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Molecular genetics of berry colour variation in table grape

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Abstract The genetics and biochemistry of anthocyanins and flavonol biosynthesis and their role in plant organ pigmentation is well established in model species. However, the genetic basis of colour variation is species specific and understanding this variation is very relevant in many fruit and flower crop species. Among grape cultivars, there is a wide genetic variation for berry colour ranging from yellow-green ("white" cultivars) to dark blue berries. Berry colour results from the synthesis and accumulation of anthocyanins in the berry skin, which in plants is commonly regulated by transcription factors belonging to the MYB and bHLH families. In this work, we aimed to identify the major genetic determinants of berry colour variation in a large collection of table grape cultivars and somatic variants. The genetic analyses of berry colour in a few grape segregating progenies had previously identified a single locus on linkage group 2 responsible for colour variation. Furthermore, somatic variation for berry skin colour in cultivar Italia had been associated with the presence of a *Gret1* retrotransposon in the promoter region of *VvmybA1*, a *Myb* gene whose expression is associated to skin colouration. The results show that *VvmybA1* is the gene underlying the mapped locus controlling berry colour in grape. Additionally, the molecular analyses indicate that genetic and somatic berry colour variation can be associated to molecular variation at *VvmybA1* in more than 95% of the analyzed cultivars. Thus, *VvmybA1* is a major determinant of berry colour variation in table grape and its instability is the major cause of somatic variation for this trait.

Keywords Berry colour · MYB genes · Grapevine · Somatic variation · Colour variation

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Introduction

Anthocyanin pigments are responsible for a large range of colours displayed by different plant organs such as leaves, flowers, fruits and seeds. Colour has been shown to play a role in the attraction of vertebrate and invertebrate animals required for pollination and seed dispersal (Gautier-Hion et al. 1985; Kevan and Baker 1983). The genetics and biochemistry of the anthocyanin biosynthetic pathway, a major branch of

flavonoid metabolism, has been well characterized in maize, *Petunia*, snapdragon, and more recently in *Arabidopsis* (Holton and Cornish 1995; Winkel-Shirley 2001). Main regulatory genes associated with the control of anthocyanin biosynthesis in plants have also been identified as belonging to two major groups of transcription factors, the MYB and bHLH families (Borovsky et al. 2004; Holton and Cornish 1995; Kobayashi et al. 2002; Ozeki et al. 2003; Ramsay et al. 2003; Robbins et al. 2003; Sainz et al. 1997; Schwinn et al. 2006; Spelt et al. 2000). The genetic basis of colour variation is species spcific and different genes in the pathway have been found responsible for colour variation in different species (Arakawa et al. 1999; Awad et al. 2000; Ben-Yehudah et al. 2005; Jaakola et al. 2002; James and Howard 1989; van Dyk et al. 2005). In this work we aimed to identify the major determinants for colour variation in table grape cultivars.

Berry colour in grapes (*Vitis vinifera* L.) displays a wide range of variation between yellow-green ("white" cultivars) and dark blue. Colour is used in some cases to differentiate cultivars being a relevant trait influencing both table grape and wine qualities. Pigmentation of red/blue berry grapevine cultivars results from vacuolar accumulation of anthocyanins in berry skin cells. Analysis of berry colour segregation in a few grape mapping populations have identified a single locus as responsible for the presence or absence of skin colour (Doligez et al. 2002; Fisher et al. 2004) on the currently known linkage group 2 (LG2) (Adam-Blondon et al. 2004). Furthermore, molecular analyses of structural genes involved in the anthocyanin biosynthetic pathway showed that all structural genes tested, except the UDP-glucose:flavonoid 3-O-glucosyltransferase (*UFGT*) gene, were expressed in most berry tissues; while expression of *UFGT* was only detected in the berry skin and was always associated to anthocyanins accumulation (Boss et al. 1996a, b; Kobayashi et al. 2001). Thus, regulation of *UFGT*, encoding one of the latest steps of the pathway, was identified as a key point in the control of berry colour although no differences were observed in either coding or promoter sequences of this gene between coloured and white cultivars (Kobayashi et al. 2002). Somatic variation for berry skin colour in cultivar Italia was later associated with the presence of the *Gret1* retrotransposon in the promoter region of *VvMybA1* (Kobayashi et al. 2004), a *Myb* gene whose expression is associated to skin colouration and which seems to be required to positively regulate *UFGT* expression (Kobayashi et al. 2002). These authors genotyped 22 grape cultivars of different origins and showed the presence of homozygous *Gret1* insertions in the promoter region of the *VvmybA1* gene in the 12 white berries bearing cultivars. The same insertion allele was also observed in the hemizygous state in the 10 coloured cultivars analyzed (Kobayashi et al. 2004). These results suggested that *VvmybA1* could be a major determinant of berry colour variation in grapes.

