3	ATCCAAGGGTGGGTTCTTTG	–18	TGTCAACACAATACACACATTACACA	+91	112
VvUBI	AGTAGATGACTGGATTGGAGGT	–2	GAGTATCAAAACAAAAGCATCG	+174	179

meristems being formed (Fig. 2B). These formations collapsed a few weeks after their initiation. This reiteration was probably the origin of the high number of inflorescence ramifications, hence the 'ramose bunch' (RB) description (Fig. 2C, E). This reiteration process caused a delay of ~30 d in the cluster development of the variant with respect to its corresponding cultivar (Fig. 2D). By the end of cluster development some flowers could reach full regular development, although it was frequently observed that the basic floral organization was altered with some reiteration of petals or formation of petaloid anther filaments.

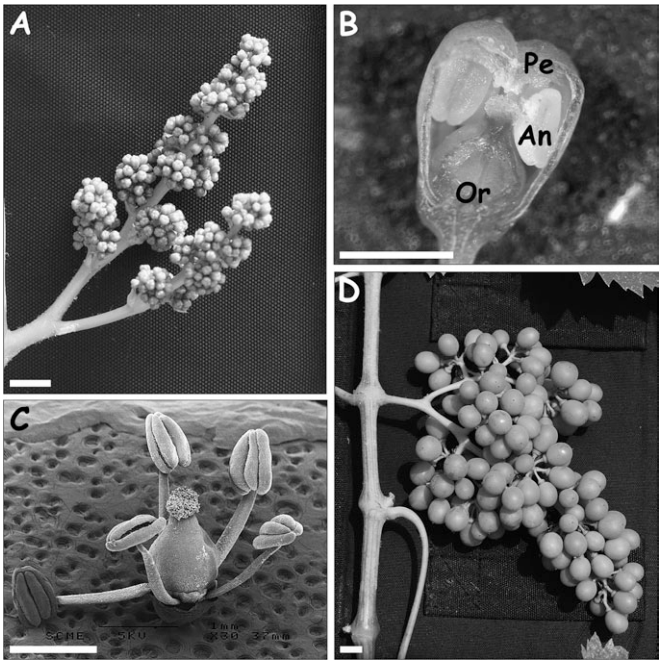


Fig. 1. Wild-type *V. vinifera* inflorescence, flower, and grapes. (A) Fully expanded inflorescence just before anthesis (bar=1 cm). (B) Side view of a flower bud (petals partially removed) just before anthesis displaying petal cap (Pe), folded anthers (An), and ovary (Or) (bar=1 mm). (C) Fully opened flower (bar=1 mm). (D) Berry cluster 6 weeks after flower set (bar=1 cm).

In general, most regular flowers eventually differentiated at the most distal inflorescence positions that corresponded to the ones developing later. Regular mature flowers were fertile and some berries developed normally up to the ripening stage, although with the delay accumulated in the previous developmental phases. At ripening, mutant vines were therefore observed to bear long, very large bunches with a high number of ramifications and berries (Fig. 2F).

Multiple perianth whorls (MPW)—Gamay, Morrastel, and Pinot variants

Inflorescence and flower primordia initiation in these variants followed the regular pattern observed in normal plants (data not shown). However, in these variants, after the flower primordia differentiated (stages 2–3), the organ primordia generated by the flower meristem reiteratively differentiated as whorl 1 (double, short sepals could sometimes be observed in Pinot) and especially whorl 2 organs. Furthermore, the number of whorls produced was not limited to four, as the flower meristem continued to generate additional whorls of perianth organs (Fig. 3A, E, I, H). As a result of this altered developmental pattern,

