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THE EFFECT OF SHADING ON THE ON THE FLAVONOID PATHWAY DURING GRAPE BERRY RIPENING IN THREE AGLIANICO BIOTYPES

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Abstract

Polyphenols play a crucial role in wine making: they are involved in the oxidation reactions and in the determination of the sensorial quality of wine, particularly the astringency and the structure (tannins) and the color of red wines (anthocyanins). Some polyphenols have nutraceutical properties and are responsible for the benefit of moderate consumption of red wine on human health. Anthocyanins are a very well known group of phenolic compounds responsible for red, blue and purple pigmentation in plants, particularly in some flowers (eg, Petunia) and in many fruits, including red grape berries. Anthocyanins are synthesised in the flavonoid pathway. Previous studies (Downey et al, 2004; Fujita et al 2007; Rustioni et al, 2006) have demonstrated that cluster shading may significantly influence anthocyanin synthesis and, in general, the whole flavonoid pathway. More studies are necessary to elucidate the role of shading in the regulation of the pathway. For this reason we have chosen to study the response to shading in very closely related biotypes of a red berry cultivar of Vitis vinifera.

Aglianico is a very famous red cultivar traditionally grown in Southern Italy in many separated areas. Recent study (Costantini et al, 2005) have demonstrated that several byotipes of Aglianico, although showing clearly different phenotypes, they are originated from the same original genotype. For this reason, Aglianico is considered a good model for intra-variety variability. Three main biotypes of Aglianico (Vulture, Taurasi and Taburno) were selected to carry out this work.

A vineyard collecting the three biotypes was chosen as experimental site. In the experimental vineyard the biotypes are grown in the same environmental and agronomic conditions. Before veraison, clusters from each biotype underwent two different treatments: 12 clusters were covered with a shading screen designed to exclude light

without modifying temperature and relative humidity; other clusters were completely exposed to sunlight through defoliation of the bottom leaves of the canopy.

Physiological and technological variables such as sugars, pH and titrable acidity, and the accumulation kinetics of the each polyphenol species were measured. The relative expression of CHS2, F3'5'H, F3'H, F3H, DFR1, LDOX1, UFGT, OMT, AM1, AM3, GST4, LAR2, FLS4, MYB5a, MYB5b, MYB12 and MYBA1 was analysed by means of Real Time PCR.

This work describes the behaviour of the three biotypes regarding both the accumulation of primary and secondary metabolites as well as the differential expression of the flavonoid biosynthethic genes.

In the mean time it describes the accumulation of primary and secondary metabolites and transcriptional expression of the genes of the flavonoid pathway in response to the grape berry shading treatment.

This work is the first report about the effect of grape bunch exposure on the expression of the F3'5'H and F3'H genes, and on the AM1, AM3 and GST4 anthocyanin transporters.

Foreword

Grape ripeness

Grape berry growth follows a double sigmoid pattern (Coombe, 1992). The berry's volume increases during stages I and III, while it is constant during the lag phase (stage II). During stage I carbohydrates are mainly used for seed development, cell proliferation and organic acid synthesis. During phase III the titrable acidity decreases while there is accumulation of sugars and secondary metabolites until ripeness.

So, grape ripening appears as the harmonious evolution of several distinct biological processes simultaneously converging towards a particular level that makes the fruit edible and/or ready for wine making. Grape ripeness, or grape maturity, is the physiological stage in which this level is achieved, and it is the ideal moment for harvest. The achievement of ripeness is the result of the natural interaction between a genotype (the variety) and its environment, but it is also the result of the ability of farmers choosing the best viticultural practices.

