Expression of genes associated with anthocyanin synthesis in red-purplish, pink, pinkish-green and green grape berries from mutated 'Sangiovese' biotypes: A case study

S. Ramazzotti, I. Filippetti and C. Intrieri

Dipartimento di Colture Arboree, Sezione Viticola del Centro Interdipartimentale di Ricerche Viticole ed Enologiche, University of Bologna, Bologna, Italy

Summary

Using normal red-purplish grape bunches and pink, pink-green and green berry colour-mutated biotypes of cv. Sangiovese (*V. vinifera* L.), we investigated their anthocyanin metabolism via biochemical and molecular assays. The number and composition of the different types of anthocyanins were analysed by spectrophotometry and chromatography. The expression of six structural genes of the biosynthetic pathway (chalcone synthase [CHS], chalcone isomerase [CHI], flavanon-3-hydroxylase [F3H], dihydroflavonol 4-reductase [DFR], leucoanthocyanidin dioxygenase [LDOX] and UDP-glucose 3-O-flavonoid:glucosyltransferase [UFGT]) was determined over the four weeks subsequent to veraison via Northen blot and Real Time PCR.

The grapes from the non-mutated biotype showed a prevailing accumulation of monoglycoside anthocyanin fractions, with only traces of acetyl and p-coumaroyl derivatives. The berries of the mutated biotypes showed a gradual berry pigment loss associated with a reduction in total anthocyanin content, although anthocyanin composition was the same of the non-mutant biotype. Indeed, the Northern blot assay data, as confirmed by the quantitative Real Time PCR tests, showed a differential expression in the berries of the non mutated and mutated biotypes for the UFGT gene, proving normal in the red-purplish, lower in the biotypes with pink and pink-green berries and wholly lacking in the green one. Thus, the UFGT gene in berry skin of colour-mutated 'Sangiovese' biotypes is controlled independently of the other structural genes encoding enzymes in the anthocyanin biosynthetic pathway and its capacity of expression is a critical factor in the synthesis and storage of these compounds.

K e y   w o r d s : *V. vinifera* L., 'Sangiovese', mutation, anthocyanins, Northern blot, Real time PCR, gene expression.

Introduction

The accumulation of anthocyanins in grape berry skin is one of the main features of red berry ripening and, hence, is fundamental for characterising grapes and wines. Indeed, the number and type of these compounds at harvest are significant parameters in evaluating crop quality. In the past, mutation affecting berry colour in various *Vitis vinifera* cultivars like 'Malvasia rosé', 'Chardonnay red', 'Sultana rosé', 'Moscato red', 'Pinot grey', and 'Cabernet bronze' have been subjected to a number of studies (Fregoni 1974, Galet 1979, Cravero *et al*. 1994, Boss *et al*. 1996 c). Mutations, especially those linked to berry colour, can evince different onset features and dynamics depending on whether they involve loss of pigmentation in red or the acquisition of colour in white cultivars. For example, Considine and Knox (1981) suggested that the cause of pale-coloured berries might be a chimeric status of skin tissues with alternating layers of mutated and fully normal cells. More recently, biomolecular studies of such species as *Zea mays* and *Arabidopsis thaliana* have found that anthocyanin synthesis is largely controlled by transcription factors, particular proteins which operate according to a highly conserved mechanism among plant species (Martin and Gerats 1993).

In grapes, two specific transcriptor factors of the Myb family (VvMybA1 and VvMybA2) were found to be involved in colour regulation (Kobayashi *et al*. 2002). In particular, a retrotransposon insertion in VvMybA1 of a black cultivar appears to be responsible for bud sports bearing white berries (Verries *et al*. 2000, Kobayashi *et al*. 2004 and 2006). For 'Sangiovese' (*V.vinifera* L.), mutant biotypes with various degree of depigmentation (from pink to green berries) were described by Intrieri (1981) and Intrieri and Silvestroni (1985) but their physiological and biomolecular traits were not yet evaluated. Given the importance of this variety, which is one of the most intensively cultivated in Italy, we investigated the causes of berry colour change in some mutated biotypes.