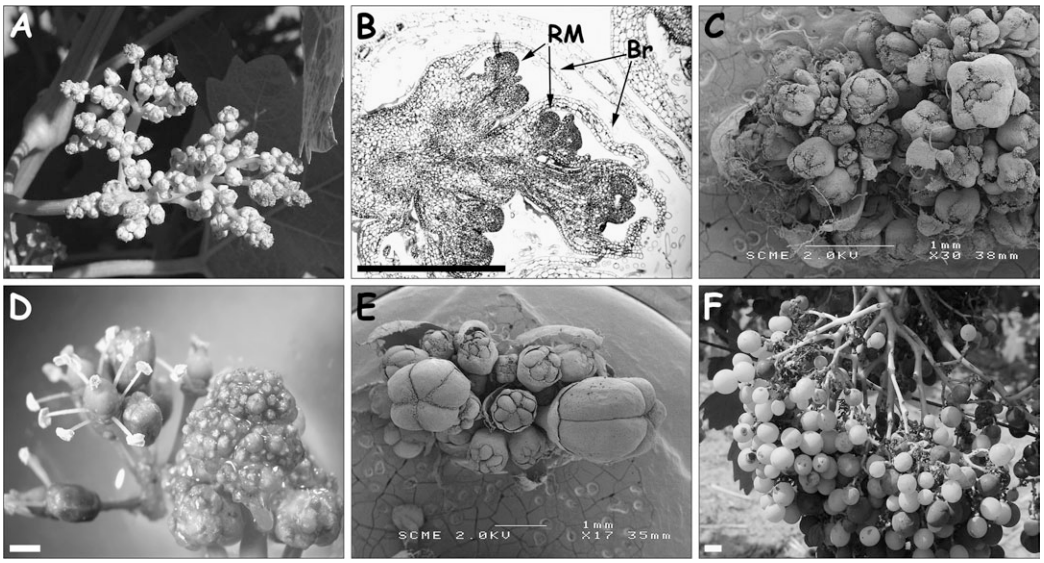


Fig. 2. Phenotype of the RRM Carignan somatic variant. (A) General view of the inflorescence (bar=1 cm). (B) Longitudinal section in a terminal cluster with supernumerary bracts (Br) and reiterated floral meristems (RM) (bar=2 mm). (C) SEM view of a cluster with multiple buds (bar=1 mm). (D) A comparison of wild-type (left) and RRM variant (right) flower clusters (bar=2 mm). (E) Part of an inflorescence displaying regular flowers eventually developing in the RRM variant (bar=1 mm). (F) Variant 'ramose bunch' phenotype at véraison (bar=1 cm).