Ripeness is a very broad concept, and it isn't easy to give a universal practical definition for it. It is even more difficult in *Vitis vinifera,* because grapes are non climateric fruits and the physiological status of the whole plant influences the ripening process. Biotic and abiotic stresses alter the kinetics of some processes, thus some metabolites may not reach the optimal level with the best timing. The definition of "optimal level" is also difficult: it strongly depends on the winemaking objective (i.e., the optimal acidity level of the fruits is different weather the grapes are for making sparkling wine or for making a long ageing red wine), and it may also depend on what a farmer regards as typical for a certain variety

in a certain area: so, in some way, we can say that *"ripeness is in the eye of the beholder"* (Hellman, 2004):

The concept of ripeness itself can be seen from different points of view:

- "*Evolutionary ripeness*". The only purpose for a grapevine to develop berries is reproduction. The berries' functions are to carry and protect the seeds until they are ready to germinate, and then attract disseminating animals. All the physiological processes going on in the berry follow this logic. When the seeds are not ready, berries need to be hidden, unsavoury and hard to digest. When the seeds are mature berries needs to visible, fragrant, appetising and nutrient for animals (e.g. the European starling) that eat grapes and eventually scatter the seeds originating new plants.

- "*Physiological ripeness*". From a strict physiological point of view, ripeness is achieved when seeds are ready to germinate. All the following processes can be interpreted as a sort of cellular senescence phenomenon involving the lysis of the middle lamella and the production of secondary metabolites.

- "*Technological ripeness*". From a classic enological point of view, ripeness is the optimal ripening stage for wine production. The concept of "technological ripeness" normally refers to the sugars/acids ratio. This is a basic quality index and it is a fundamental requirement for any wine, but it is not sufficient for high quality products.

- "*Aromatic ripeness*". Grape is ripe when it has the highest content of odour and flavour active compounds and of their precursors. The level of these compounds must guarantee the aromatic quality of wine over time.

- "*Phenolic ripeness*". Phenolic ripeness refers to the amount and structure of phenolic compounds in the grape, and their potential extractability during the maceration process. From this point of view, grapes are ripe when anthocyanins reach the maximum concentration, tannins have the optimal structure, all other polyphenols reach the ideal concentration and the extractability of the polyphenols is highest. Hence, from this point

of view, the lysis of the middle lamella and the production of secondary metabolites are not considered as senescence phenomena, but as main processes leading to ripeness.

When technological, aromatic and phenolic ripeness are achieved simultaneously there has been an ideal interaction between the plant and the territory, the season and farmer's ability. Biotic or abiotic stresses, poor seasonal weather, mistakes in the choice of the cultivar or in the farming practices and a low viticultural suitability of the territory cause the asynchronous achievement of the different levels of ripeness, compromising wine quality (Ribereau-Gayon et al., 1998).

Polyphenols and Flavonoids

Polyphenols, and flavonoids particularly are a crucial group of compounds in wine making. They are responsible for the sensorial differences in colour and taste between white and red wines. Polyphenols are also very interesting from a nutritional and health care point of view, particularly in the prevention of heart diseases.

High quality polyphenols are one of the keys to the achievement of high quality wines. Good vineyard management is crucial to this purpose. Polyphenols are extracted mainly from grape seeds and skins during the wine making process, and subsequently they undergo several oxidation, hydrolysis and condensation reaction during wine ageing. This way polyphenols change their original structure forming very complex molecules which can be very difficult to study with chemical and physical methods. The quantity and quality of the polyphenols contained in grapes, their extraction during wine making and the conditions in which they undergo all the chemical processes modifying their structure determine the quality of the wine.

In the past wine makers had a very simple and pragmatic concept of grape ripeness, taking into account only a few variables easy to measure. Nowadays is more important to

understand grape ripeness deeply, as quality variables such as polyphenols and aroma-active compounds are becoming more and more important.

Molecular biology is a powerful tool to investigate grape ripening, and particularly, the analysis of the expression levels of the genes involved in the synthesis of polyphenols may give us new insight, allowing us to understand better a complex phenomenon such as grape ripening.

A "colourful model for genetics"

Flavonoids are the major red, blue and purple pigments in plants. For this reason they have have been a research topic for many centuries and they have played a main role in many important scientific breakthroughs.

Robert Boyle (1927 - 1691) long studied the colour of plants. In 1664 he first published an essay about the chemical properties of plant pigments in Experiments and considerations touching colours. The colour of flowers was one of the major characters studied by Gregor Mendel (1823 - 1883) leading him to the postulation of the principles of inheritance. Barbara McClintock (1902 - 1992) first discovered the "jumping genes" (transposons) studying the flavonoid pathway in *Zea mais*, and she was awarded with the Nobel Prize in 1983.

