Divergent gene expression patterns in the berry skin cells of Sicilian grapes.

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ABSTRACT

We present the transcription patterns of some flavonoid genes, expressed during late development, by the berries skin cells of grapes (cv Nero d'Avola, Nerello Mascalese and Syrah) cultivated in two areas of Sicily (Marsala in the west and Mount Etna in the east). The expression of ten genes encoding for metabolic enzymes and of five genes encoding for Myb-family transcriptional activators was measured in real-time quantitative PCR experiments. The patterns we observed differed in part from those reported in the scientific literature: during late development (post véraison), berry skin cells of Sicilian grapes express *mybPA1*, *myb5a*, *lar* and *anr* genes that are typical of earlier (pre-véraison) stages. This might have consequences for the quality of the grapes cultivated in this area.

RIASSUNTO

In questo lavoro presentiamo i profili di espressione di alcuni geni coinvolti nella sintesi dei flavonoidi, espressi durante lo sviluppo tardivo, dalle cellule della buccia degli acini di uve (cv Nero d'Avola, Nerello Mascalese e Syrah) coltivate in due aree della Sicilia (Marsala nella parte occidentale ed Etna nella parte orientale). In esperimenti di PCR quantitativa real-time, è stata studiata l'espressione di dieci geni codificanti per enzimi metabolici e di 5 geni codificanti fattori di regolazione trascrizionale della famiglia Myb. I profili che abbiamo osservato differiscono da quelli riportati in letteratura: durante lo sviluppo tardivo (post-invaiatura) le cellule della buccia delle uve siciliane esprimono geni tipici di stadi precoci, pre-invaiatura, quali *mybPA1*, *myb5a*, *lar* e *anr*. Questo potrebbe avere conseguenze per la qualità delle uve coltivate in quest'area.

INTRODUCTION

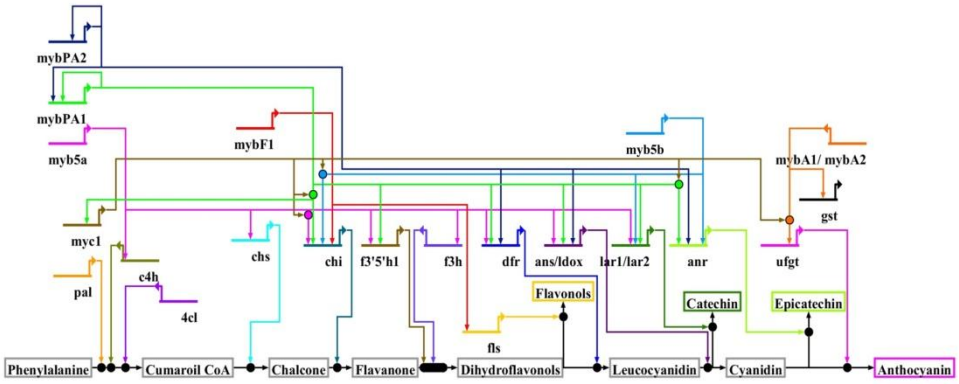
Flavonoids are secondary metabolites produced in the skin and in the seeds of maturing grape berries, which play important roles in the definition of the color, confer antioxidant and anti UV-damage properties and help protect the seeds from herbivores by making the grapes astringent until full maturation.

These different functions are executed in a time-regulated fashion during berry development, thanks to the carefully orchestrated activation of the genes encoding for the enzymes in the flavonoid synthesis pathway and for their transcriptional regulators (*e.g.* Bogs et al., 2005; Boss et al., 1996; Cutanda-Perez et al., 2009; Czemmel et al., 2009; Ford et al., 1998).

A number of laboratories worldwide have been involved in elucidating the details of the flavonoid genes expression regulation; a general picture has emerged in which flavanols

(which confer astringency) are synthesized before véraison; anthocyanins (which define the color) are synthesized afterward; flavonols (which participate in color stabilization reactions) can be synthesized at different times. Also, their synthesis can also be affected by environmental and climatic factors (*e.g.* Castellarin et al., 2011; Cohen et al., 2012; Jeong et al., 2004). These three classes of compounds are produced from branches of a common (central) pathway (fig. 1).

Figure 1



A simplified view of the grape flavonoid pathway. Genes are represented by horizontal lines topped by a bent arrow. Metabolic products, by grey boxes. Activations by arrows impinging on other genes (transcriptional activation) or on black ovals (enzymatic catalyses).

