Vitis **54** (Special Issue), 45–48 (2015)

Analysis of *VvMybA1* and *VvMybA2* genes in grape bud sports

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Summary

Berry skin colour is a fundamental qualitative trait of grape varieties, which has become largely diversified during the centuries of viticulture. Colour mutations in grape berry, resulting in black, red, pink, grey, green and yellow fruits, were relatively frequent events. In the Carpathian Basin there are several berry colour variant groups named *conculta*, members of which contain bud sports differing in skin colour. In most cases, this difference cannot be detected by microsatellite analysis, therefore we examined the variations of the *VvMybA1* and *VvMybA2* genes related to anthocyanin biosynthesis. Based on the results of the allelic polymorphisms, we have discriminated the *conculta* members among the old Hungarian and several other foreign varieties. Based on our results, it was possible to find molecular differences in 10 out of 14 *concultas*.

K e y w o r d s : berry colour; *conculta*; *Gret1*; SNP; SNaPshot; *Vitis vinifera.*

Introduction

Anthocyanins represent a major group of the flavonoid family and they are responsible for red and blue colours of many plant tissues (Harbone *et al*. 2000). Mutations affected important traits of horticultural plants (Holton *et al*. 1995). Skin colour is one of the most important quality traits in wine and table grapes (Boss *et al*. 1996). Black and red grape cultivars accumulate anthocyanins in their skins, whereas white cultivars do not synthesize them (Kobayashi *et al*. 2002). A single gene cluster, located on chromosome 2, is responsible for most colour variations (Fournier-Level *et al*. 2009). *VvMybA1* and *VvMybA2* genes determine the berry pigmentation (Kobayashi *et al*. 2002, Walker *et al.* 2007). *VvMybA1* gene silencing is due to the insertion of *Gret1*, a gipsy-type retrotransposon in its promoter (Kobayashi *et al*. 2004). This allele is called *VvMybA1a*. Presence of *Gret1* retrotransposon in the promoter of the *VvMybA1* gene was firstly identified as a loss-of-function mutation in cultivars 'Italia' and 'Muscat of Alexandria' (Kobayashi *et al*. 2004), and further studies showed that the colouring of grape skin depends on the *VvMybA1* polymorphism (Lijavetzky *et al*. 2006, Azuma *et al*. 2007). Regarding *VvMybA2*, the single-nucleotide polymorphism (SNP) *K980* in the coding sequence (T instead of G), led to a non-functional allele (Walker *et al*. 2007). Several *VvMybA1* alleles have been identified: *VvMybA1a*, containing *Gret1*; *VvMybA1b*, harbouring 3' *Gret1* LTR as a consequence of retrotransposon excision; *VvMybA1c*, *Gret1*-less, (wild type allele); *VvMybA1d*, null allele, due to the deletion of the functional *VvMybA1* gene (Kobayashi *et al.* 2004, Yakushiji *et al.* 2006, Azuma *et al*. 2007). Besides, the altered colouration of berries can be affected by different mutation patterns of the skin cell layers (Vezzulli *et al.* 2012, Migliaro *et al.* 2014).

Zhukovsky (1950) introduced the term *conculta* for the colour mutant grape varieties. Based on Zhukovsky's work, Hungarian ampelographer Márton Németh adapted and integrated the term *conculta* into the morphological and phenological characterization systems of the varieties found in Hungary (Németh 1966, 1967). Not only the berry skin colour is the difference between the *conculta* members, but also the colouration of autumn leaf and prostrate hairs of the shoot tips. In the Institute of Genetics and Biotechnology (Szent István University, Gödöllő, Hungary) in earlier studies (in the frame of GrapeGen 06 project), based on microsatellite (SSR) analysis of the Carpathian Basin cultivars, 6 *concultas* were examined: 'Lisztes' (piros/red and fehér/white), 'Bakator' (piros/red and tüdőszínű/pink), 'Gohér' (piros/red, fehér/white, változó/varying), 'Muskotály/Muscat' (fekete/black, piros/red, sárga/yellow and csíkos/stripy), 'Furmint' (piros/red, fehér/white and változó/varying), 'Barátcsuha' (kék/blue and szürke/grey). The berry colour variants gave identical SSR allele patterns (Szőke *et al*. 2012).

In order to determine the genetic background of berry colour in several Carpathian Basin and other putative *concultas* grown or maintained in other countries, we analyzed the variation of *VvMybA1* gene examining the presence, absence or excision of *Gret1* retrotransposon in the promoter of *VvMybA1*gene and two SNP in the coding region of *VvMybA2* gene.