Breeders and farmers, in the search for new and original ornamental plants and crops, created a vast variety of individuals with a complete range of tones and colours in fruits and flowers using traditional breeding techniques. Modern breeding and transformation techniques produced even more variability. The complexity of phenotypic expressions is a very valuable resource for scientists. The flavonoid pathway is a well characterised, multi-branched metabolic pathway that leads to the production of several different metabolites that are stored in different parts of the cell.

This pathway presents many features that makes it an ideal model for several kinds of scientific studies:

• it is active in many different species;

• it is differentially regulated from a spatial and temporal point of view;

• it is organ and tissue specific;

• it is under a strong transcriptional control;

• its regulation system involves the interaction of different transcription factor families;

• it features a complex transport network involving different mechanisms and transporters;

• it responds to a number of endogenous and environmental stimuli.

• Nevertheless, many of the molecular mechanism are still not completely characterised, and others are still far from being really understood.

For all these reasons, the flavonoid pathway may well be considered a "colourful model" (Winkel-Shirley, 2001) for the genetic spatial and temporal control of a metabolic pathway and for the intracellular compartmentation of secondary metabolites.

The purpose of this work

The grapevine germoplasm is a good example of a vast resource of phenotypical variation, making it is a good model-species to study the flavonoid pathway in fruit trees. Flavonoids are crucial compounds determining the quality of grapes and wine. Furthermore they determinate of the colour of berry skin and they are considered a reliable tool in grape chemotaxonomy. Among many environmental factors influencing flavonoid synthesis, light is clearly one of the most important. The quantity of light received by grape berries is, to a certain extent, easy to manage through proper viticultural practices. The training system, the row's orientation and the number of leaf layers present in the fruit zone are key factors influencing the grapes' sunlight exposure, hence the grapes'

flavonoid accumulation. Researchers devoted a lot of work to elucidate the effect of light on the flavonoid metabolism in grape berries. However, many aspects are far from being completely understood.

Aglianico is one of the most important and ancient red grape cultivars of Southern Italy. It is well known not only for the excellent wine it produces, but also for its plasticity and its ability to adapt to different environments and training systems. Aglianico shows a great range of biotypes with different phenotypic expressions, making it a good resource to investigate intra-variety variability.

This work analyses the effect of cluster shading in three closely related biotypes of Aglianico, with a particular focus on the kinetics of flavonoid accumulation and on the transcription of the key genes of the flavonoid pathway. In the mean time, this work investigates the intra-variety variability of Aglianico biotypes.

The first part of the introduction to the experimental work will regard the up-to-date knowledge about the chemical properties of the grapes' phenolic compounds, their metabolism and their ripening kinetics. An important part will be devoted to analysis of the molecular aspect of flavonoid metabolism, regulation and transport in model species and in the grapevine. It will follow a review over the effects of sunlight and shading on the flavonoid pathway in *Vitis vinifera*. Eventually, the cultivar Aglianico will be described, focusing on its important role as a model for intra-varietal variability.

Introduction

Grapevine Flavonoids

Polyphenol and Flavonoid chemistry

Phenols

A phenol is an organic compound made of an aromatic ring bound to a oxydrilic group (Russo et al., 1998).

They can form very strong hydrogen bonds and they have very high melting and boiling points (e. g. 41°C and 182°C for the phenol) and they are moderately soluble. Phenols react very easily with sodium hydroxide forming sodium phenoxide, while they are scarcely reactive towards carbonates.

Phenol is a weak acid (Ka = 10^{-11}), but its acidity is much stronger when electrophilic substituents are present the *ortho* and *para* positions. Nevertheless, in musts' and wines' pH conditions, phenols are hardly dissociated to phenates.

The canonical forms stabilising the phenol create a negative charge density in the *ortho* and *para* positions. For this reason phenols react very easily in those positions with electrophilic compounds such as the carbocations. As it happens for alcohols, The oxydrilic group of the phenols may react with anhydrides and chlorides acids forming an ester (Russo et al., 1998).

