Ethanol triggers grape gene expression leading to anthocyanin accumulation during berry ripening

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Abstract

Recent studies have shown that low doses of ethanol stimulate the maturation of some fruits. The present work showed that spraying Cabernet Sauvignon grapes, with 5% ethanol at veraison enhances the anthocyanin accumulation. Veraison is the time when the berries turn from green to purple. HPLC analysis showed a marked increase in the total concentrations of the derivatives of delphinidin, cyanidin, petunidin, peonidin and malvidin from the fourth day after the ethanol treatment until harvest. This was not linked to a difference in berry weight in comparison to controls. Two distinct expression patterns were found for anthocyanin biosynthesis genes in the treated and untreated berries. For one group, consisting of chalcone synthase, flavanone-3-hydroxylase, dihydroxyflavonol-4-reductase and leucoanthocyanidin dioxygenase, the expression was inhibited or unchanged by the ethanol treatment, whereas for UDP glucose-flavonoid 3-*O*-glucosyltransferase (UFGT) there was a marked increase in expression from 1 to 20 days after ethanol treatment. These results suggest that the UFGT gene is a key factor in the observed anthocyanin accumulation following ethanol treatment.

Keywords: *Vitis vinifera*; Maturation; Ethanol; Anthocyanins; Glucosyltransferase

1. Introduction

The application of exogenous ethanol has been reported to inhibit or promote tomato fruit ripening in a dose-dependent manner, correlated to ethylene evolution [1]. Farag et al. [2] showed that ethanol in combination with an ethylene precursor enhanced anthocyanin content of cranberries. In a recent study [3], we observed that spraying an aqueous solution of ethanol onto grapes at veraison enhanced internal ethylene production. We also noticed that berry skin extracts resulting from the ethanol treatment showed increased absorbance at 520 nm. This is the wavelength at which anthocyanins, the main colored pigments of grapes [4], have the maximum absorption. In this paper, we report HPLC analyses of berry skin extracts, performed to determine which anthocyanin species accumulated after the ethanol treatment.

In grapes a number of structural genes involved in anthocyanin biosynthesis have been cloned (PAL, CHS, CHI, DFR, F3H, LDOX, and UFGT) [5]. PAL stands for phenylalanine ammonia-lyase, CHS for chalcone synthase, CHI for chalcone isomerase, F3H for flavanone-3-hydroxylase, DFR for dihydroxyflavonol-4-reductase, LDOX for leucoanthocyanidin dioxygenase and UFGT for UDP glucose-flavonoid 3-*O*-glucosyl-

Abbreviations: CHS, chalcone synthase; DFR, dihydroxyflavonol-4-reductase; F3H, flavanone-3-hydroxylase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP glucose-flavonoid 3-*O*-glucosyltransferase.

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transferase. Their transcription rates change according to the berry development state, and a marked increase was observed at veraison for some of them, depending on the cultivars [6,7]. It has been shown that some of these genes are regulated by treatments with auxins which inhibit ethylene action [8]. In addition, the exposure of fruits other than grapes to ethylene, resulted in an increased transcription of CHS and F3H [9,10].

Thus, in addition to the HPLC analysis, we checked the effect of ethanol on the transcription of CHS, DFR, F3H, LDOX, and UFGT during berry development after veraison.

2. Material and methods

2.1. Grapes and treatments at veraison

Treatments were carried out in 1999, on 10 individual vines of Cabernet Sauvignon (*Vitis vinifera* L.), grafted on 110 Richter (*Vitis Berlandieri* × *Vitis rupestris*), in a vineyard close to Toulouse (France). The treatments took place 8–9 weeks after anthesis when 50% of berries changed color (first fortnight of August). Clusters were sprayed (back pump) with ethanol at 5% in water. Spraying was performed at a rate equivalent to 200 l/ha. Control vines were sprayed with water. Five clusters per treatment were sampled, each on a separate vine, at various times after spraying (0, 1, 4, 20 and 56 days 'at harvest'). Batches of twenty randomly chosen berries were used to calculate the berry weight. Berries were then kept frozen at −80 °C.