Material and Methods

O r i g i n   o f   p l a n t   m a t e r i a l : During the 1977 harvest two bunches with yellow-green berries were observed by Intrieri (1981) in a fruiting cane of a 'Sangiovese' vine (*Vitis vinifera* L.) trained to spur-pruned cordon (Fig. 1 A). This cane was then used as a lead in 1978 and pruned to 8 nodes (Fig. 1 B). While the fruiting shoot that grew in 1978 from node 1 of the retained cane gave rise to normal grapes, the shoots from the seven remaining nodes produced grape clusters of varying colour loss (Fig. 1 C). Single-bud cuttings from each shoot at all 8 nodes were taken during the winter 1978-1979, which
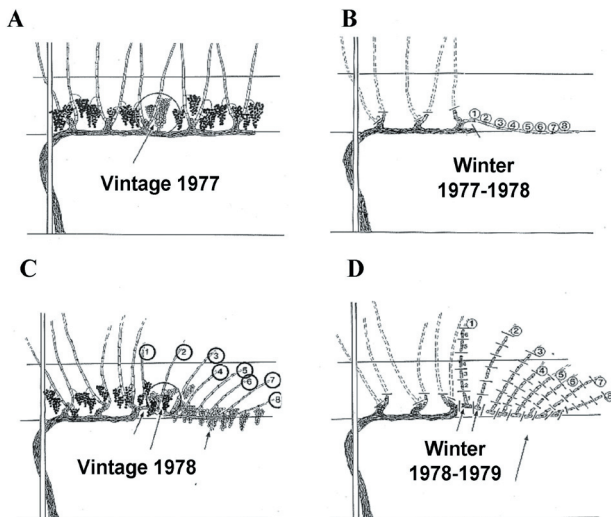
Fig. 1: (**A**) Original 'Sangiovese' vine (*V. vinifera* L.) found in 1977 to have de-coloured grape clusters on a cane growing from a spur towards the middle of the permanent cordon. (**B**) Pruning in winter 1977-78: Part of the permanent cordon was eliminated and the cane with the observed mutation was pruned at 8 nodes and laid out in its place. (**C**) distribution of the mutated and non-mutated bunches from the shoots of the 8-nodes cane in 1978. (**D**) Multiplication via single-bud cuttings in 1978-79 season: These cuttings from the shoots of the original cane were tagged with a progressive identification number as per reference shoot followed by a progressive number as per reference node (e.g. 4-6 = shoot 4, cutting from node 6).

Fig. 2: Clusters exemplifying the different colour gradations of the mutant 'Sangiovese' biotypes. The photos were taken at harvest.

in turn originated self-rooting vines each having bunches of varying colour intensity (Fig. 1 D). While all the buds from shoot 1 produced normal, red-purplish berries, all those from the other seven shoots produced mutated biotypes with pink, pinkish-green and green berries (Intrieri 1981). Although the self-rooting vines were later multiplied a number of times via grafting, and since the de-pigmented bunches retained their specific traits, it was thus assumed that these mutations were 'stable' and most likely periclinal. Other details about all the above mentioned biotypes can also be found in Intrieri and Silvestroni (1985) and Solari *et al.* (1985). The plants used in the present study were taken from a collection field housing the mutated (tagged SGM and assigned number codes) and non-mutated biotypes from the original vine (tagged SG and assigned number code).

M e t h o d o l o g y : The assays were run on the vines of biotype SG 1-1, which was taken as control, and on biotypes SGM 3-2, 5-2 and 8-2 marked by their respective pink, pinkish-green and green berry colour (Fig. 2). All the tested biotypes were pruned to uniform bud and shoot numbers and, after fruit set, to uniform bunch number both in 2003 and again in 2004, the later being the study year.