Polyphenols

Polyphenols are similar to phenols, but the aromatic ring is bound to more than one oxydrilic group. An example of a simple polyphenol is phloroglucinol (1,3,5-

trihydroxybenzene): a aromatic ring with three oxydrilic substituents in *meta* position. This compound is stabilized by 10 contributing structures delocalizing a negative charge density in the *ortho* and *para* positions, hence phloroglucinol is very reactive towards carbocations (Russo et al., 1998).

Phenolic compounds in grapes and wines.

Wine contains several kinds of different phenolic compounds which can be classified into two main groups: *flavonoids* and *non-flavonoids*. Flavonoids are molecules characterized by a particular 15 carbons structure formed by two polyphenolic rings with an tetrahydropyran heterocycle between them. *Flavonoids* can be classified depending on the oxidation degree of the heterocycle. The most common flavonoids in grapes and wines are flavonols, flavans and anthocyanins. *Non-flavonoids* are polyphenols with a different kind of structures: phenolic acids, stilbenes and hydrolysable tannins. The latter derive from the wood of barrels, thus they can be found in wine but they do not naturally occur in grapes (Ribereau-Gayon et al., 1998; Monagas et al., 2005).

Non-flavonoid polyphenols

Phenolic Acids

Phenolic acids are organic acids made of an aromatic ring directly or indirectly bound to a carboxylic group. Benzoic and hydroxycinnamic acids are two types of phenolic acids naturally occurring in the mesocarp and in the skin of grape berries. Their concentration in wines strongly depends on the wine making process and varies between 10 and 20 mg/l in white wines up to 100-200 mg/l in red wines. Although phenolic acids are not very important from a sensorial point of view, they can be the substrate for certain micro-organisms (e.g. *Brettanomyces*) giving origin to volatile phenols (vinyl and ethyl phenols) which may cause major aroma problems in wine because of their distinctive unpleasant odour (Ribereau-Gayon., 1998).

Benzoic (or hydroxybenzoic) acids have a simple C6-C1 structure. In this model the carboxylic group is directly bound to the benzenic ring. Benzoic acids are distinguished by the number and the type of substituents in the aromatic ring. The most important benzoic acids in grapes are the protocatechuic, vanillic, siringic and p-hydroxybenzoic acids, while salicilic and gentisic acids are found only in traces. The ratio among the benzoic acids content in grapes, and particularly the ratio between siringic and vanillic acids, is variety dependent (Kallithraka et al., 2007). Benzoic acids in grapes are found as heterosides, but in wine they are released in the free form by acid hydrolysis. Sometimes, benzoic acids are found as esters of Hydrolysable tannins, in this case they are released by alkaline hydrolysis. Benzoic acids in wine may derive also by the thermic degradation of more complex compounds such as the anthocyanins. Many benzoic acids derivates have been found in wine such as metyl and etyl- vanillate, etyl-p-hydroxybenzoate metyl and etyl - protocatechuiate, etyl- gallate and the glucose esters of vanillic acid. (Monagas et al., 2005).

Cinnamic (or hydroxycinnamic) acids have a C6-C3 structure. Caffeic, p-coumaric, ferulic and sinapic acid are the cinnamic acids found in grapes and wines, and they differ from one another for the substituents of the aromatic ring. The tartaric esters of these acids may cause wine darkening in presence of polyphenoloxydases. Cinnamic acids are more common in grapes in the *trans* form rather than the *cis one*. The cinnamic acids can form esters with the glucose of monoglucosidic anthocyanins, producing acilated anthocyanins. In wine also glucose esters of the p-coumaric and ferulic acids, *trans*-etyl-caffeoate, *trans*-etyl-coumarate and the corresponding tartaric esters. Cinnamic acids are also present in the *cis* and *trans* 4-O-glucosides forms.

Cinnamic acids are contained in the vacuole of the cells of the pulp and skin of the grape berries. Higher concentrations are found in the pulp. In this tissue caffeic acid is the

most present, followed by p-coumaric acid and ferulic. In the skin the ratio among the different types of cinnamic acids is cultivar dependent (Ribereau-Gayon et al., 1998; Monagas et al., 2005).