2.2. RNA and protein extractions, Northern and Western blots

Total RNA was extracted from the samples according to Boss et al. [7]. Aliquots of 15 μg of RNA were denatured and run on a 1.2% agarose gel (containing 3% formaldehyde and 10% MOPS). Northern blot analysis was performed using the available probes of CHS, DFR, F3H, LDOX, and UFGT genes cloned by Sparvoli et al. [5]. The method of Boss et al. [7] was used with the following modifications. The RNA was transferred to a Gene Screen Plus® hybridization transfer membrane (NEN™ Life Science Products, Boston, MA) with a vacuum blotter (mod. TDNA, Appligene, Illkirsch, France) for 3.5 h at 45–50 mbars. Then prehybridization was run for 4 h at 42 °C in 5x SSPE, 50% (w/v) deionized formamide, 1% SDS, 10% dextran-$NaSO_4$ MW 500 000 and 10 μg/ml denatured salmon sperm. The membranes were hybridized for 15 h at 42 °C with the addition of denatured ^{32}P-labelled cDNAs probes prepared by random primer labeling (Ready-To-Go DNA Labeling Kit, Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was then washed twice for 10 min at 42 °C and then once for 15 min at 42 °C and exposed to the autoradiography film (Hyper film, Amersham Pharmacia Biotech). The membranes were also hybridized with a 18S probe. The hybridization signal intensity was assessed with the SigmaScan software (SPSS Inc, Chicago). To enable the comparison, the hybridization signals were adjusted according to the mean 18S signal of each membrane, then the adjusted values were expressed as percentages of the means of the day 0 signal (100%). Blots were performed in duplicates with different RNA samples, extracted from berries of different vines.

Proteins were extracted as described previously [11] and Western blots and immunodetection were performed according to Ford et al. [12]. Equal protein amounts (10 μg) were loaded onto each lane (data not shown).

2.3. Grape skin extracts and HPLC analyses

Methanolic extraction was performed according to Boss et al. [7]. Briefly, berries were individually thawed in warm water and skins were frozen immediately after peeling, then ground in mortar and pestle with liquid nitrogen; we used skins of 20 berries per replicate randomly chosen among each batch of 5 clusters. Separation of the anthocyanins was performed by direct HPLC analysis on a reversed stationary phase as described by Cheynier et al. [13]. Anthocyanins were analysed at 520 nm with a diode array detector and identified according to the retention times and UV spectral data. Quantifications were based on peak areas at 520 nm using a response factor calculated with malvidin-3-glucoside. This compound was purchased from Extrasynthese (Genay, France).

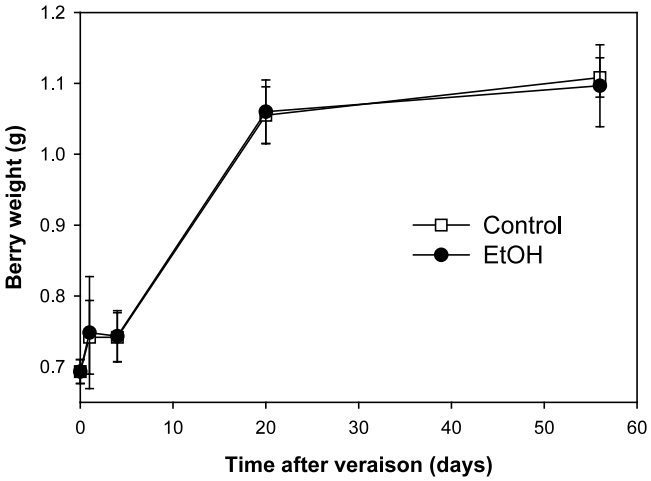

Fig. 1. Effect of a 5% ethanol (EtOH) treatment at veraison (day 0) on berry weight up to harvest (day 56); $n = 3$, error bars represent S.E.

3. Results and discussion

3.1. Berry weight and color of skin extracts

The berry weight did not changed between the controls and the treated berries over the ripening period (Fig. 1). These results confirmed our observations [3], which showed that ethanol induced an increase in the absorbance at 520 nm of berry skin extracts without a change of berry weight at harvest.