Four berry samplings were taken at regular intervals from the onset of veraison in 2004. The fourth was taken when the berries of the non-mutated SG 1-1 had reached a sugar concentration of about 17 °Brix. At harvest the mutated biotypes had a comparable °Brix level. At each sampling 60 berries were randomly collected from each biotype, immediately sealed in plastic bags and kept in ice until their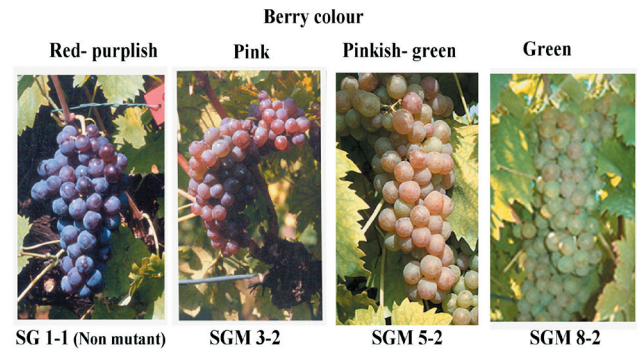 delivery to the laboratory. Each sample was divided into two groups: one was used for standard juice quality analyses (pH, soluble solids, acidity) and the second for anthocyanins biochemical and molecular analysis.

A n t h o c y a n i n   b i o c h e m i c a l   a n a l y s i s v i a   c h r o m a t o g r a p h y   a n d   s p e c t r o - p h o t o m e t r y : Skins of berry samples were manually separated from the flesh and seeds, weighed and frozen in liquid nitrogen and stored at -80 °C. The colour substance was extracted and assayed by HPLC after Downey *et al.* (2004) to determine the type of anthocyanins. Spectrophotometric analysis at 520 nm was then used to determine the total number of anthocyanins (expressed as mg of malvidin-3-monoglucoside per kg of fresh skin). For this analysis the ANOVA statistical procedure was performed to estimate the minimum significant difference in the mean percentage composition of the individual anthocyanins via Student-Newman-Kuels test.

T o t a l   g r a p e   s k i n   R N A   e x t r a c t - i o n   a n d   N o r t h e r n   b l o t   a n a l y s i s : All the RNA in the berry skin was extracted after the Hot Borate or XT Buffer protocol (Wan and Wilkins 1994), which was modified according to Boss *et al.* (1996 a). RNA yield and quality were determined by the absorbance spectrum from 220 to 320 nm with a Beckman DU-64 spectrophotometer. Northern blot analysis was run under standard procedures starting from 5 µg of total extracted skin RNA. The probes used correspond to the partial or complete cDNA clones of the genes isolated and described by Sparvoli *et al.* (1994) and Boss *et al.* (1996 b). The inserts were extracted from the cloning vector pBluescript SK+/- (Promega). The DNA bands matching each insert were isolated from agar gel after electrophoresis using the QIAquick Gel Extraction kit (QIAGEN Gel purification). Subsequent radioactive marking of the probes was carried out via random priming (Feinberg and Vogelstein 1983) with GigaPrime DNA Labelling kit (Besatec).

R e t r o t r a n s c r i p t i o n   o f   R N A   i n   c D N A : Because of their marked colour differentiation (Fig. 2), the retrotranscription of RNA in cDNA was performed for all biotypes using the SuperscriptTM II RNase H- Reverse Transcriptase (by Invitrogen) according to the protocol suggested by the manufacturer.

R e a l   t i m e   P C R : Real Time PCR was used for determining the expression of the *ufgt* gene in all biotypes. The expression values of the target gene are related

to those of an internal standard, *i.e.* the cytoplasmatic α-tubulin gene, that is constitutively expressed in all vine tissues and detected in the reaction conditions.

The amplification reactions were run in triplicate for both the standards and samples, with a final reaction volume of 25 μl and an amount of cDNA ranging from 50-100 ng. Each primer (VvUFGTf- 5'AGC TGG TAC TTC TTG GAC TAC GTC G- 3' and VvUFGTr- 5'ACT CCG TGA TCA GTC AGT CAG TTA TG-3') was used in the reaction mixture at 0.3 μM concentration. The amplification cycle included a 3-min first stage at 50 °C to activate the uracyl-N-glycosylase enzyme (UNG), a 3 s at 95 °C to denature UNG and activate Taq Polymerase, and then 35 cycles of 30 s for denaturation at 95 °C, 30 s for pairing at 57 °C and 30 s for extension at 72 °C; the emitted fluorescence was measured during these runs.