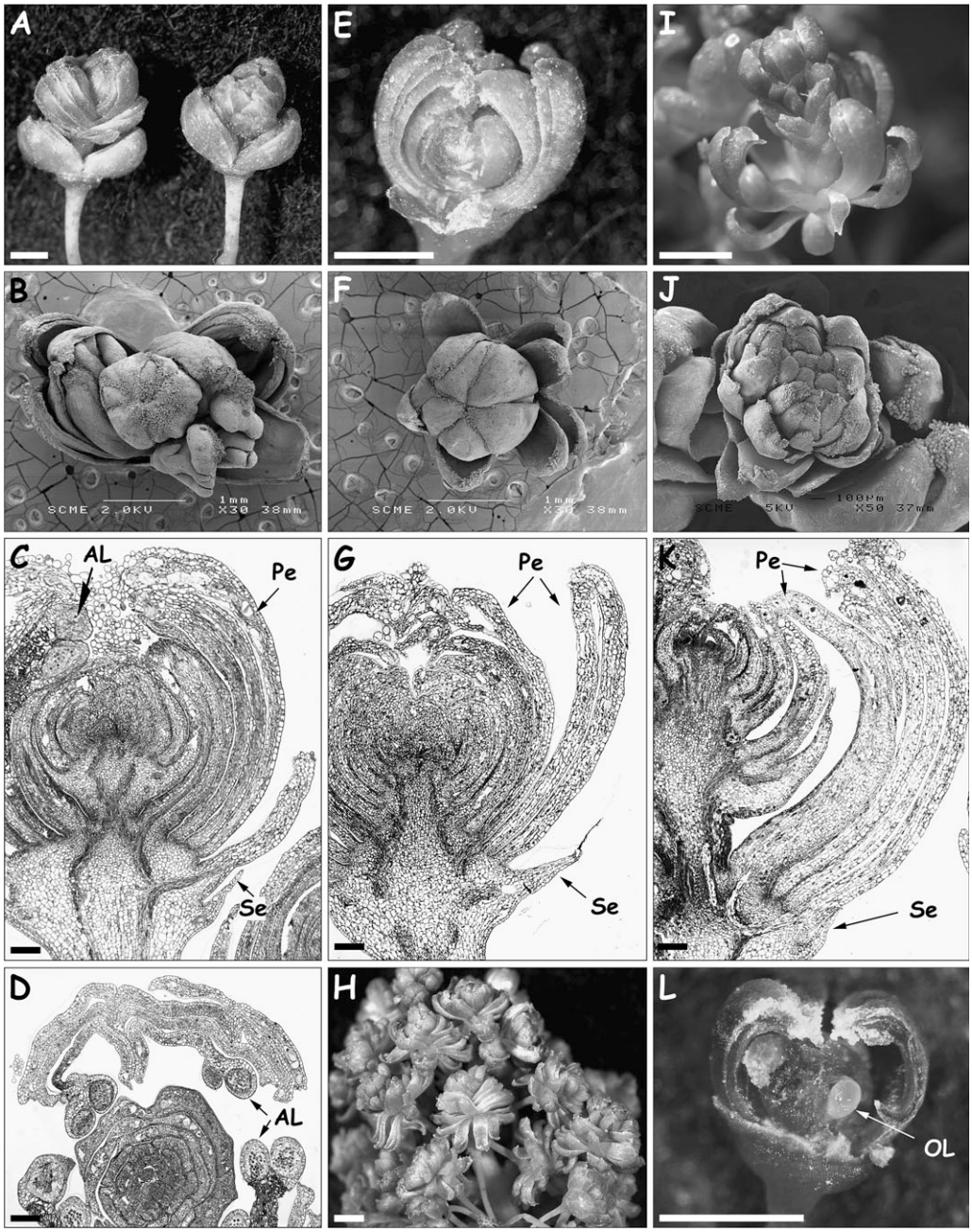


Fig. 3. Flower phenotypes of MPW Pinot (A–D), Morrastel (E–H), and Gamay (I–L) somatic variants. (A, E, I) Close-up of flowers showing multiple petals with occasional flower reiterations (A, left flower; I) (bar=1 mm). (B, F, J) SEM illustrations showing multiple petals in all three variants. Note the presence of a reiterated, central flower in Pinot (B). (C, G, K) Longitudinal sections of isolated flowers showing anther-like structures (AL), sepals (Se) and multiple petals (Pe) (bar=0.1 mm). (D) Cross-section in a variant Pinot flower showing an abnormally positioned anthers-like structures (AL) (bar=0.1 mm). (H) Exacerbated petal formation in Morrastel flowers (bar=1 mm). (L) Isolated ovule-like (OL) structure in MPW Gamay (bar=1 mm).

these somatic variants generated flower structures consisting of multiple reiterations of sepal and petal whorls lacking reproductive organ whorls (Fig. 3B and C, F and G, J and K). In addition to these common features, anther-like structures were occasionally found fused to the upper part of inner petals of MPW Pinot (Fig. 3C, D) and some ovule-like formations appeared in the innermost organs of MPW Gamay (Fig. 3L). Moreover, the MPW Gamay inflorescence exhibited additional specific features such as lack of secondary rachis and very short main rachis and pedicels. Sometimes, MPW Gamay flowers displayed the formation of a secondary pedicel separating reiterated flowers in the position of whorls 3 and 4 (Fig. 3K). Somatic variants reached their most advanced

inflorescence and flower development at the anthesis time of the corresponding wild-type plants. After this stage, somatic variant inflorescences necrosed within a few weeks without berry formation.

Abnormal reproductive whorl development variants

UFC Bouchalès: Inflorescence and flower primordia developed normally in this mutant. However, later flower development was altered as some flowers displayed a double sepal or petal whorl (Fig. 4C), while all of them showed anomalies in the development of stamens and carpels. Stamens were normal in number and position but filaments were shorter than in the wild type or sometimes absent with anthers fused to ovaries. The abnormal anther structure did not allow a normal closure of the petal cap (calyptra). Furthermore, ovary development was also compromised and most ovaries exhibited only partially fused carpels (Fig. 4C). The most conspicuous phenotypic modification in this variant was premature flower opening (Fig. 4A, B) as a result of abnormal stamen and carpel development. The premature opening of flowers prevented complete development and decreased flower set. Still, after open pollination, some ovaries were able to develop into normal, seeded berries but, at ripening, bunches were mainly composed of small seedless berries as sometimes observed for female grapevine genotypes with stamens or pollen not fully functional (Fig. 4D).

CLS Mourvèdre: This somatic variant displayed larger flower buds than its wild-type plant counterpart (Fig. 5A). Microscopic analysis did not reveal alterations in the initiation of inflorescence and flower primordia, which initiated in regular positions and developed normally in the first and second whorls. However, primordia in whorls 3 and 4 did not differentiate regular reproductive organs but only carpelloid structures (Fig. 5B, D). Regular carpels with normal ovaries could not be observed, but ovule-like structures were often found fused to carpelloid structures in the fourth central whorl. These ovule-like structures displayed some normal ovule features such as recognizable integuments (Fig. 5C). Anthers were absent or displayed a completely abnormal shape, although microspores could be observed (Fig. 5E, F). Developed flowers were not functional and necrosed a few weeks after full development of the inflorescence. This variant was completely sterile and did not develop fruit, even under open pollination, indicating that the carpelloid structures were not functional.