Hydrolyzable tannins

Tannins are compounds able to form stable bonds with proteins and other vegetal polymers such as polysaccharides. From a chemical perspective, tannins are large phenolic molecules deriving from the polymerization of monomeric units containing phenolic groups. A tannin polymer must reach a certain dimension to form a stable complex with proteins, anyway, if the polymer is too large it cannot reach the active site of proteins and, thus, there is no complex formation. Molecular mass of tannins ranges between 600 and 3500 Da (Ribereau-Gayon et al., 1998; Jöbstl et al., 2004).

In wine there are two classes of tannins: hydrolyzable and condensated. The latter belong to the group of flavonoid polyphenols and they derive from the grapes. On the other hand, hydrolyzable tannins do not derive from grapes, but from the oak of the wooden barrels where wine is conserved and aged. Hydrolyzable tannins can also artificially added to the wine as commercial products. Hydrolyzable tannins can be classified into gallotannins and ellagitannins depending on weather they release gallic or ellagic acid by acid hydrolysis. Hydrolyzable tannins are very important during the aging of both white and red wines for their oxydability and for their organoleptic properties (Ribereau-Gayon et al., 1998).

Stilbenes

Stilbenes are low molecular mass phenolic compounds with a particular kind of structure: two aromatic rings linked by a ethylenic or an ethanic group. Each aromatic ring has one or more phenolic functions.The most well-known stilbene is trans-resveratrol (3,5,4'-trans-trihydroxystilbene) and its cis isomer. Other important stilbenes in grapes and wines are the heterosides of resveratrol (piceids), piceotanonl (4',3,4,5-

tetrahydroxystilbene), pterostilbene (trans-3,5 dimetil-4'-hydroxystilbene) and the cyclic dimers and trimers of resveratrol (viniferines) (*Monagas et al., 2005; Ribereau-Gayon et al. 1998b; Bavaresco and Fregoni, 2000*). Recently, several other stilbenes have been identified: 3,5,3',4'-tetrahydroxystilbene-3-O-glucoside (trans-astringin), 2,4,6-trihydroxy-phenanthrene-2-O-glucoside, resveratrol-2-C-glucoside, E-viniferin-diglucoside, pallidol-3-O-glucoside, pallidol-3,3''-diglucoside and partenocissin-A (*Monagas et al., 2005*). The relative proportion of stilbenic compounds is cultivar specific (Kallithraka et al., 2007)

Significative concentration of resveratrol can be found in wine. In red wines cis and trans resveratrol ranges between 0,3 and 12 mg/l, depending on agronomical, technological and environmental factors and on the cultivar. In grapes stilbenes are concentrated mostly in the seeds and in the skin, hence the wine making technique greatly influences the final concentration in wine of these compounds.

General interest for stilbenes, and more specifically for resveratrol, is caused by their nutraceutical properties. In 1992 Renaud and De Lorgeril publish a paper revealing the famous *french paradox*. Studying data from several european countries, the authors showed a direct link between daily consumption of animal fats and the incidence of heart disease deaths. France represented an exception to this rule, having a paradoxical situation showing a low frequency of deaths related to heart diseases and a high consumption of animal fats. The only variable able to solve this contradiction is the regular consumption of red wine. The positive effect of red wine is connected also to the presence of resveratrol. (German and Walzem, 2000)

Flavonoids

Flavones

Flavones, also known as anthoxantines, are yellow pigments widespread in plants. Flavones are often found in the as glucosides. The aglicones goes under the name of

Anthoxantidin. Flavones are also found forming complexes with tannins. Several flavones have been identified in plants, however these compounds are not frequently found in fruits. In the grapevine they are mainly present in the leaves, mainly apigennin-8-C-glucoside, apigenin-7-O-glucoside, Luteolin and Luteolin-7-O-glucoside (*Monagas et al., 2005*).