To analyze the role of the *VvMybA1* gene in berry colour variation in table grape we performed a cosegregation analysis of berry colour and *VvMybA1* genotypes in a selfcross progeny derived from the red table grape cultivar Ruby Seedless. Moreover, we genotyped this locus in a large collection of table grape cultivars to ascertain the existent allelic variation for this gene and its association with berry colour. Finally, we analyzed independent events of colour somatic variation in several cultivars to understand the molecular basis of this variation. The results show that *VvMybA1* is likely the gene underlying the mapped locus controlling berry colour in grapes and that genetic and somatic colour variation analyzed is associated to molecular variation at *VvMybA1* in over 95% of the analyzed cultivars.

Materials and methods

Plant materials and colour analyses

The molecular basis of colour variation was analyzed in a collection of 189 grape cultivars used for table grape production or with double usage, table and wine, which are mostly maintained at the germplasm collection of "El Encín" (IMIDRA, Alcalá de Henares, Madrid, Spain). Their name and collection code are described in Table S1 of the Supplementary Materials. The molecular basis of colour somatic variation was analyzed in a subgroup of 23 table grape cultivars corresponding to 9 original genotypes and 14 derived somatic variants. Most of them were also from the germplasm collection of "El Encín". Their names and origin are also listed in Table S1. The genetic identity between the original cultivars and their derived somatic variants was verified by genotyping 6 microsatellite loci: VVMD5, VVMD7, VVMD27, VrZAG62, VrZAG79 and VVS2 (This et al. 2004). Cosegregation between the colour phenotype and *VvmybA1* was analyzed in 19 plants derived from selfcross of the coloured cultivar Ruby Seedless. This progeny was generated and maintained at the IMIDA (Murcia, Spain). In this progeny, colour was quantified using a colourimetric value (CIRG) derived from the colour evaluation. $CIRG = (180\text{-}H)L + C$, where H = hue angle, L = lightness and C = chroma (Carreño et al. 1995). This procedure assigns the following mean

threshold values for the different berry colours: yellow-green = 1.55; pink = 2.49; red = 3.66; violet = 4.75; dark violet = 5.57. Statistical analyses were performed using the STATISTICA v7.0 software package (StatSoft).

DNA extraction and analysis

Total genomic DNA was isolated from young leaves using the DNeasy Plant Mini Kit (Qiagen). The *VvmybA1* genotypes were first scored using a PCR assay originally based on the amplification of the 5′-flanking region and coding sequences of the insertion allele of *VvmybA1* and its functional reversion allele, named as *VvmybA1a* and *VvmybA1b* by (Kobayashi et al. 2004), with minor modifications. For simplicity in the use of the allele names, and given the number of natural alleles that can be found for *VvmybA1*, we will refer to them, from here on, as the gene followed by a three letter code representing the name of the cultivar were the allele was first described. In this way the two mentioned alleles will be named as $VvmybA1^{ITA}$ (for cultivar Italia insertion allele) and $VvmybA1^{RUO}$ (for cultivar Ruby Okuyama reversion allele). Primers used for $VvmybA1^{ITA}$ were *a* (5′-AAAAAGGGGGGCA ATGTAGGGACCC-3′) and *d3* (5′-CCTGCAGCTT TTTCGGCATCT-3′), and those used for $VvmybA1^{RUO}$ were *b* (5′-GGACGTTAAAAAATGGTTGCACGT G-3′) and *d3* (Fig. 4b). Amplification of the 5′-LTRs and 3′-LTR from Italia and Sugraone $VvmybA1^{ITA}$ alleles were performed with primers *b* and *e1* (5′-GTCTTTCGCTTGCCAACTGT-3′) and *a* and *d3*, respectively, (Fig. 4b). The solo-LTRs present in somatic variants Ruby Okuyama, Ralli Seedless and Super Red were amplified with primers *b* and *d3*. The $VvmybA1^{AFL}$, $VvmybA1^{SUB}$ and $VvmybA1^{ROD}$ alleles were PCR amplified using primers b and cDNAs (5′-TCAGATCAAGTGATTTACTTGTGT G-3′) (Fig. 4b), with the exception of $VvmybA1^{ROD}$ which required a 3′ RACE approach for the 3′ end characterization, as described below. PCR amplifications were performed with an initial denaturing step of 95°C for 5 min followed by 35 cycles of 94, 65 and 72°C for 1 min each and a final extension step of 72°C for 10 min. Amplification products were separated by electrophoresis in 1% agarose gels. Cloning of PCR products was carried out with the pGEM-T easy vector (Promega) and sequenced at the Genomic Unit of the Parque Científico de Madrid. Sequencing analysis and alignments were performed with BIOEDIT v7.0.5.3 (Hall 1999). Additional primers used in the somatic variants analysis were: *3s* (5′-TCGAAAATCAGTGA GGGTAACAAAG-3′), *4as* (5′-CGACACGTTTCT TATGGTGCATT-3′) and *c* (5′-GAACCTCCTTTT TGAAGTGGTGACT-3′). SSR analyses were performed according to previously described conditions (Cabezas et al. 2003).