Material and Methods

The plant material was composed of 21 Carpathian Basin cultivars from Hungary and 15 other foreign varieties, including 3 reference cultivars: 'Barbera' (no *Gret1* insertion), 'Pinot noir' (heterozygous for *Gret1*) and 'Chardonnay' (homozygous for *Gret1*) (Walker *et al*. 2007, Szőke *et al*. 2012). Young leaves were collected from University of Pécs, Research Institute of Viticulture and Enology, Pécs

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(Hungary) and genomic DNA was isolated using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The Table shows the cultivar names and their berry colour (N: noir, black; B: blanc, white; Rg: rouge, red; Rs: rose, pink; G: gris, grey).

Presence or absence of the *Gret1* retroelement in the *VvMybA1* promoter region was detected using the primers according to KOBAYASHI *et al*. (2004): a (5′-AAAAAGGGGGGCAATGTAGGGACCC-3′); c (5′-GAACCTCCTTTTTGAAGTGGTGACT-3′); b (5′-GGACGTTAAAAAATGGTTGCACGTG-3′). PCR was performed in a Bio-Rad iCycler (Bio-Rad) in a 25 µL volume. The reaction mixture contained 20 ng DNA template, 1 µM of each primer, 75 µM of each dNTP, 2 mM $MgCl_2$, 1x reaction puffer and 1 unit Taq polymerase (WestTeam Biotech, Hungary). The following PCR profile was applied: precycle: 2 min at 95 °C; 40 cycles of denaturation 30 s at 95 °C, 30 s annealing at 56 °C and 1 min extension at 72 °C; postcycle: 5 min at 72 °C. Amplified fragments were separated in agarose gel according to BODOR *et al*. (2014).

For the loci *K980* (WALKER *et al*. 2007) and *C22*, a recently discovered *VvMybA2* SNP (CARRASCO *et al*. 2015)

Table

Results of *VvMybA1* and *VvMybA2* allelic polymorphism analysis of grapevine varieties

Varieties	*VI*VC numbers	Berry colour	*VvMybA2*						*VvMybA1*		
			K980			*C22*			*VvMybA1a Gret1*	*VvMybA1c* No *Gret1*	*VvMybA1b* 3'LTR
			T/T	G/G	T/G	T/T	G/G	T/G			
Barbera	974	N		x			x			x	
Pinot noir	9279	N			x			x	[x]	[x]	
Chardonnay	2455	B	x			x			x		
Bajor szürke/grey*	14783	G	x			x			[x]	[x]	
Bajor kék/blue	765	N			x	x				[x]	
Bajor feketefájú/black wooded*	765	N	x			x			[x]	[x]	
Bakator tüdőszínű/pink*	906	Rs	x			x			[x]	[x]	
Bakator piros/red*	905	Rg	x			x			[x]	[x]	
Barátcsuha szürke/grey*	17431	G			x			x	x	x	
Barátcsuha kék/blue*	17430	N			x			x	x	x	
Csiljaki krasznüj/red*	2564	Rg	x			x			x		
Csiljaki belüj/white*	2561	B	x			x			x		
Furmint piros/red	17047	Rg	x			x			[x]		[x]
Furmint fehér/white*	4292	B	x			x			[x]		
Furmint változó/varying*	17471	B	x			x			x		
Gohér piros/red	4861	Rg			x			x	[x]		
Gohér fehér/white*	767	B	x			x			[x]		
Gohér változó/varying*	14912	B	x			x			[x]		
Huszajne krasznüj/red	6209	Rg			x			x	x		x
Huszajne belüj/white	6203	B	x			x			x		
Kéknyelű	16421	B	x			x			x		
Kéknyelű piros/red		Rg	nd	nd	nd	nd	nd	nd	x		x
Korinthusi fekete/black	6410	N	x			x			x	x	
Korinthusi piros/red	6411	Rg			x	x			x	x	
Lisztes fehér/white	17061	B	x			x			[x]		
Lisztes piros/red	14999	Rg	x			x			[x]		x
Muskotály/Muscat fekete/black	8238	N	x			x			x	x	
Muskotály/Muscat csíkos/stripy*	8193	B	x			x			x		
Muskotály/Muscat sárga/yellow*	8193	B	x			x			x		
Muskotály/Muscat piros/red	8248	Rg	x			x			x		[x]
Oportó szürke/grey*	9623	G			x			x	x	x	
Oportó kék/blue*	9620	N			x			x	x	x	
Piquepoul gris	9297	G	x			x			x	x	
Piquepoul noir	9298	N			x			x	x	x	
Szilváni zöld/green	11805	B	x			x			x		
Szilváni piros/red	11807	Rg	nd	nd	nd	nd	nd	nd	x	x	