Flavones are formed by a 15 carbon skeleton, configured following a C_6-C_3-C_6 pattern featuring three typical rings. The A ring is similar to a molecule of phloroglucinol bound to a oxygen unsaturated heterocycle known as the C ring. The B ring is the lateral 6 carbon cycle. The heterocycle's carbons 2 and 3 have a double bond. These carbons have a sp^2 hybridisation. In this hybridisation one *s* and two *p* orbitals are combined forming three hybrid sp^2 orbitals while one *p* orbital is not hybridised. The three sp^2 orbitals are on the same plane, and they form a 120° angle among each other, while the *p* orbital is perpendicular to the plane. (Russo et al., 1998). This kind of hybridization is responsible for the planarity and conformational rigitdity of the flavone and of its derivatives. Therefore, they do not have *cis* or *trans* diastereoisomers.

Flavonoids are phenolic compounds that share the same C_6-C_3-C_6 structure as the flavones, and they are classified depending the oxidation degree of the heterocycle.

Anthocyanins

Anthocyanins are important plant pigments responsible for the red, violet and blue colours of several fruit and flowers of many plants. They are responsible for the colour of the skin of red grapes and of the colour of the pulp of some grape cultivar (*teinturier* cultivars). Anthocyanins are heterosides, the corresponding aglycones are called anthocyanidins. Anthocyanins are amphoteric substances: in acid environments they are red, in a neutral solution they are violet and their metallic salts are blue. The core of the molecule of anthocyanins is benzopyrilium, a molecule formed by a benzenic ring bound to a heterocycle with 3 carbons and an oxygen. A phenol is bound to the position 2 of the

heterocycle, forming the 2-phenyl-benzopyrilium cation, also known as flavylium (Riberau-Gayon et al., 1998; Russo et al., *1998*).

Anthocyanidins derive from 3,5,7-trihydroxyflavylium and the differ from each other for the hydroxyl and/or methoxyl substituents of the lateral ring. Six main species of anthocyanidins are known: cyanidin (3,3',4',5,7-pentahydroxyflavylium), peonidin (3-methyl-3,4',5,7-tetrahydroxyflavylium), delphinidin (3,3',4',5',5,7-esahydroxyflavylium), petunidin (5'-methyl-3,3'4',5,7-pentahydroxyflavylium), malvidin (3',5'-dimethyl-3,4',5,7-tetrahydroxyflavylium), pelargonidin (3,4',5,7-tetrahydroxyflavylium). The latter is not found in *Vitis,* and therefore is absent in grapes and wine. The color of the anthocyanidins depends on the kind and number of substituents and on the solvent's pH. The substituents give different chemical properties to each anthocyanidin: malvidin is more sensible to thermal degradation as the methyl substituents activate the B ring, making the aliphatic chain easier to break down to a chalcone, while cyanidin is more resistant to high temperatures. On the other hand, the methyl substituents of malvidin protect it from oxydation, while cyanidin is more easily oxidized (Ribereau-Gayon et al., 1998, Monagas et al., 2005) .

Anthocyanins are found bound to several sugars, mono, di- and tri- saccharides. The most common of them is glucose. There are monoglycosylated and diglycosylated anthocyanins. The glucose is usually bound to the 3 or 3,5 positions with a beta-glycosidic bond. *Vitis vinifera* grapes only contain mono-glycosylated anthocyanins, while diglycosylated anthocyanins are found in american species such as *V. riparia* and *V. rupestris*. The "diglycosylated anthocyanins" character is dominant, and it is found in *Vitis vinifera* hybrids with american species. The sugar residue of the anthocyanidin may form an ester with an acetic, p-coumaric or caffeic acid, forming acylated anthocyanins. Each grape variety has a typical anthocyanin profiling, in other wordsa specific pattern of the

relative concentration of each anthocyanin. The anthocyanin profiling is under a strong genetic control, ant the influence of the environment is limited. For this reason, red berry grapevine cultivars can be classified into groups (Mattivi et al., 2006):

• cultivars with a higher proportion of disubstituted anthocyanins (e.g. Nebbiolo);

• cultivars with a higher proportion of trisubstituted anthocyanins (e.g. Merlot);

• cultivars with a high p-coumarates / acetates ratio (e.g. Dolcetto);

• cultivars with a low p-coumarates / acetates ratio (e.g. Barbera);

• cultivars with no acylated anthocyanins (e.g. Pinot Noir);

• cultivars with high proportion of acylated anthocyanins (e.g. Cabernet Sauvignon).