DNA blot hybridization analyses were performed according to Sambrook et al. (1989). Briefly, genomic DNA (10 μg) was digested with the restriction enzyme *Apa* I and the digestion products were separated by electrophoresis in 0.7% agarose gels, transferred and immobilized into Hybond-N + (Amersham Bioscience) nylon membranes and hybridized with a ^{32}P labeled probe. The *VvmybA1* probe was generated by PCR amplification with primers *Ps* (5′-TCACGGGG TTTAGAAAGTGG-3′) and *Pas* (5′-ATCAATTGG GGAATTGGTGA-3′) using Muscat Hamburg genomic DNA as template. The hybridized membranes were scanned with a STORM PhosphorImager (Molecular Dynamics).

RT-PCR and 3′-RACE

Total RNA extractions from berry skin tissues were performed according to Zeng and Yang (2002) with a final purification step using the RNeasy Plant Mini Kit (Qiagen). RT-PCR assays were carried out using the conditions and primers described in Kobayashi et al. (2004). Isolation of 3′-end of Roditis cDNA was performed using the BD SMART RACE cDNA Amplification Kit (BD Bioscience, Clontech).

Results and discussion

VvmybA1 co-localizes with the locus responsible for berry colour in grape segregating progenies

The genetic determinism of grapevine berry colour has been analyzed in two F_1 progenies ([Dattier de Beyrouth × 75 Pirovano] × [Alphonse Lavallée × Sultanine] and [Regent × Lemberger]) segregating for this trait (Doligez et al. 2002; Fisher et al. 2004). In both cases the presence or absence of colour segregated as a monogenic trait determined by a locus on LG2. Independently, Kobayashi et al. (2004) showed that insertion of retrotransposon *Gret1* upstream of the *VvmybA1* gene was responsible for the lack of colour in berries of cultivar Italia. The results suggested that recombination between *Gret1* LTRs and lost of the retrotransposon in a *VvmybA1* allele was responsible for berry colouration in the Italia somatic variant known as Ruby Okuyama. Moreover, homozygosity for the insertion of this retrotransposon upstream of *VvmybA1* was shown to be associated with the lack of berry colour in 12 additional white berry cultivars

(Kobayashi et al. 2004). Thus, it is possible that *VvmybA1* could be the gene underlying the previously mapped colour locus. To confirm this hypothesis, we analyzed the segregation of both, the *VvmybA1* gene and the colour trait in an F_1 progeny derived from the selfcross of the red cultivar Ruby Seedless. The progeny was selected because Ruby Seedless is hemizygous for the presence of the retrotransposon insertion ($VvmybA1^{ITA}/VvmybA1^{ALF} - ALF$ = allele found in Alphonse Lavallé) and displayed berry colour segregation. Nineteen F_1 plants were phenotyped for berry colour and their CIRG values were scored as described in Materials and methods. The same plants were also genotyped for the presence of *Gret1* in the *VvmybA1* locus (Fig. 1a) and for microsatellite loci VMC6B11, VMC5G7, VMC7G3 and VMC8C2 linked to the colour locus on LG2 and found heterozygous in Ruby Seedless. Colour segregated as a monogenic dominant trait ($\chi^2 = 1.9$, df = 1; n/s). Linkage analysis indicated a close linkage between the *VvmybA1* sequence and SSR markers on LG2 [recombination frequency (r), $r = 0.026 \pm 0.025$, $r = 0.026 \pm 0.025$, $r = 0.052 \pm 0.036$ and $r = 0.078 \pm 0.043$], respectively, for VMC5G7, VMC6B11, VMC7G3 and VMC8C2 as previously described for the colour locus. There was a strong association between berry colouration and the presence of the *ALF* allele at the *VvmybA1* gene ($P < 0.00001$). All plants bearing white-skinned berries presented CIGR values bellow 1.55 and were homozygous for the *Gret1* insertion allele $VvmybA1^{ITA}$, while all plants yielding coloured berries carried one or two copies of the functional $VvmybA1^{ALF}$ allele and presented CIRG values above 2.49. The ANOVA analysis of average CIRG values for the corresponding genotypic classes (*ALF/ALF*, *ITA/ALF* and *ITA/ITA*) showed highly significant differences ($P < 0.0001$) between white and coloured berry plants (Fig. 1b). Individuals homozygous for the $VvmybA1^{ALF}$ allele (*ALF/ALF*) showed slightly higher CIRG values than the heterozygous (*ITA/ALF*), which suggests the existence of certain level of haploinsufficiency. Altogether, the results indicate that variation at the *VvmybA1* gene is responsible for the berry colour phenotypic variation observed in previous genetic analyses (Doligez et al. 2002; Fisher et al. 2004).