A synoptic view of the main morphological alterations observed in the six somatic variants described herein is presented in Fig. 6.

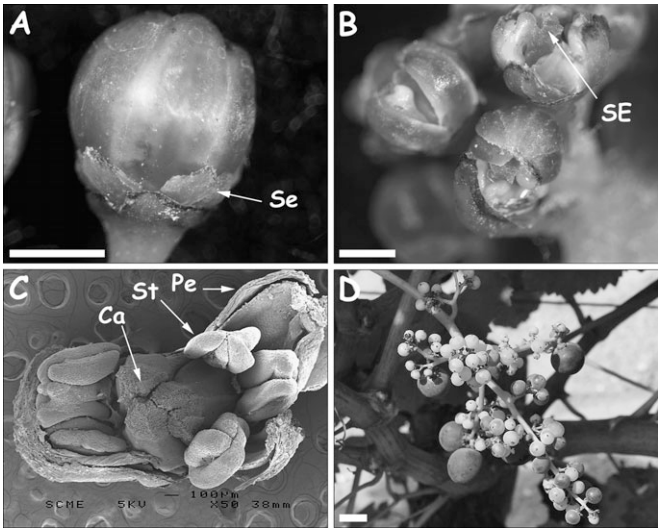


Fig. 4. Phenotype of a UFC Bouchalès somatic variant. (A) Unfused sepals (Se) on an oversized bud (bar=1 mm). (B) Premature petal opening exposing the stylar extremity (SE) (bar=1 mm). (C) SEM view of an opened bud displaying a double petal whorl (Pe), abnormal stamens (St), and unfused carpels (Ca). (D) Bunch displaying mainly undeveloped berries (bar=1 cm).

Expression of VvMADS- box genes during flower development of somatic variants

VvMADS1, *VvMADS2*, and *VvMADS3* were initially identified as the putative orthologues of *AG*/*SHP*, *SEP1*/*2*, and *AGL13*, respectively (Boss *et al*., 2001, 2002). In *Arabidopsis*, *SEPALLATA* genes are involved in the specification of petal, stamen, and carpel identity (Pelaz *et al*., 2000), *AGAMOUS* is required to establish the identity of reproductive organs (Drews *et al*., 1991), while *SHATTERPROOF* seems to be more involved in carpel development (Savidge *et al*., 1995). Finally, the role of *AGL13* is not yet clear, although based on its expression pattern it could be involved in ovule formation. Expression of the corresponding grapevine orthologous genes was analysed by real-time PCR in the somatic variants to obtain information on the molecular events associated with the described flower developmental phenotypes. The results are shown in Fig. 7.

The correspondence between inflorescence development stages used for RNA extraction sampling and floral organ development was: stage 1, formation of first flower meristems and initiation of outermost whorl primordia; stage 2, more advanced flower primordia initiating sepals; stage 3, most flowers developing petals and stamens, and initiating ovary development; stage 4, sepals fully developed in most flowers, petals and stamens finishing development, and ovaries developing; stage 5, petals and stamens formed and ovary development completing in most flowers; stages 5+, most flowers with dehiscing anthers. Stages 1 and 2 were analysed together as the corresponding inflorescence samples were combined.

Expression profiles and levels of *VvMADS1*, *VvMADS2*, and *VvMADS3* were very similar in the six original cultivars. *VvMADS1* and *VvMADS2* showed a clear increase