In red grape cultivars, anthocyanins are located in the inner layer of the skin. At veraison, when anthocyanin synthesis begins, they are strongly bound to the tonoplast. During the grape ripening, anthocyanins are released into the vacuole, making color extraction easier during wine making. Anthocyanins have a primary role in winemaking as they are responsible for the colour of red wines. The colour of these pigments depends on the molecular structure, on the pH and on the interaction with other substances present in wine such as SO_2 (Ribereau-Gayon et al., 1998).

The molecular structure of anthocyanins depends on the number and on the nature of the B-ring substuents: Pelargonidin is monosubstituted and it is orange while Malvidin is trisubstituted and it is mauve. The increase in the number of substituents causes a batochromic shift (red shift), moving the absorbance peak to a longer wavelenght. Glycosylation and acylation cause the opposite phenomenon, an ipsochromic shift: the molecule's absorbance peak moves to a shorter wavelength and the colour becomes more orange. (Ribereau-Gayon et al. 1998; Monagas et al 2005).

Anthocyanins change colour depending on the pH: in acid solution they are red and they loose colour as pH rises up to pH 3.5, at pH 4 they are blue and they eventually

become yellow in an alkaline environment. Anthocyanins may assume four different structures in wine and equilibrium occurs among them: the flavylium cation (A^+), the quinoidal base (AO), the carbinol base (AOH) and a *cis* or *trans* chalcone (C). The flavylium is a red cation and it has a positive charge localised on the oxigen and it is stabilised by six resonance structures. The quinoidal base is blue and it is defined by a ketonic group with a fast proton transfer reaction formed from one of the phenolic hydroxyl groups of the flavilyum. The flavylium may undergo a proton transfer after a hydration reaction, as a result a colourless carbinol base is formed with an hydroxyl functional group in position 2 or 4. The heterocycle of the carbinol base may form a *cis* or a *trans* chalcone by tautomerization. There are three equilibria occuring among these molecules:

- $A^+ + H2O$ <----> $AOH + H^+$ ($pK_a = 2,93$)

- A^+ <----> $AO + H^+$ ($pK_a = 3,41$)

- AOH   <---->   C   ($K_t = 0,61$)
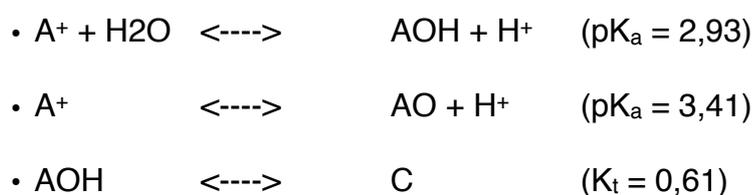
Low pH favours the formation of the more coloured and stable forms, while at higher pH the equilibrium moves towards the colourless and unstable forms (AOH and C). Chalcones are very unstable compounds and they easily break down to 2,4,6-trihydroxybenzaldeyde and syringic acid. This causes the definitive loss of anthocyanins, as this reaction is irreversible in wine conditions. Anthocyanins are in the vacuole, where the pH condition are optimal for stabilising the colour (Bruillard and Dubois, 1977), (Chen and Hrazdina, 1982), (Monagas et al., 2005).

In the vacuole of the grape's skin cell   anthocyanins interact also with other molecules (cofactors) such as organic compounds (i.e. flavonols) or metal cations forming molecular associations or complexes. This may result in an enhancement in the absorbance and in some cases, a shift in the wavelength of the maximum absorbance of the pigment. This phenomenon is known as copigmentation . Copigmentation explains the

differences in colour between the berries' skins and the must, and it may account up to 50% of the colour of young wines. (Boulton, 2001).

Free anthocyanins undergo many reactions during wine ageing. They form stable red complexes with condensed tannins. If the anthocyanin and the tannin are linked by an ethanal bridge, the resulting complex is particularly stable. The formation of these molecules prevents the complete decoloration of red wine due to the complete loss of free anthocyanin caused by chemical degradation or by other causes (i.e. the decolorating action of SO2, precipitation), (Brouillard et al., 1997), (Ribereau-Gayon et al., 1998).