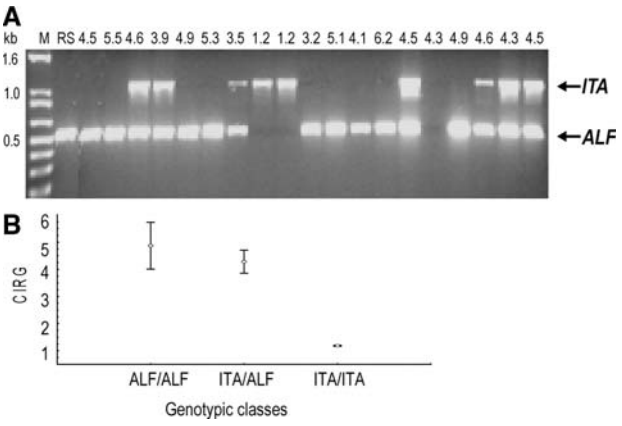

Fig. 1 Association between *VvmybA1* genotype and berry colour in a selfcross progeny of Ruby seedless. **a** PCR analysis of *VvmybA1* alleles for the Ruby Seedless selfcross population. *Numbers* on each lane represent the CIRG values for the corresponding samples. *M* molecular weight marker, *RS* Ruby Seedless, *ITA* ($VvmybA1^{ITA}$); *ALF* ($VvmybA1^{ALF}$). **b** Graphical representation of mean CIRG values and their standard deviations calculated for each *VvmybA1* genotypic class

Berry colour is always associated to *VvmybA1* functional alleles

To evaluate the extent of berry colour variation explained by *VvmybA1* in table grape we analyzed the allelic composition for this locus in a representative sample of 189 table grape cultivars containing an equivalent number of coloured and white-skin accessions. The first step in the analysis consisted in the PCR detection of the insertion $VvmybA1^{ITA}$ allele. This insertion allele was considered non-functional or null allele based on Kobayashi et al. (2004). Additionally, amplification of PCR products with primers specific for the 5′ upstream and coding regions of *VvmybA1* was considered an indication of the existence of a functional allele, as shown in Fig. 2, where six different genotypes for *VvmybA1* could be identified. Interestingly, in 91 out of 96 white cultivars analyzed (exemplified by Dominga in Fig. 2) we only detected the presence of the null *ITA* allele (Table 1). Furthermore, in 88 out of 93 coloured cultivars (exemplified by Muscat Hamburg, Sultanine Roseé, Alphonse Lavallée and Black Seedless in Fig. 2) we found the presence of at least one putative functional allele (Table 1 and Fig. 2). This PCR assay allowed the detection of three putative functional alleles $VvmybA1^{ALF}$, $VvmybA1^{SUB}$ and $VvmybA1^{RUO}$ (see somatic mutation section), with $VvmybA1^{ALF}$ being the most frequent in the colour table grape cultivars analyzed (present in 71 out of 93 cultivars). Two coloured cultivars (Black Monukka and Black Seedless) were heterozygous for the putative functional alleles $VvmybA1^{ALF}$ and $VvmybA1^{SUB}$ (i.e., Black Seedless in Fig. 2). Thus, in 179 cultivars out of the 189 tested (95%) the berry colour phenotype can be explained on the basis of the PCR established *VvmybA1* genotype, being white when no functional allele can be amplified and coloured when at least one functional allele is detected. These results confirm the original proposal of

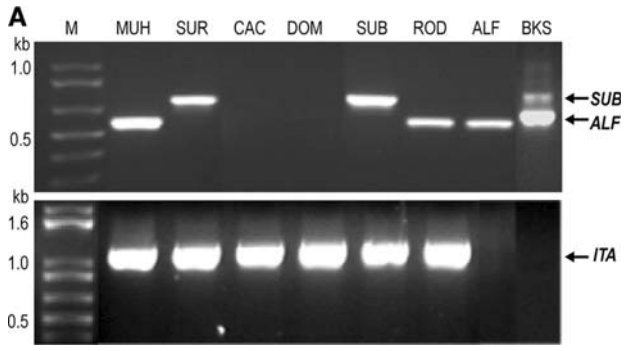

Fig. 2 Molecular phenotypes of table grape cultivars at the *VvmybA1* locus. **a** PCR assay for the functional (-*ALF* and *SUB*-above) and null (-*ITA*-below) alleles. Eight cultivars were chosen to illustrate all the different molecular phenotypes found: *MUH* Muscat Hamburg (*coloured*); *SUR* Sultanine Rosée (*coloured*); *CAC* Cape Currant (*coloured*); *DOM* Dominga (*white*); *SUB* Sultanine (*white*); *ROD* Roditis (*white*); *ALF* Alphonse Lavallée (*coloured*); *BKS* Black Seedless (*coloured*). *M* molecular weight marker