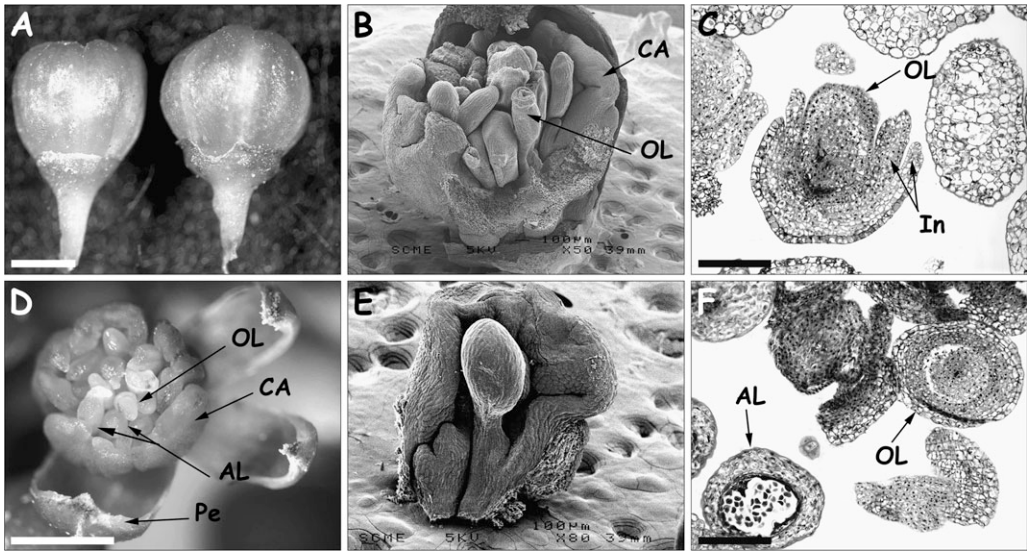


Fig. 5. Phenotype of a CLS Mourvèdre somatic variant. (A) Variant bud (right) with size increased compared with the wild-type bud (left) (bar=1 mm). (B) SEM view of a flower (most petals removed) displaying a carpelloid anther and ovule-like structures. (C, F) Variant flower cross-sections displaying a nude ovule-like structure with integuments (In) and anther-like structures with microspores (bar=0.1 mm). (D) Mature flower (petals moved aside) to reveal carpelloid anthers, anther-like and ovule-like structures (bar=1 mm). (E) An isolated carpelloid anther with attached ovule-like structure. Carpelloid anthers (CA), anther-like (AL), and ovule-like (OL) structures, petals (Pe).

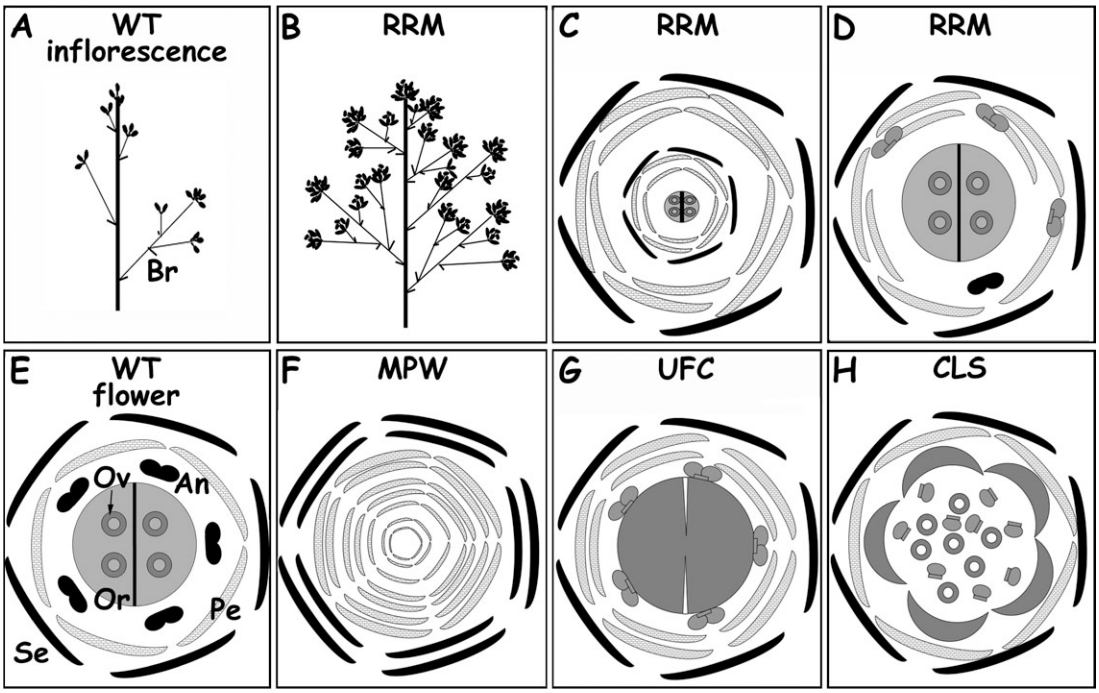
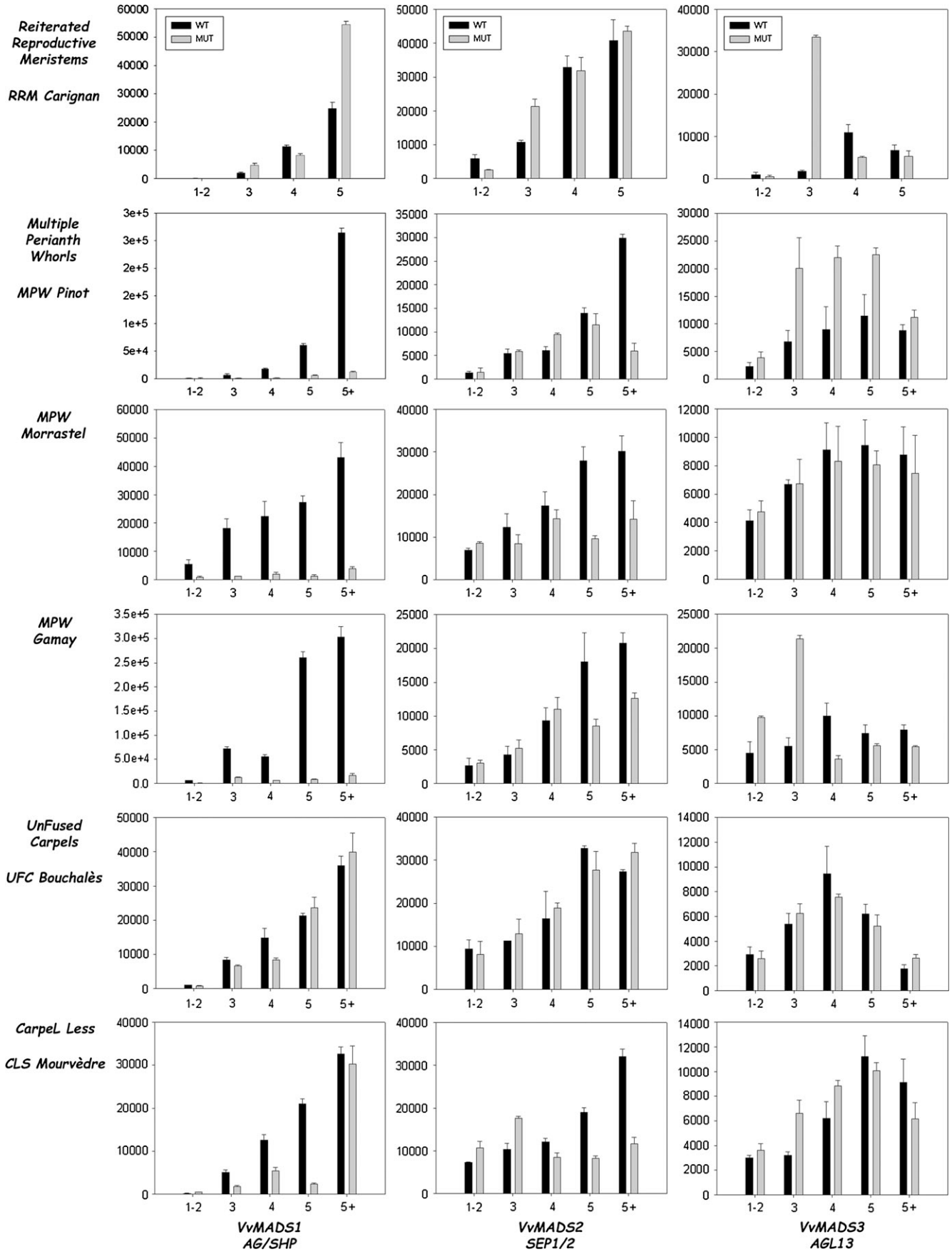