3.2. HPLC analysis

The HPLC analysis of berry skin extracts showed that ethanol sprayed at veraison stimulated the accumulation of the five main anthocyanins present in berry skins, namely delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside, from 4 days after treatment until harvest (Fig. 2). Malvidin-3-glucoside derivatives were the main pigments at harvest and their levels were nearly

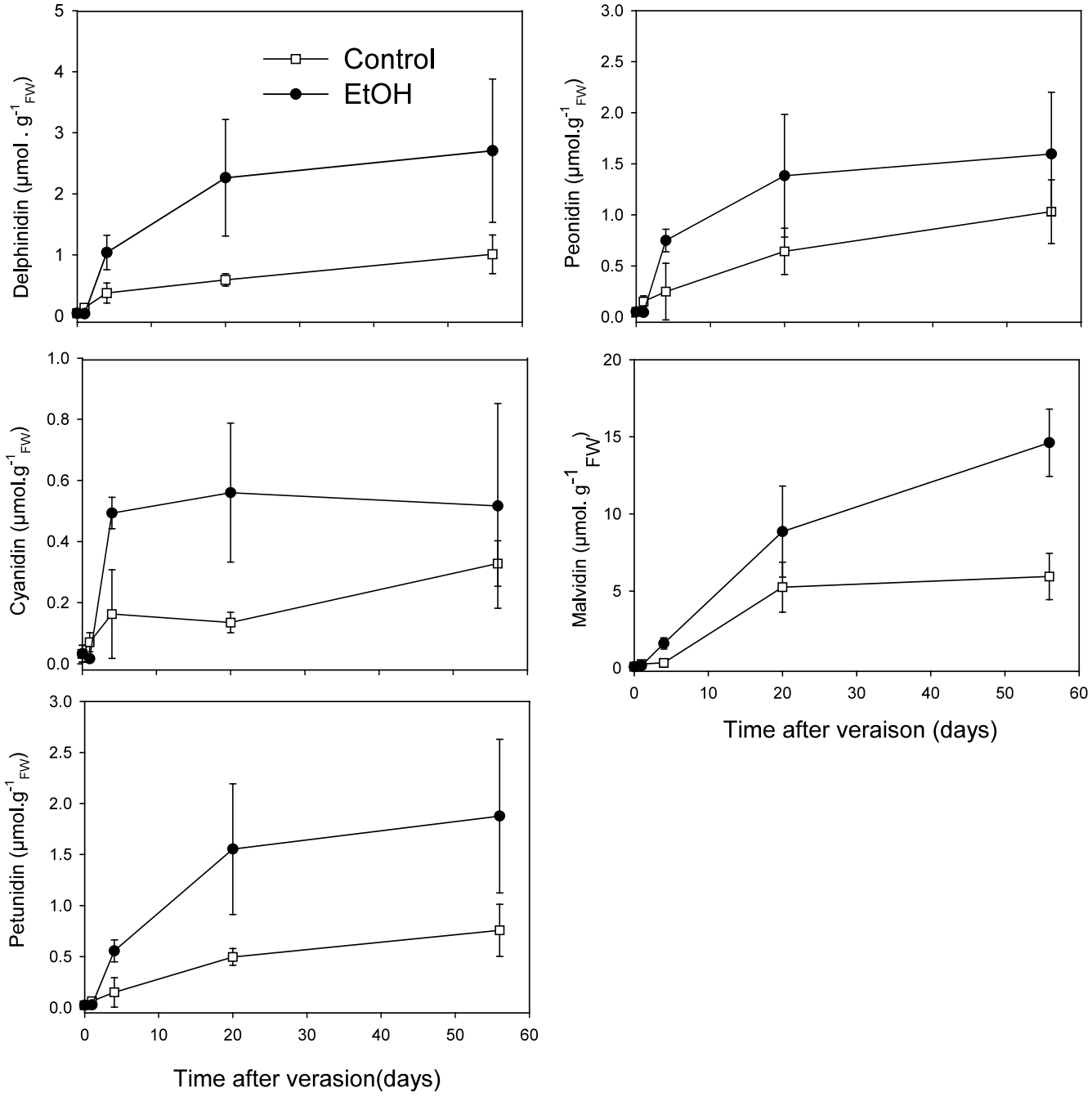

Fig. 2. Accumulation of the main anthocyanins in grape berry skins after spraying at veraison with 5% ethanol (EtOH). Each point is the sum of monoglucosides, acetylglucosides and p-coumarylglucosides; $n = 3$, error bars represent S.E.

three fold higher in ethanol treated berries than in controls. There was no change in the relative distribution between monoglucosides, acetylglucosides and *p*-coumarylglucosides (data not shown), which were present in percentages similar to those previously described [4].