Table 1 *VvmybA1* genotypes and colour phenotypes observed in the analyzed table grape cultivars

VvmybA1 PCR based genotypes					Colour phenotypes[b]		Total
Genotype	Alleles[a]				W	C	
	ITA	ALF	SUB	RUO			
1	+				91	5	96
2	+	+			1	52	53
3	+		+		4	10	14
4		+			0	17	17
5		+	+		0	2	2
6	+			+	0	7	7
				Total	96	93	189

[a] Alleles based on the PCR amplification of null (*ITA* *VvmybA1*^{ITA}) and/or functional (*ALF* *VvmybA1*^{ALF}, *SUB* *VvmybA1*^{SUB} or *RUO* *VvmybA1*^{RUO}) alleles (see Materials and methods)

[b] Berry skin colour: *W* white (yellow or yellow-green); *C* coloured (pink to dark blue)

Kobayashi et al. (2004) suggesting that variation in grape berry colour could largely be due to *VvmybA1* variation. They show a strong parallelism with what has been already reported in plant species such as maize, pepper or petunia where variation at a putative *MYB* orthologous gene is responsible for colour variation at seeds, fruits and flowers (Borovsky et al. 2004; Quattrocchio et al. 1993; Spelt et al. 2000).

The existence of five coloured cultivars in which no functional *VvmybA1* allele could be amplified as well as five white cultivars, which apparently carry *VvmybA1* functional alleles (Table 1), might suggest the existence of additional loci controlling grape berry colour and deserved further molecular analyses.

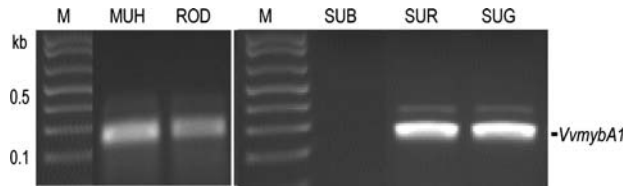

Fig. 3 *VvmybA1* expression in the berry skin of particular cultivars. RT-PCR amplification on total RNA from Muscat Hamburg (*MUH*), Roditis (*ROD*), Sultanine (*SUB*), Sultanine Rosée (*SUR*) and Sultanine Rouge (*SUG*). *White* cultivar Roditis and *coloured* cultivar Muscat Hamburg (both displaying the same PCR molecular phenotype) showed identical expression for *VvmybA1*. Despite presenting identical coding sequences as Sultanine Rosée and Sultanine Rouge, no *VvmybA1* expression was detected in Sultanine. *M* molecular weight marker

The results regarding the five white cultivars will be described in the next paragraph, while the five coloured cultivars that were known somatic variants will be further described in the somatic variation section.

The existence of five white cultivars carrying putative functional alleles *VvmybA1*^{ALF} (Roditis) or *VvmybA1*^{SUB} (Sultanine, Autumn Seedless, Kischmisch Ali Blanc and Pirovano 166A) (Table 1 and Fig. 2) might provide support for the existence of a second locus responsible for berry colour variation. Interestingly, all these cultivars are hybrids derived directly or indirectly from Sultanine. To evaluate the involvement of *VvmybA1* in this phenotype we first analyzed the expression of the *VvmybA1* alleles present in those white cultivars. Cultivar Roditis carries a PCR typified *VvmybA1*^{ALF} allele (Fig. 2a) and RT-PCR analyses on berry skin total RNA showed regular *VvmybA1* expression (Fig. 3). On the other hand, Sultanine, consistently with its white phenotype, did not display any expression of *VvmybA1* in the berry skin. Nevertheless, two coloured somatic variants (Sultanine Roseé and Sultanine Rouge) did show expression of the *VvmybA1*^{SUB} allele.