Fig. 6. Summary of the wild-type (WT) and somatic variant reproductive organ schemes. (A) WT inflorescence with bracts (Br), terminal and secondary flowers; and (E) WT floral diagram showing the position of sepals (Se), petals (Pe), anthers (An), ovary (Or), and ovules (Ov). (B) Inflorescence type of RRM Carignan variant. (C, D) Floral diagram of RRM Carignan. (F) Floral diagram of MPW variant type (Pinot, Morrastel, and Gamay). (G) Floral diagram of UFC Bouchalès variant. (H) Floral diagram of CLS Mourvèdre variant.

in their expression from inflorescence initiation to the mature flower stage (Fig. 7). On the other hand, the expression profile of *VvMADS3* showed a maximum at developmental stages 4–5 (Fig. 7).

The RRM somatic variant of Carignan was characterized by an overexpression of all *VvMADS-box* genes at stage 3 of flower development as well as at stage 5 for *VvMADS1*. All the MPW variants, independently of their


Reiterated Reproductive Meristems
RRM Carignan
Multiple Perianth Whorls
MPW Pinot
MPW Morrastel
MPW Gamay
UnFused Carpels
UFC Bouchalès
CarpeL Less
CLS Mourvèdre
WT
MUT
1-2
3
4
5
5+
VvMADS1 AG/SHP
VvMADS2 SEP1/2
VvMADS3 AGL13

origin, were characterized by a strong reduction in *VvMADS1* expression throughout flower development. They also shared coincident expression of *VvMADS2*, which was expressed at levels similar to the corresponding original cultivars during the initial stages of flower development but showed a significant reduction during late stages (4–5). Finally, these three different somatic variants displayed slightly different phenotypes regarding the expression of *VvMADS3*. Expression of this gene did not seem affected in the MPW Morrastel somatic variant, with respect to its wild-type cultivar. However, *VvMADS3* was overexpressed in the initial flower developmental stages of MPW Gamay and throughout flower development in the MPW Pinot variant (Fig. 7).