Results

S p e c t r o m e t r y   a n d   c h r o m a t o-g r a p h y : Fig. 3 shows the evolution of the anthocyanin concentration values recorded in samples. The biotypes whose absorbance value topped the threshold showed an increasing anthocyanin trend over the four weeks. The SG 1-1 control registered a final anthocyanin concentration of 925.75 mg·kg^{-1} skin, *i.e.* within the average for this cultivar. Absorbance was zero for SGM 8-2, the one with no berry pigmentation, and notably lower than control for the biotypes with a fairly marked colour SGM 3–2 (518 mg·kg^{-1}) and SGM 5-2 (124 mg·kg^{-1}).
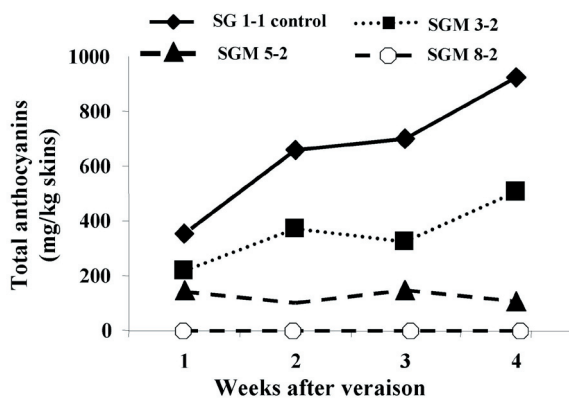

Fig. 3: Variation in total anthocyanin concentration in the skins of the 'Sangiovese' mutant biotype berries over the four weeks subsequent to veraison.

Reverse-phase HPLC was performed only on SG 1-1, SGM 3-2 and SGM 5-2 since no pigmentation was detected in SGM 8-2. The results are reported in Fig. 4 and expressed in per cent of total anthocyanin content per biotype. The non-mutated SG 1-1 registered seven anthocyanin types: 3-monoglucoside delphinidin, cyanidin, petunidin, peonidin and malvidin, the 3-acetylglucosides and the 3-p-coumaroylglucosides; the later two being present at the lowest concentration (0.5 and 2.5 % of the total respectively), thus confirming previous studies for the same variety growing in different conditions (Bucelli *et al.* 1992, Baldi *et al.* 1992, Bellincontro *et al.* 2004).
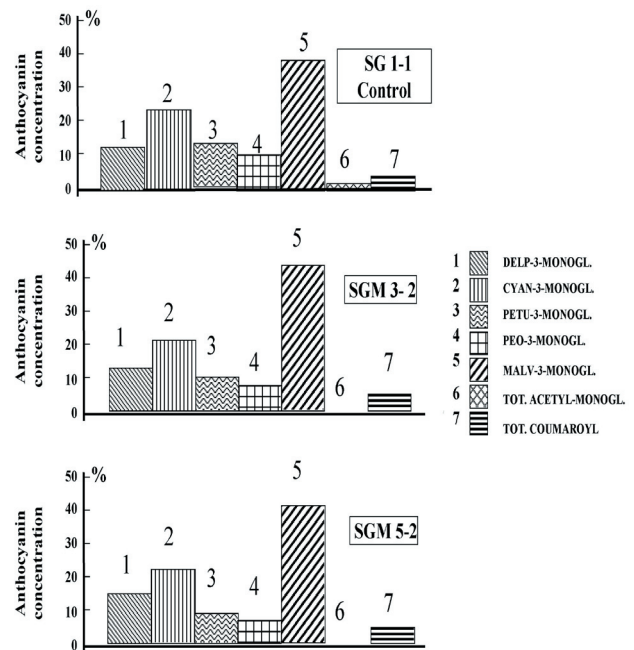

Fig. 4: Anthocyanin profile of the skins of biotypes SG 1-1, SGM 3-2 and SGM 5-2 as determined by HPLC on the samples collected 4 weeks after veraison: 1 = delphinidin 3-monoglucoside; 2 = cyanidin 3-monoglucoside; 3 = petunidin 3-monoglucoside; 4 = peonidin 3-monoglucoside; 5 = malvidin 3-monoglucoside; 6 = total acetyl-3-monoglucosides; 7 = total coumaroyl 3-monoglucosides. No statistical differences among the profile of the tested biotypes were found