To obtain more information about the molecular nature of those alleles we PCR amplified and sequenced the corresponding *VvmybA1* genome region from Alphonse Lavallé, Sultanine, Sultanine Rosée and Roditis using primers b, cDNAas and a 3′ RACE strategy as described in Materials and methods. Based on sequence differences the *VvmybA1*^{ALF} from Roditis was renamed as *VvmybA1*^{ROD} (Fig. 4). Nucleotide sequence comparisons with *VvmybA1*^{ITA} and *VvmybA1*^{RUO} allele sequences (Kobayashi et al. 2004) showed that *VvmybA1*^{ALF} and *VvmybA1*^{ROD} alleles were more related with the *VvmybA1*^{ITA} allele, while the *VvmybA1*^{SUB} sequence was quite divergent from all of other. Apart from the existence of a large number of SNPs between *VvmybA1*^{SUB} and the other

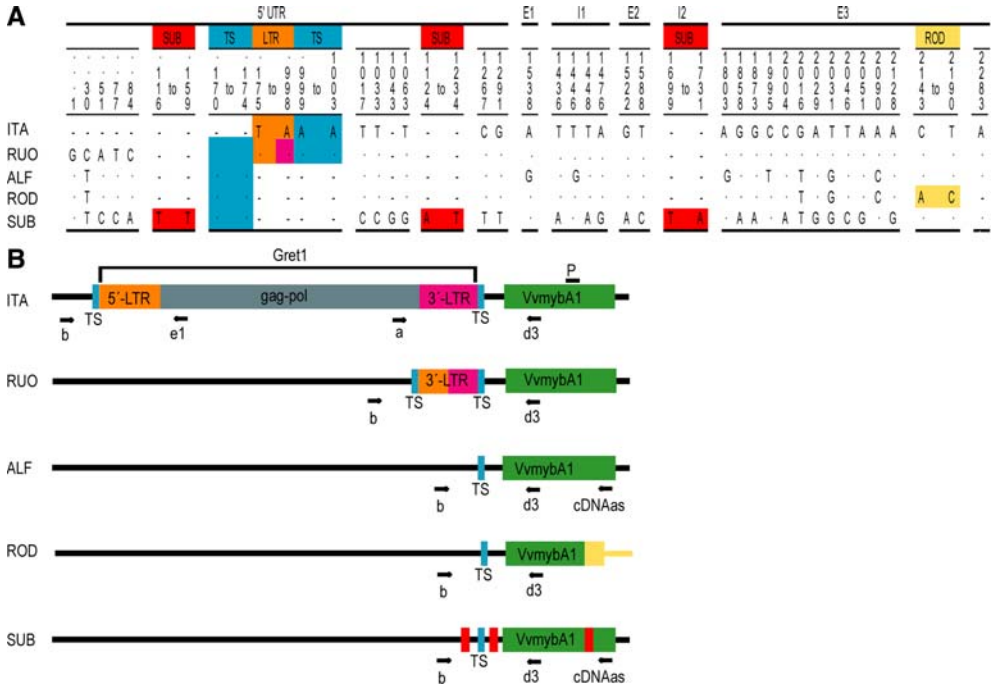

Fig. 4 Comparison of nucleotide sequences for *VvmybA1* alleles. **a** Sequence alignment of *VvmybA1* alleles. All sequences are compared to the reference allele $VvmybA1^{ITA}$ (*ITA*) (Kobayashi et al. 2004). The position of the polymorphic sites and the $VvmybA1^{SUB}$ (*SUB*) and $VvmybA1^{ROD}$ (*ROD*) specific indels, and their locations in the 5′-UTR region, introns (*I*) and exons (*E*) are indicated on top. Start position corresponds to the location of primer *b* (see *panel* **b**). *TS* duplicated target site for the *Gret1* insertion. **b** Schematic representation of the alleles and approximate position of the primers used in the different PCR assays

VvmybA1 alleles, the Sultanine allele carries three short insertions, two in the 5′-UTR region and one in the second intron (Fig. 4). Comparison of DNA sequences between $VvmybA1^{ALF}$ and $VvmybA1^{ROD}$ revealed six SNPs differences and the existence of a deletion of the last 135 coding nucleotides of the Roditis gene (Fig. 4). The Roditis allele ($VvMybA1^{ROD}$) encodes a truncated protein and might represent a loss of function allele responsible for the absence of colour in the skin of Roditis berries. In fact, this carboxyl terminus has been shown to function as activation domain in C1 (Goff et al. 1991) and is also proposed to have a relevant functional role in VvMYBA1 (Kobayashi et al. 2005). Still, without a functional proof, we cannot completely discard the possibility that this allele is partially active and Roditis's lack of colour results from genetic variation at some other locus.

Comparative analysis of $VvmybA1^{SUB}$ sequences from coloured and white berry cultivars showed that they were identical, indicating that the colour difference could not be explained by sequence variation at the *VvMybA1* coding sequence. These results might suggest either the existence of an uncovered tranposon insertion in the upstream promoter region of *VvMybA1* or of a second regulatory locus controlling berry colour in grape. In any case, the transposon insertion or the second locus would regulate *VvMybA1* expression (Fig. 3).