Expression patterns of *VvMADS-box* genes in the two somatic variants altered in the development of reproductive organs, i.e. CLS Mourvèdre and UFC Bouchalès, differed significantly, as did the morphology of their flowers. CLS Mourvèdre displayed reduced expression of *VvMADS1* expression during early flower developmental stages. In contrast to this *VvMADS1* expression profile, *VvMADS2* and *VvMADS3* appeared to be overexpressed in earlier stages (1–2 and 3 for *VvMADS2* and 3–4 for *VvMADS3*). In the case of *VvMADS2*, there was a strong reduction in its expression in the later stages of 5 and 5+. For UFC Bouchalès, the expression of all three *VvMADS-box* genes was found to be very close to that of the corresponding wild type.

Discussion

It is significant that out of the 200 variants contained in the 4500 *V. vinifera* accessions of the Vassal repository, the largest grapevine collection of Vitaceae germplasm, only six variants displayed altered flower morphology (This *et al*., 2006). This could indicate that little attention so far has been paid to flower development and/or that such perturbations are rare events in *Vitis*. Indeed, while many reports describe somatic variation for leaf morphology (Durquety and Houbart, 1982), berry form, or colour (Pires *et al*., 2003; Kobayashi *et al*., 2004; Fernandez *et al*., 2006), few authors have reported *Vitis* variants with abnormal flower morphology. Previous reports have mentioned the existence of multiple whorled flowers (Oprea, 1965; Branas, 1974), but lacked detailed descriptions. Caporali *et al*. (2003) described flowers with abnormal development of anthers in wild, hermaphrodite *V. vinifera* Additionally, the Mourvedre and Bouchalès variants have been only briefly reported recently (Sreekantan *et al*., 2006).

The genetic basis of somatic variants

The utility of somatic grapevine variants in the study of gene function depends largely on the understanding of the mechanisms involved in the generation of somatic variation. In the highly probable case that the described phenotypes are the result of somatic mutations, several considerations are required when interpreting the variant phenotypes. First, the somatic mutation takes place in a single cell belonging to a specific cell layer (L1 and L2 layers are distinct in grapevine shoot apical meristems; see Thompson and Olmo, 1963; Franks *et al*., 2002; Fernandez *et al*., 2006). For the mutant phenotype to be observed, the mutant cell has to 'colonize' the corresponding cell layer in at least one shoot apical meristem and derived organs. However, unless the mutant cell layer colonizes the other cell layer and this situation is again stabilized, the somatic variant will be a chimera. This in fact has been the case in most grapevine somatic variants described previously (Boss and Thomas, 2002; Franks *et al*., 2002; Ageorges *et al*., 2006; Fernandez *et al*., 2006). This situation means that the observed phenotype results from chimerism and cannot be directly associated with the phenotype of solid mutant plants. Secondly, somatic mutations will only take place in one copy of the target gene. Unless the mutation takes place in the functional allele of a heterozygous locus, an unlikely situation, the resulting phenotypes are more likely to be due to the occurrence of gain-of-function mutations causing an observable change. This has been the case in the two somatic variants that have so far been characterized at the molecular level in grapevine [*GAI1* by Boss and Thomas (2002) and the berry colour *MYBA1* gene by Kobayashi *et al*. (2004)]. Thus, it is highly probable that the phenotypes observed in the described variants are the result of gain-of-function mutations in a chimeric state, something that should be considered when attempting to understand the phenotypes under current genetic models of flower development. Thirdly, it is not known which molecular mechanisms are responsible for the generation of these mutant phenotypes and so far the available information is scarce. At least for berry colour, retrotransposable elements through homologous long terminal repeat (LTR) recombination or illegitimate recombination are responsible for a large proportion of the somatic variation observed for the trait (Lijavetzky *et al*., 2006). If these mechanisms were responsible for some of the flower phenotypes described,