These results suggest that the increased anthocyanin levels, previously observed in cranberries when treated

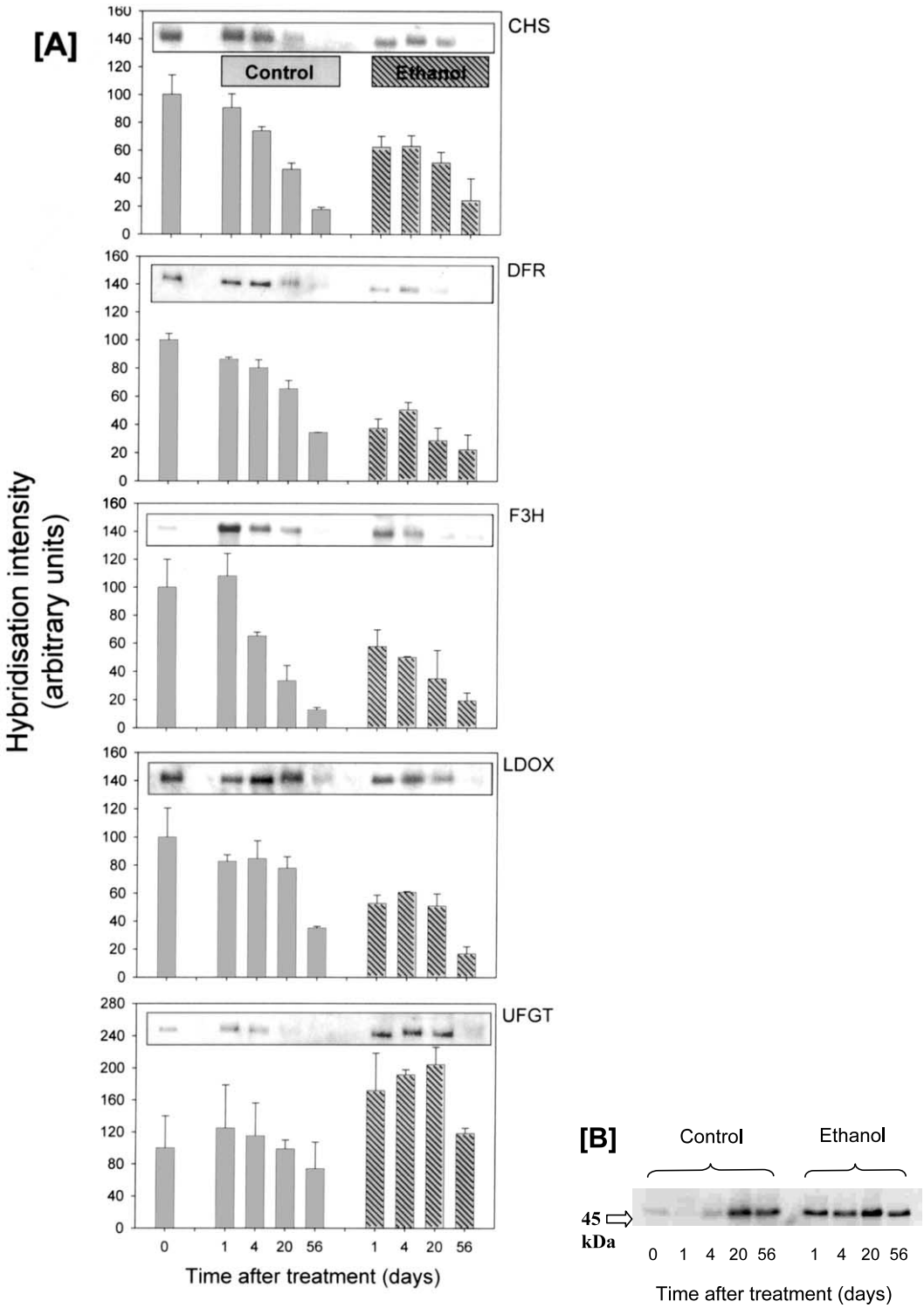

Fig. 3. (A) Relative transcription of the five of anthocyanin genes, CHS, F3H, DFR, LDOX and UFGT, after an ethanol (5%) treatment at veraison (day 0) up to harvest (day 56), in comparison to controls. The inserted images show the most representative blots; $n = 2$, error bars represent S.E. (B) Immunoblot of total soluble protein extracts with rabbit antibodies raised against rUFGT as described in Ford et al. [12].

with a combination of an ethylene precursor and ethanol [2], were not only due to ethanol increasing the ethylene precursor diffusion through cell membranes as suggested by their authors, but may be partly due to an effect of ethanol itself on anthocyanin biosynthesis. In the next part of our work, we studied the transcription rate of some of the genes involved in the anthocyanin synthesis, using available cDNA probes.