In summary, with the exception of the Roditis's truncated mRNA, all white berry cultivars analyzed show lack of *VvmybA1* expression either due to the insertion of *Gret1* in the *VvmybA1* promoter region as in the $VvmybA1^{ITA}/VvmybA1^{ITA}$ cultivars or to the down-regulation of functional alleles like in the white $VvmybA1^{ITA}/VvmybA1^{SUB}$ cultivars.

Molecular basis of colour somatic variation

Colour somatic variation is a well studied phenomenon in plants and has been studied in many fruit crops including apple (Arakawa et al. 1999; Awad et al. 2000; Ben-Yehudah et al. 2005), bilberry (Jaakola et al. 2002), citrus (James and Howard 1989) and pear (van Dyk et al. 2005). The generation of somatic coloured variants from white cultivars is a relatively common phenomenon in table grape (Franks et al. 2002; Hocquigny et al. 2004; Thompson and Olmo 1963). In Ruby Okuyama, a red berry somatic variant of Italia, the coloured phenotype was associated with the recovery of *VvmybA1* expression and related to the intra-LTR recombination of the *Gret1* retrotransposon (Kobayashi et al. 2004).

We have investigated the molecular basis of somatic colour variation in seven additional white berry cultivars homozygous for the *VvmybA1*^{ITA} alleles (Beba, Chasselas Doré, Jaén Blanco, Moscatel de Grano Gordo, Moscatel de Grano Menudo, Sugraone and Xarel.lo) and their colour somatic variants. As shown in Fig. 5a, in six somatic variants, colour recovery was associated with the amplification of the solo-LTR PCR fragment (*RUO*) initially identified in Ruby Okuyama (Kobayashi et al. 2004). Interestingly, in the remaining variants analyzed (Benitaka, Cape Currant, Chasselas Rouge, Moscatel de Grano Menudo Rojo and Jaén Rosado-Fig. 5a) colour recovery was not related to the appearance of the solo-LTR PCR fragment. Both classes of somatic variants were further analyzed to understand the molecular basis of colour recovery. For the first class, two independent spontaneous colour variants of Sugraone, Ralli Seedless and Super Red were analyzed. Both cultivars displayed *VvmybA1*^{RUO} alleles (Figs. 4b, 5a). Sequencing of the solo-LTRs from Ralli Seedless and Super Red and comparison with the 5′ and 3′-LTR sequences in Sugraone showed that these two red somatic variants derived from recombination events taking place at two different positions within the LTRs. This result was obtained thanks to the identification of 5 SNPs between the 5′ and 3′ LTRs of Sugraone that were enough to trace the recombination events giving rise to each colour cultivar (Fig. 5b). It proofs that intra-LTR recombination is the colour recovery mechanism for these somatic variants and confirms the independent nature of recombination events giving rise to them.

For the remaining colour somatic variants neither a solo-LTR nor any other functional allele could be PCR amplified (Fig. 5a), even when the six different primer combinations detailed in Fig. 5c (left) were used. A DNA blot hybridization analysis with Benitaka, Chasselas Rouge, Italia and Ruby Okuyama genomic DNA allowed for the detection of the restriction fragment corresponding to the *VvmybA1*^{ITA} allele as well as additional hybridizing bands with different size in each genotype that could be attributable to the presence of *VvmybA1* functional alleles (Fig. 5c right). Retrotransposon insertions are known to frequently generate deletions, likely through mechanisms of illegitimate recombination. In rice and Arabidopsis these deletions have been proposed as a possible cause for genome size reduction (Ma et al. 2004). We argue that illegitimate recombination in the region of *Gret1* insertion could produce deletion of the retrotransposon and additional sequences in the 5′ region of *VvmybA1* preventing PCR primers hybridization.