In the mutated SGM 3-2 and 5-2 all the acetyl-glucoside forms were completely absent, while their anthocyanin profiles show a distinct prevalence of the monoglucosides malvidin and cyanidin, which alone account for 60 % of the total, followed by delphinidin, peonidin and petunidin monoglucosidates. No statistical differences (p >0.05) among the profiles of the tested biotypes were found by ANOVA analysis, thus demonstrating that the differences among them are rather quantitative than qualitative.

N o r t h e r n   b l o t   a n a l y s i s   o f   t h e   s t r u c t u r a l   g e n e s   f o r   a n t h o c y a n i n   b i o s y n t h e s i s : The results of these assays, which refer to the fourth post-veraison week, are shown in Fig. 5 and indicate a marked expression in the tested biotypes for the *ufgt* gene, which at that time was clearly detectable in the SG 1-1 control and in the partially de-coloured SGM 3-2 and 5-2. At the same time this gene was either not expressed, or not detectable, in SGM 8-2. By contrast, the CHS, CHI, F3H, DFR, LDOX biosynthetic genes were more or less expressed in the tested biotypes although the samples from the most de-coloured skins registered a lower level of expression.

R e a l   t i m e   P C R : Fig. 6 shows the test results for the *ufgt* gene, expressed in per cent of peak value registered among all biotypes at the four sampling dates. The data are derived from the relationship between the concentration of the *ufgt* gene and that of the endogenous reference α-Tubulin gene, which was amplified in parallel analysis for each sample and with the same conditions. The reported value at each point is the average from three replicates per each sample.
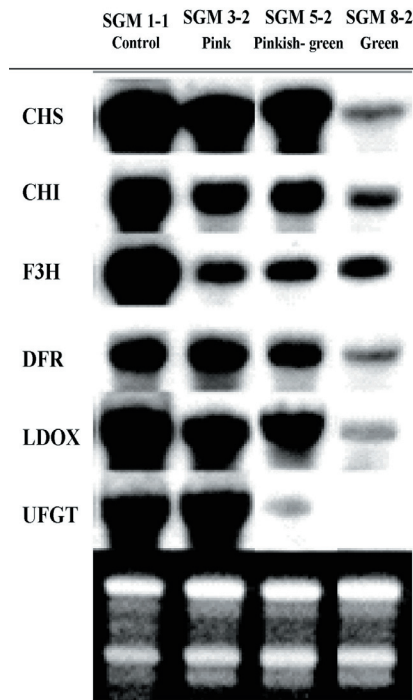
Fig. 5: Northern blot results from hybridization of RNA extracted from biotypes SG 1-1, SGM 3-2, SGM 5-2 and SGM 8-2 at post-veraison week 4 using probes matching the structural genes chs, chi, f3h, dfr, ldox and ufgt. Bottom: the RNA of the same samples separated on agar gel and visualised under ultraviolet light.

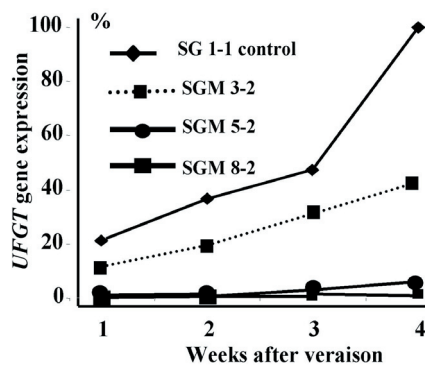Fig. 6: The ufgt gene expression pattern in the mutated 'Sangiovese' biotypes and in non-mutated control over the four post-veraison weeks: The values are expressed as percentage of peak recorded value, measured via Real Time PCR and calculated on the threshold cycle recorded for each reaction.