Myb-family factors participate in the transcriptional regulation of the flavonoid synthesis genes; among them *mybPA1* and *mybPA2* regulate the expression of *lar* and *anr* genes (flavanols synthesis), typically expressed before the onset of véraison. Several general activators have been also identified; among these, *myb5a* which regulates a number of genes in the pathway and which is activate before véraison (reviewed in (Czemmel et al., 2012)).
Despite its importance, there are no studies concerning flavonoid gene expression in grapes cultivated in Sicily, the fourth wine producing region of Italy. Therefore we have began an investigation on flavonoid gene expression in two areas of Sicily, characterized by different climate: the Marsala area in the West (semi-arid climate) and Mount Etna in the East (mild continental climate). We monitored the expression of ten genes for metabolic enzymes and five genes for Myb-family transcription factors, from véraison until ripeness in three *cv*: Nero d'Avola (Marsala), Nerello Mascalese (Mount Etna) and Syrah (cultivated in both sites). Our results show that in Sicily genes typically expressed only before véraison, continue to be expressed afterward.

MATERIALS AND METHODS

Sampling

Marsala samples. Nero d'Avola and Syrah grape berries (both *V. vinifera* L.) were sampled during summer of 2012, from field-grown vines of the "Istituto Regionale del Vino e dell'Olio" vineyard, 37°47'12'' North; 12°33'43'' East, Biesina district, Marsala, Trapani, Italy. The vineyard were located at about 95 mt above sea level (a.s.l.). Climate was semi-arid, with no rainfalls recorded during the observation period. Plantation year was 1997 for the Nero d'Avola and 2007 for the Syrah grapes. Soil was of average composition, clayey. The rows were oriented North-South, the vines were trained with a vertical trellis system and pruned according to the Guyot system. Distance between the rows was 2.5m; within each row, plants were positioned every 1.2 m (Syrah) or 1.5 m (Nero d'Avola); rows were 1.5 m

tall, had 3000 and 2666 plants/ha respectively, and produced about 100 quintals/ha. Locally selected clones were grafted on "140 Ruggeri" wild vines rootstocks in both cases. No irrigation was provided to the Syrah, while auxiliary irrigation (120 liters/plant in the first few days of July) was provided to the Nero d'Avola vines. Sampling began at 50% berries coloration (July 20 and July 24 for the Syrah and Nero d'Avola respectively) when the ^{o}Brix was 11.6±0.1 and ended when the ^{o}Brix was 20.8±0.7 (August 24).
Mount Etna samples. Grape berries of the Nerello mascalese and Syrah cultivars (*V. vinifera* L.) were sampled during summer of the same year, from field-grown vines of the "Cottanera" winery in Catiglione di Sicilia, 37^{o}53'10'' North; 15^{o}1'49'' East; Catania, Italy. The field was 650 m a.s.l., on a sandy, volcanic, mildly acidic soil. Climate is mild-continental. No rainfalls were recorded during the observation period. Rows were oriented North-South; vines were trained using the "spurred cordon" system, with no pruning. Distance between the rows was 2.3m and plants were positioned every 0.8m. Rows were 1.9 m tall. Syrah vines were implanted in 1999 (clones 525, 383, 174 on 420A rootstocks); Nerello mascalese vines were implanted in 2000 (Nerello M. standard on 110R rootstocks). Production was 5680 plants/ha, 70-100 quintals/ha; no irrigation was provided. Sampling began August 6 (40-50% coloration for the "Nerello mascalese", 50-60% coloration for the Syrah) when the ^{o}Brix was 14.2±0.5 and ended September 5 when the ^{o}Brix was 19.85±0.95.

RNA extraction, cDNA synthesis, real time PCR and data analyses

About 25 berries (in triplicate) were randomly picked at each sampling and the skins were immersed in 20 ml of "RNAlater" buffer (Life Technologies), transferred to the laboratory and processed immediately or stored at -80^{o}C until processing. Skins were frozen in liquid nitrogen and pulverized in mortar. About 150 mg of powder were dissolved in 2 ml of RLT buffer (Qiagen), with the addition of 1% Peg 20000 and 1% β-mercaptoethanol, warmed ad 56^{o}C for 3 minutes, and processed using the columns and the reagents in the RNAeasy "Plant Mini Kit" (Qiagen). A second extraction was performed and the genomic DNA was eliminated using the reagents of the "RNAse-free DNAse set" (Qiagen). Total RNA was quantified using a Nanodrop ND 2000 apparatus (Thermo Scientific) and visualized by electrophoresis on agarose gel. To make sure that no genomic DNA contamination was present in the samples, real time PCR reactions were performed on aliquots of about 100 ng of total RNA, using the *ubiquitin* gene primers. This procedure was repeated until no amplification signal was obtained. For each sample, 1 μg of total RNA was reverse transcribed, using the reagent in the "QuantiTect Reverse Transcrition Kit" (Qiagen). A dilution 1:20 of each cDNAs was prepared and 10 μl were used in a 25μl real-time PCR reaction (in triplicate), using QuantiFast Syber Green (Qiagen) in a IQ5 apparatus (Biorad) with 1μM final concentration of each primer. A single primer pair was designed to detect both *mybA1* and *mybA2* mRNAs. This would anneal in a region common to both transcripts (accession numbers FJ687553.1 and DQ886419.1). This primer pair was then used to detect the expression of the two genes, whose expression is therefore noted as *mybA1-2*. For the *myb5a* gene, primers were designed using the information available at accession number AY555190.1. For the *pal* gene, primers were designed using the information available at accession number X75967.1. The "Primer3" software (http://frodo.wi.mit.edu/) was utilized to design these primer pairs. Primer sequences were as follows:

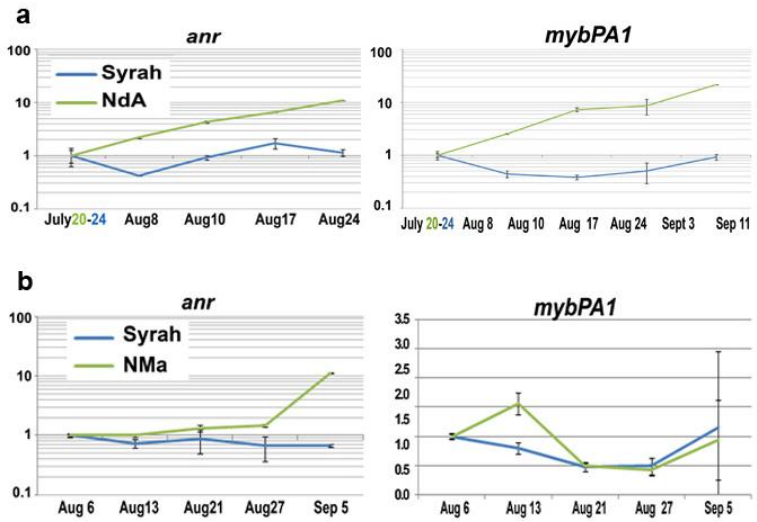
MybA1-2 F: GGGTTGAATAGATGCCGAAA
MybA1-2R: TGTTCCCCAACAAATTGTGA
Myb5aF: CCGCCTAACCTGGATCAGTA

Myb5aR: AGGGAATGTATCGTCGTTGC
VvPALF: ACAACAATGGACTGCCATCA
VvPALR: CACTTTCGACATGGTTGGTG
To detect the expression of all the other genes, the primer sequences (Bogs et al., 2007, 2005, 2005; Czemmel et al., 2012; Downey et al., 2003; Ford et al., 1998; Jeong et al., 2004; Mori et al., 2007; Terrier et al., 2009; Walker et al., 2007) were used.
Gene expression levels were obtained after normalization against the amplification signal of the *ubiquitin* mRNA and after correcting for the efficiency of each primer pair. The expression level of each mRNA was then calculated as a ratio over the level measured at the first sampling date (véraison), which was arbitrarily considered equal to 1.

RESULTS AND DISCUSSION

During the summer of 2012 we monitored the expression of five genes in the central pathway: *pal*, *dfr*, *ldox*, *chi* and *chs*. The analysis of the transcript levels demonstrated that the pathway was active after véraison in all the *cv* and at both sites, in a way that was consistent with the data reported in the scientific literature (not shown).
On the other hand when the expression of five genes in the side branches was investigated (*lar1*, *lar2*, *anr*, *fls* and *ufgt*) we noticed that the genes involved in the flavanols (catechin and epicatechins) synthesis were expressed at a time when they are normally off. Consistently with this, the expression of the *mybPA1* transcriptional regulator remained at level similar to those seen at véraison or was even increased. Furthermore we noticed the the *myb5a* gene remained expressed in both cultivars at the Marsala site and in the Syrah grapes of Mount Etna. An exemplification of these trends is provided in fig 2.

Figure 2



Expression patterns for the anr and mybPA1 genes, in grapes from the Marsala site (a) and from the Mount Etna sita (b). Expression levels are normalized to those measured at véraison.

Overall the data we collected suggest that in grapes grown in Sicily part of the energy flowing through the flavonoid metabolic pathway, might be directed toward the production of flavan-3-ols, even late in development of the berries. Further experiments will be needed to verify such hypothesis.

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