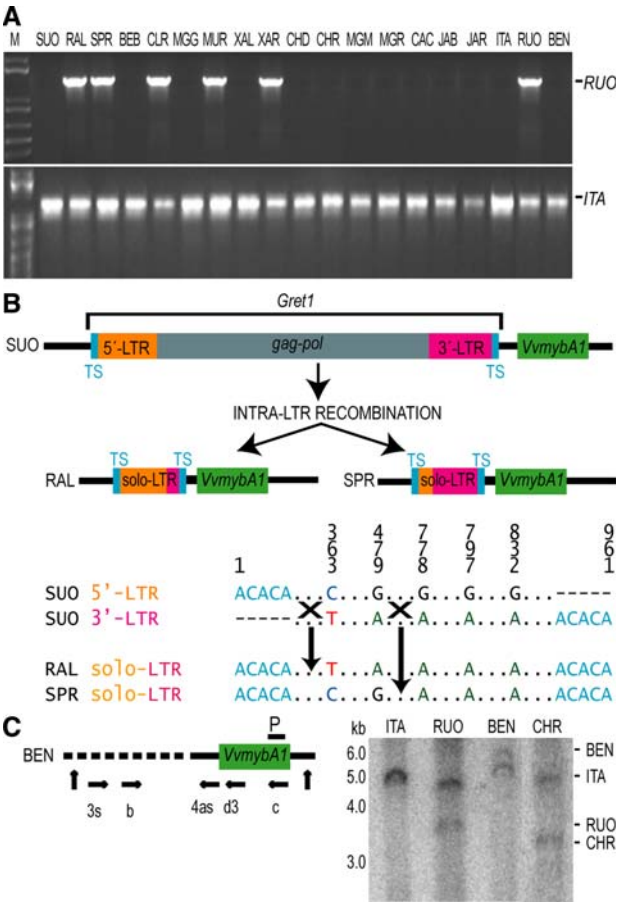

Fig. 5 Molecular basis of somatic variation for grape skin colour. **a** PCR assay for the functional (-*RUO*-above) and null (-*ITA*-below) alleles from eight white cultivars and their colour somatic variants: [*SUO* Sugraone/*RAL* Ralli Seedless and *SPR* Super Red], [*BEB* Beba/*CLR* Calop Rojo], [*MGG* Moscatel de Grano Gordo/*MUR* Moscatel Rosa], [*XAL* Xarel'lo/*XAR* Xarel'lo Rosado], [*CHD* Chasselas Doré/*CHR* Chasselas Rosé], [*MGM* Moscatel de Grano Menudo/*MGR* Moscatel de Grano Menudo Rojo and *CAC* Cape Currant], [*JAB* Jaén Blanco/*JAR* Jaén Rosado], [*ITA* Italia/*RUO* Ruby Okuyama and *BEN* Benitaka]. *M* molecular weight marker. **b** Schematic representation of the intra-*LTR* recombination events that took place in the white cultivar Sugraone (*SUO*) to generate somatic coloured variants Ralli (*RAL*) and Super Red (*SPR*) (*top*). Sequence alignment of *SUO* 5′-*LTR* and 3′-*LTR* together with the solo recombinant *LTR*s of *RAL* and *SUR*. *Arrows* indicate the region where recombination events took place (*bottom*). *TS* duplicated target site (ACACA in *blue*). **c** Schematic representation of the putative *VvmybA1* organization in the *coloured* cultivar Benitaka and primers used in the corresponding PCR assay. *Vertical arrows* indicate the approximate position of the *Apa* I restriction sites used for the DNA blot hybridization assay. *P VvmybA1* probe (*left*). DNA blot hybridization analysis of Benitaka (*BEN*) and Chasselas Rose (*CHR*). Italia (*ITA*) and Ruby Okuyama (*RUO*) were used as controls (*right*)

Somatic variation is a very relevant phenomenon creating berry colour variation in grape. We have shown that this variation is mostly based on the genetic instability associated with the presence of *Gret1*at locus

VvmybA1 and can result from homologous recombination between LTRs as well as putative illegitimate recombination events.

Conclusions

In summary, berry colour variation in grapevine is strongly associated with genetic variation at the *VvmybA1* gene. We have shown that this gene underlies the major colour locus mapped in segregating populations. Three major haplotypes, *ITA*, *ALF* and *SUB*, with allelic frequencies of 69, 23 and 4% respectively, have been identified in the analyzed sample (Table S1). Among them *ALF* seems to represent a major functional allele while *ITA* and *SUB* are present in colourless cultivars. Interestingly, all cultivars carrying two functional *VvmybA1* alleles such as Black Seedless, Almeria Nera, Dattier Noir or Negra Tardía are known by names that allude to their dark skin colour (see Table S1 and synonymies at the Vitis International Variety Catalogue: http://www.genres.de/idb/vitis/). Sequence variation at the *VvmybA1* locus explains berry colour in over 95% of the analyzed cultivars. While in the remaining cultivars, berry colour is always associated to *VvmybA1* expression. The null *Gret1* insertion allele, *ITA*, behaves as an unstable allele generating additional derivative alleles from *Gret1* related recombination events. *Gret1* related instability can be associated to most cases of somatic colour variation with the exception of the Sultanine derived somatic variants that suggest instability at a second transposon insertion or a second locus. Additional loci will further contribute to berry colour variation, either through the regulation of *VvmybA1* or through their role in the anthocyanin biosynthetic pathway, such as those encoding flavonoid hydroxylases (Bogs et al. 2006; Castellarin et al. 2006) which could be responsible for dark blue colours.

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