Bold letters indicate the putative wild type varieties;
Abbreviation for berry colours: N: noir/black, B: blanc/white, Rs: rose/pink, Rg: rouges/red, G: gris/grey;
x: presence of the appropriate alleles;
[x]: data already published by BODOR *et al*. (2014);
*: Varieties cannot be discriminated by either *VvMybA1* or *VvMybA2* gene;
nd: not detected.

the SNaPshot assay was applied. The *VvMybA2* gene sequence was amplified using the primers and conditions reported in Walker *et al*. (2007). PCR fragments were separated by electrophoresis in 0.8 % agarose gel in TBE buffer and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. SNP markers were detected according to the protocol provided with the ABI PRISM SNaPshot Multiplex kit (Life Technologies Corporation, Carlsbad, California). The purified SNaPshot PCR products were detected on capillary electrophoresis instrument (ABI PRISM® 310 Genetic Analyzer, Life Technologies Corporation) and data analysis was performed by GeneMapper 4.0 software (Life Technologies Corporation).

Results and Discussion

Based on the analysis of *VvMybA1* gene with primers described by Kobayashi *et al*. (2004) for detection of the *VvMybA1a, VvMybA1b*, and *VvMybA1c* alleles, two varieties out of 36 did not contain the *Gret1* retrotransposon insertion: 'Barbera' and 'Bajor' kék/blue.

In our earlier work, based on *VvMybA1* allele polymorphism, it was possible to successfully discriminate 'Bajor' kék/blue from 'Bajor' szürke/grey and feketefájú/black wooded, 'Furmint' piros/red from fehér/white and változó/varying, and 'Lisztes' fehér-piros/white-red (Bodor *et al*. 2014). In the present study, the Table showed that *VvMybA1* allele polymorphism was found in 'Huszajne' krasznüj-belüj/red-white, 'Kéknyelű'-'Kéknyelű' piros/red, 'Muskotály/Muscat' *conculta* members and 'Szilváni' piros-zöld/red-green. Since the other samples remained undistinguishable, *VvMybA2* SNP polymorphisms were also examined. According to Fournier-Level *et al*. (2010), berry colour variation can be the consequence of mutations in *VvMybA1* (*Gret1*) or *VvMybA2* (*K980* locus) genes. Several haplogroups were defined based on the presence or absence of the *Gret1* in the *VvMybA1* promoter, and the presence of a functional G allele or a mutated T allele at the *K980* locus in the *VvMybA2* coding sequence. These haplogroups consist of "coloured", "altered colour" and "white" variations.

Two SNPs of the *VvMybA2* gene were tested by SNaPshot method and carried out the sequencing in 34 grape cultivars. The *K980* point mutation has been identified based on Walker *et al*. (2007), called *VvMybA2R44*, and a new point mutation which has not been identified before, called *C22* (Carrasco *et al*. 2015). Twenty-four cultivars were homozygous for *K980* (23 T/T and 1 G/G) and 10 samples were heterozygous (G/T), where T is the non-functional allele and G the functional one. Based on the new single nucleotide polymorphism (*C22*), 26 cultivars were homozygous (25 T/T and 1 G/G) and 8 samples were heterozygous (G/T), with G and T, wild type and mutated allele, respectively. Considering both genes (*VvMybA1* and *VvMybA2*), all the analysed varieties became arranged corresponding to their berry colour. All the white cultivars showed the homozygous *VvMybA1a* allele for *VvMybA1* gene and the homozygous T nucleotide for both *VvMybA2* SNPs, resulting in the B haplogroup described by Fournier-Level *et al*. (2010). Among the pigmented cultivars, only 'Barbera' showed the typical haplogroup (N) of black berry varieties, holding no *Gret1* but the functional G allele at *K980* locus (Fournier-Level *et al*. 2010). For the altered colour berry samples (Rs and G), new allele combinations were identified.

'Gohér' piros/red differed from változó/altering and fehér/white in both SNP, 'Korinthusi' fekete-piros/black-red in *K980* locus and 'Piquepoul' gris-noir in both SNPs. Neither *VvMybA1* nor *VvMybA*2 gave different patterns for 16 *conculta* members (Table).

Since the grapevine berry develops from different layers of the apical meristem (L1, berry skin, and L2, berry flesh), a layer-specific approach (Migliaro *et al*. 2014) will improve our knowledge in determining the genetic background of bud sports not yet discriminated, as well as in understanding the evolutionary events leading to their origin.

Acknowledgements

This work was supported by COST Action FA1003 "East-West Collaboration for Grapevine Diversity Exploration and Mobilization of Adaptive Traits for Breeding" and the Research Center of Excellence of Faculty of Agricultural Science at Szent István University-9878/2015/FEKUT projects.

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