The mRNA pairs of *ufgt* were found at peak value in the SG 1-1 control, at about 60 % and 95 % less in the SGM 3-2 and SGM 5-2 respectively but all were missing in the colourless 8-2. Real Time analysis thus confirms the qualitative data from Northern blot while quantifying the tested gene's transcripts and providing an estimation of the time course of its expression level in the analysed biotypes.

Discussion

According to Boss *et al.* (1996 c), the *ufgt* gene in grapevine evinces a markedly differential expression pattern between red and de-coloured or white biotypes,

whereas all the other genes of the anthocyanin biosynthesis pathway are expressed in all biotypes regardless of their berry colour. Our results for 'Sangiovese' mutants confirm this finding and support the idea that the control system of anthocyanin biosynthesis in berry grapevine is further upstream than in species like arabidopsis or maize (Martin and Gerat 1993). Indeed, anthocyanin synthesis in our de-pigmented grape bunches is impeded because the *ufgt* fails to gain expression despite the fact that a complete and non-mutated copy of the correspondent gene is present (data not showed). No mutations were detected in the promoter region of the *ufgt* gene in the different biotypes (data not shown). Thus, as found in recent works on other colour mutated cultivars (Kobayashi *et al.* 2004 and 2006, Walker *et al.* 2007), the berry depigmentation of 'Sangiovese' bud sports can also be ascribed to a mutation affecting one or more transcription factors that activate the *ufgt* gene expression.

The tested biotypes featuring partial or full loss of colour, as is usually the rule with white cultivars, also showed a weaker expression of all the biosynthesis genes implicated in anthocyanin metabolism than the non-mutated red- purplish biotypes. This response may be explained by the role of the mutated element at varying metabolic levels, whether via direct action on the other genes involved in biosynthesis or via the indirect action of an inhibitory feedback loop generated by accumulation of the substrate upon which the enzyme encoded by the *ufgt* gene is supposed to act.

Our biomolecular data on the UFGT enzyme appear to support the view that there is only one form of the enzyme in 'Sangiovese' acting on all the different types of anthocyanins produced by a given tissue in proportion to the action of the transcription factors. This assumption is reinforced by the fact that the biotype chromatographic assays (Fig. 4) show anthocyanin profiles marked by quantitative but not qualitative variations. Thus, as reported by Walker *et al.* (2006) for 'Cabernet Sauvignon' color mutants, the depigmented 'Sangiovese' biotypes could equally derive from a bud sport leading to lack of anthocyanins in sub-epidermal cell layers (as for the pale colored berries) and in the whole skin tissues (as for the green berries).

Further researches in 'Sangiovese' biotypes should move a step closer to disclosing the molecular basis at the origin of the bud sports either mutation in regulatory genes (Walker *et al.* 2007) or their allelic variations (This *et al.* 2007). This should help to isolate and characterise transcription factors such as VvMybA1 e VvMybA2 and make possible a far greater understanding of how they 'drive' anthocyanin synthesis towards greater colour expression for superior quality grapes.

References

Baldi, A.; Romani, A.; Mulinacci, N.; Vincieri, F.; Casetta, B.; 1992: HPLC/MS application to anthocyanins of *Vitis vinifera* L. J. Agric. Food Chem. **43**, 2104-2019.

Bellincontro, A.; De Santis, D.; Botondi, R.; Villa, I., Mencarelli, F.; 2004: Different postharvest dehydration rates affect quality char-

acteristics and volatile compounds of Malvasia, Trebbiano and Sangiovese grapes for wine production. J. Sci. Food Agric. **84**, 1791-1800.

Boss, P. K.; Davies, C.; Robinson, S .P.; 1996 a: Expression of anthocyanin biosynthesis pathway genes in red and white grapes. Plant Mol. Biol. **32**, 565-569.

Boss, P. K.; Davies, C.; Robinson, S.P.; 1996 b: Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol. **111**, 1059-1066.

Boss, P. K.; Davies, C.; Robinson, S. P.; 1996 c: Anthocyanin composition and anthocyanin pathway gene expression in grapevine sports differing in berry skin colour. Aust. J. Grape Wine Res. **2**, 163-170.