3.3. Gene transcription and expression

Treatment of Cabernet Sauvignon grapes with 5% ethanol at veraison resulted in some altered regulation of the transcription of anthocyanin biosynthesis genes compared to the controls. Ethanol treatment had no stimulating effect on the transcription of genes in the early part of the anthocyanin biosynthesis pathway, including CHS, DFR, F3H and LDOX (Fig. 3). However, both Northern and Western blot analyses indicated that the UFGT gene was more highly expressed than in controls from the first day after ethanol treatment until 20 days later (Fig. 3). This ethanol effect, observed for the first time in grapes, could be indirect through an increase of C_2H_4 production [3], which is known to induce UFGT activity in fruit [14]. Moreover, this effect could be partly due to the conversion of some ethanol into acetaldehyde, which is more efficient than ethanol in inducing ethylene production in fruit, [1,15,16] or could involve a stress response in the treated plants.

These results provide new evidence that UFGT plays an important role in increasing the accumulation of anthocyanins in red cultivars, as well as its role in determining the difference between red and white grapes [6,17].

In apples, it was reported that chalcone synthase does not play a regulatory role in anthocyanin synthesis, whereas like UFGT expression in grapevine, the UFGaIT activity was correlated with anthocyanin synthesis during fruit maturation [18]. This may be due to its effect on anthocyanidin glycosylation, which usually confers stability to anthocyanins [19].

The pattern of gene expression in grape berry could be explained in relation to anthocyanin regulatory genes (i.e. those that control the expression of the structural genes of the pathway). Two types of regulatory genes may be present, one that controls the expression of PAL, CHS, CHI, F3H, DFR and LDOX and another that induces UFGT gene expression [7]. Sequence analysis showed that there was no difference either in the coding region or in the promoter of the UFGT gene between white and the red grape cultivars, suggesting differences in the UFGT regulatory factors [17]. These factors which control the expression of the anthocyanin structural genes, including UFGT, have been identified in other species, but have not been yet isolated in grapes. Thus, a possible explication of our results is that ethanol treatment can affect the expression of UFGT regulatory factors.

It is clear that UFGT genes, whose expression is correlated with anthocyanin synthesis, are regulated by regulatory genes that can respond to exogenous treatment such as auxin in grapes [8] and ethylene in apples [18]. However, the only paper describing the induction of glycosyltransferase activity by ethanol comes from a study on rats [20].

In conclusion, the effect of ethanol sprayed at veraison on anthocyanin accumulation in grape berries seems to be due to its stimulatory effect on UFGT gene transcription rather than on the other anthocyanin genes studied (CHS, DFR, F3H and LDOX). This increase did not change the relative distribution among the different anthocyanin derivatives. However, we cannot exclude that other genes or cellular functions are involved in this response, and we have not checked the enzyme activities corresponding to the genes for which northern analysis were conducted.

These results offer new perspectives to improve berry color of grape varieties showing anthocyanin deficiencies or of grapes cultivated in climate areas where full maturity is hard to reach. Ethanol, a simple organic compound, could match the sustainable viticulture needs. However, it is essential that we determine the optimal ethanol dose according to the sprayer type. More work has been initiated to study the best dose and the best moment for application of ethanol on grape berries in order to achieve greater anthocyanin accumulation.

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References

[1] J.C. Beaulieu, M.E. Saltveit, Inhibition or promotion of tomato fruit ripening by acetaldehyde and ethanol is concentration dependent and varies with initial fruit maturity, J. Amer. Soc. Hort. Sci. 122 (1997) 392–398.

[2] K.M. Farag, J.P. Palta, E.J. Stang, Ethanol enhances the effectiveness of ethephon on anthocyanin production in cranberry fruits in the field, HortScience 27 (1992) 411–412.