Fig. 7. *VvMADS1–3* real-time PCR expression profiles during inflorescence and floral development of six somatic variants versus their respective wild-type counterpart. The *x*-axis shows reproductive organ development with stages 1–2 (young inflorescence a few days after bud burst) to stages 5+ (flowers opening). The *y*-axis shows arbitrary expression units for each variant/wild-type pair. SE bars correspond to the variation for technical triplicates.

one would expect some phenotypes to appear repeatedly in specific cultivars as well as in genetically related cultivars. The MPW phenotype, found in Pinot and Gamay, a derived hybrid variety, would be in agreement with this possibility. Slight phenotypic differences between both variants could be attributed to different genetic backgrounds. The similarity of phenotypes observed even in non-closely related cultivars such as Morrastel and Pinot could also be explained by the presence of those mutable loci in the genome.

Mutant phenotypes and altered gene functions in grapevine flower development

The somatic variants presented here are affected in the specification of identity, of either flower meristems or organ primordia. However, no earlier events regulating flower initiation appear to be affected. The RRM variant exhibited the earliest phenotypic alteration, apparently causing a delay in flower meristem specification. This alteration resulted in clusters with exacerbated ramifications and delayed development. In *Arabidopsis*, a similar phenotype is caused by lack-of-function mutations in genes required for flower meristem specification such as *AP1* (Bowman *et al*., 1993), *LFY* (Parcy *et al*., 2002), *FUL* (Ferrandiz *et al*., 2000), or more extremely in the double mutant *ap1 cal* (Kempin *et al*., 1995). Similar to what was observed in the Carignan variant, in the *Arabidopsis* mutants the phenotype of the basal flowers is more strongly affected than that of the uppermost flowers that are almost normal and fertile. In fact, even strong cauliflower (*ap1 cal*) phenotypes are eventually able to elongate their inflorescence and produce regular, fertile flowers (Kempin *et al*., 1995). This suggests that both in the *Arabidopsis* mutants and in the Carignan variant the genes required for floral organ formation are fully functional. The RRM phenotype could also be explained as a result of overexpression or ectopic expression of *TFL*-like genes in the inflorescence and flower meristems, as has been shown in transgenic *Arabidopsis* using either *Arabidopsis* TFL genes or genes from other species, including grapevine (Boss *et al*., 2006).

Grapevine MPW variants are able to develop perianth organs requiring A and B functions, but are impaired in the specification of anther and carpel identity. This phenotype is consistent with a loss-of-function mutation in class C genes such as *AGAMOUS* (Coen and Meyerowitz, 1991) which is also required to prevent reiteration of whorl formation (Yanofsky *et al*., 1990). In agreement with this possibility, the expression of the *VvMADS1* gene, a putative *AG*/*SHP* homologue (Boss *et al*., 2001), was strongly reduced in the three variants during all stages of flower development.

The CLS Mourvèdre variant did not exhibit clear homeotic transformations of the ovary into other floral organs as commonly observed in *Arabidopsis*. Instead, this mutant developed normal sepals and petals, but exhibited alterations in the innermost whorls with the development of carpelloid structures. These whorls displayed ovules together with abnormal, stamen-like tissues. The fact that alterations were restricted to the two inner whorls suggests malfunction in class C or downstream genes. Interestingly, *VvMADS1* was found to be repressed in this mutant during early flower development, but later expression was found to be similar to the wild type, and ovule-like structures could develop.

The UFC Bouchalès displayed less severe flower morphology defects and similar expression patterns for *VvMADS1*, *2*, and *3* compared with the wild type. Sreekantan *et al.* (2006) recently showed a delayed overexpression of *VvMADS9* (*PI* homologue) in the UFC variant compared with the wild type. Interestingly, a *PI* temporal deregulation was also shown to be associated with some alterations of ovary shape in the *fleshless* grapevine mutant (Fernandez *et al*., 2006).

The grapevine genome sequence was recently completed (Jaillon *et al*., 2007), facilitating the identification and cloning of candidate gene sequences. Grapevine somatic variants exhibiting unusual patterns of flower or berry development represent unique material to enable the investigation of the regulation of reproductive development in grapevine (Fernandez *et al*., 2006, 2007). However, because of the origin of these phenotypes, the dissociation of L1 and L2 cell layers, through either sexual propagation (as gametes arise from the L2 cell layer) in the fertile mutants or somatic embryogenesis (Franks *et al*., 2002), will be required for subsequent genetic analysis. This report is the first comprehensive description of grapevine somatic variants showing altered early development of reproductive organs. Further investigations, based on our hypotheses, should eventually result in the identification of the gene(s) responsible for the mutant phenotypes and lead to a better understanding of their function in grapevine flower development.

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