Bucelli, P.; Faviere, V.; Riannetti, G.; Gigliotti, A.; 1992: Sviluppo e caratterizzazione degli antociani delle bacche e delle foglie di Sangiovese dall'invaiatura alla maturazione. Vignevini **19** (1/2), 61-66.

Considine, J. A.; Knox, R. B.; 1981: Tissue origins, cell lineages and pattern of cell division in the developing dermal system of the fruit of *Vitis vinifera* L. Planta **151**, 403-412.

Cravero, M. C.; Guidoni, S.; Schneider, A.; DiStefano R.; 1994: Morphological and biological characterization of coloured berry-muscat grapevine cultivars. Vitis **33**, 75-80.

Downey, M. O.; Harvey, J. S.; Robinson, S. P.; 2004: The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grape. Aust. J. Grape Wine Res. **10**, 55-73.

Feinberg, A. P.; Vogelstein, B.; 1983: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**, 6-13.

Fregoni, M.; 1974: La "Malvasia rossa" nuova mutazione gemmaria. Frutticoltura **3**, 5-8.

Galet, P.; 1979: A practical Ampelography: Grapevine Identification. Ithaca, N.Y, Comstock Pub. Associates.

Intrieri, C.; 1981: Vitigni Provenienti da Mutazione Gemmaria. Relazione sull'Attività Svolta nel 1980 dalla Sezione Viticola del CRIVE, Bologna, Italy.

Intrieri, C.; Silvestroni, O.; 1985: Primi Risultati della Caratterizzazione di Mutanti del Vitigno Sangiovese. Estratto dagli Atti dell'Accademia Italiana della Vite e del Vino.

Kobayashi, S., Ishimaru, M.; Hiraoka, K.; Honda, C.; 2002: Myb-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta **215**, 924-933.

Kobayashi, S.; Goto-Yamamoto, N.; Hirochika, H.; 2004: Retrotransposon-induced mutations in grape skin color. Science **304**, 982.

Kobayashi, S.; Yamamoto, N.G.; Hirochika, H.; 2006: Association of VvmybA1 gene expression with anthocyanin production in grape (*Vitis vinifera*) skin – color mutants. J. Jpn. Soc. Hortic. Sci. **74**, 196-203.

Martin, C.; Gerats, T.; 1993. Control of pigment biosynthesis genes during petal development. Plant Cell. **5**, 1253-1264.

Sparvoli, F.; Martin, C.; Scienza, A.; Gavazzi, G.; Tonelli, C.; 1994: Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape *Vitis vinifera* L. Plant Mol. Biol. **24**, 743-755.

Solari, C.; Giudici, P.; Intrieri, C.; Guerzoni, M. E.; Silvestroni, O.; 1985: L'impiego della microvinificazione nella selezione della vite: Esperienze su biotipi mutati di "Sangiovese" (*V. vinifera* L.). Vignevini **5**, 47-54.

This, P.; Lacombe, T.; Cadle-Davidson, M.; Owens C. L.; 2007: Wine grape (*Vitis vinifera L.)* color associates with allelic variation in the domestication gene VvMybA1. Theor. Appl. Genet. **114**, 223-230.

Verriès, C.; Bès, C.; This, P.; Tesnière, C.; 2000: Cloning and characterization of Vine-1, a LTR-retrotransposon-like element in *Vitis vinifera L.,* and other Vitis species. Genome **43**, 366-376.

Walker A. R.; Lee, E.; Robinson S.; 2006: Two new grape cultivars, bud sports of Cabernet Sauvignon bearing pale-coloured berries, are the result of deletion of two regulatory genes of the berry colour locus. Plant Mol. Biol. **62**, 623-635.

Walker, A. R.; Lee, E.; Bogs, J.; McDavid, D. A. J.; Thomas, M. R.; Robinson, S.; 2007: White grapes arose through the mutation of two similar and adjacent regulatory genes. Plant J. **49**, 772-785.

Wan, C. Y.; Wilkins, T.; 1994: A modified hot borate method significantly enhances the yield of high-quality RNA from cotton *Gossypium hirsutum* L. Anal. Biochem. **223**, 7-12.