[3] C Chervin, A. Elkereamy, J.P. Roustan, J.D. Faragher, A. Latche, J.C. Pech, M. Bouzayen, An ethanol spray at veraison

enhances colour in red wines, Aust. J. Grape Wine Res. 7 (2001) 144–145.
[4] G. Mazza, Anthocyanins in grapes and grape products, Crit. Rev. Food Nutr. 35 (1995) 341–371.
[5] F. Sparvoli, C. Martin, A. Scienza, G. Gavazzi, C. Tonelli, Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis Vinifera* L.), Plant Mol. Biol. 24 (1994) 743–755.
[6] P.K. Boss, C. Davies, S.P. Robinson, Expression of anthocyanin biosynthesis pathway genes in red and white grapes, Plant Mol. Biol. 32 (1996) 565–569.
[7] P.K. Boss, C. Davies, S.P. Robinson, Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implication for pathway regulation, Plant Physiol. 111 (1996) 1059–1066.
[8] C. Davies, P.K. Boss, S.P. Robinson, Treatment of grape berries, a nonclimacteric fruit with a synthetic auxin, retards ripening and alters the expression of developmentally regulated genes, Plant Physiol. 115 (1997) 1155–1161.
[9] R. Ardi, I. Kobiler, B. Jacoby, N.T. Keen, D. Prusky, Involvement of epicatechin biosynthesis in the activation of the mechanism of resistance of avocado fruits to *Colletotrichum gloeosporioides*, Physiol. Mol. Plant Pathol. 53 (1998) 269–285.
[10] A.K. Kanellis, G. Diallinas, Developmental and ripening regulation of gene expression of phenylpropanoid biosynthetic enzymes in melon fruit, Proceedings of 'Postharvest physiology and pathology', International Symposium, Agadir, Morocco, 16–21 January 1994, Institut Agronomique et Veterinaire Hassan II, (1995) 353–360.
[11] C.M. Ford, P.B. Høj, Multiple glucosyltransferase activities in the grapevine *Vitis vinifera* L., Aust. J. Grape Wine Res. 4 (1998) 48–58.
[12] C.M. Ford, P.K. Boss, P.B. Høj, Cloning and characterisation of *Vitis vinifera* UDP-glucose: flavonoid 3-*O*-glucosyltransferase, a homologue of the enzyme encoded by the maize Bronze-1 locus that may primarily serve to glucosylate anthocyanidins in vivo, J. Biol. Chem. 273 (1998) 9224–9233.
[13] V. Cheynier, I. Hidalgo Arellano, J.M. Souquet, M. Moutounet, Estimation of the oxidative changes in phenolic compounds of Carignane during winemaking, Am. J. Enol. Vitic. 48 (1997) 225–228.
[14] G. Hrazdina, G.F. Parsons, L.R. Mattick, Physiological and biochemical events during development and maturation of grape berries, Am. J. Enol. Vitic. 35 (1984) 220–227.
[15] F. Mencarelli, P. Savarese, M.E. Satveit, Ripening of kiwifruit exposed to ethanol and acetaldehyde vapors, Hort. Sci. 26 (1991) 566–569.
[16] E. Pesis, R. Marinansky, Carbon dioxide and ethylene production by harvested grape berries in response to acetaldehyde and ethanol, J. Amer. Soc. Hort. Sci. 117 (1992) 110–113.
[17] S. Kobayashi, M. Ishimaru, C.K. Ding, H. Yakushji, N. Goto, Comparison of UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) gene sequence between white grapes (*Vitis vinifera*) and their sports with red skin, Plant Sci. 160 (2001) 543–550.
[18] Z. Ju, L. Chenglian, Y. Yongbing, Activities of chalcone synthase and UFGA1: flavonoid-3-*O*-glycosyltransferase in relation to anthocyanin synthesis in apple, Sci. Hort. 63 (1995) 175–185.
[19] C.B. Do, F. Cormier, Y. Nicolas, Isolation and characterization of UDP-glucose cyanidin 3-*O*-glucosyltransferase from grape cell suspension cultures (*Vitis vinifera* L.), Plant Sci. 112 (1995) 43–51.
[20] C.S. Lieber, E. Rubin, Ethanol increase glycosyl transferase activity in the hepatic golgi apparatus, Nature, New Biol. 243 (125) (1973) 123–125.