Flavonols and Flavononols

Flavonols and flavononols are yellow coloured molecules. Flavonols (3-hydroxy-flavone)are the hydroxyl derivatives of the flavone, and they have the same typical 15 carbon structure with two benzenic rings connected by an oxygen hetherocycle. Flavononols derive from the flavanone: the structure is similar to the flavone, but it lacks a double bond in the hetherocycle (Russo et al., 1998).

Flavonols are more common than flavononols in grapes and wines. They are the yellow pigments present in both white and red berry grapes as well as in a large number of other fruit and flowers. There are three major species of flavonols n grapes and wines, distinguished by the substituents of the B-ring: 3-4'-dihydroxy-flavone (kaempferol), 3,3'-4-trihydroxy-flavone (quercitin) and 3,3',4',5'-tetrahydroxy-flavone (myricetin). Small quantities of isorhamnetin may be found as well. The proportion of flavonols is cultivar dependent, and myricetin is totally missing in white berry grapes (Mattivi et al., 2006). Flavonols are found as mono- or disaccharide glycoside . The sugar is bound to the hydroxyl in position 3. 8 monosaccharide and 3 disaccharide species are found in grapes. (Monagas et al., 2005). The concentration of flavonols in red wines reaches a few hundred

mg/l, while in white wines ranges between 1 and 3 mg/l. Glycoside flavonols are rapidly hydrolysed during the wine making process, so they are found only as aglycones in wine.

Flavonols are involved in the protection against UV radiation (Flint et al., 1985). They act also as cofactor in the copigmentation of several fruits and flowers. Full light exposed grapes have higher levels of flavonols. The concentration of flavonols in the grapes depends also on the variety, on the thickness of the skin, on the dimension of the berries and on the skin/berry ratio (*Monagas et al., 2005*).

Flavononols are not as yellow compared to flavonols. Dihydroquercitin (3,3',4'-trihydroxy-flavanonone) is the most important flavononol; dihydromyricetin and dihydrokaempferol (both as glycoside and aglycones), engeletin and astilbin are also found in grapes (Monagas et al., 2005).

Flavonols and flavonols contribute to the nutraceutical properties of wine (Tapas et al., 2008)

<u>Flavan-3-ols, Proanthocyanidins and Condensed Tannins</u>

Flavans are a very important class of polyphenols in enology. This class is made of several molecules: some are relatively simple, others are very heavy and complex polymers. Flavan-3-ols are the simplest and they are the monomeric units of bigger molecules. Dimeric and trimeric flavan-3-ols are called proanthocyanidins, bigger polymers are generally called condensed tannins.

Flavan-3-ols are the hydroxyl-derivatives of the flavan. The structure is similar to that of other flavonoids: two C6 benzene cycles bound to a C3 saturated oxygen heterocycle. However, the carbon 3 of the heterocycle shows an hydroxyl functional group. As opposed to other flavonoids, flavan-3-ols are not planar molecules. The carbons 2 and 3 of the flavan-3-ol show a sp^3 hybridisation (as opposed to sp^2). This kind of hybridisation allows a limited rotation in cyclic molecules, causing the formation of *cis* and *trans* diastereomers

(Russo et al. 1998). The *trans* isomers are called catechins (catechin and gallocatechin), while the *cis* isomers are called epicatechins (epicatechin and epigallocatechin) (Monagas et al. 2005). Because of the sp^3 hybrydisation, C_2 and C_3 become chirality centres. For each chirality centre there is an R and an S enantiomer. Enantiomers are optically active and they have a (+) or (-) form. Hence, each flavan-3-ol diastereomer has two enantiomers:

- (+)-catechins (C_2=R, C_3=S)

- (-)-catechins (C_2=S, C_3=R)

- (+)-epicatechins (C_2=S, C_3=S)

- (-)-epicatechins (C_2=R, C_3=R)

(+)-catechins and (-)-epicatechins are the most present isomers in grapes. Catechin and epicatechin have two hydroxyl functional groups in 3' and 4'. In the berry skin are also found the 3'-4'-5' hydroxyl flavan-3-ols, and they are called (+)-gallocatechin and (-)-epigallocatechin. In total there are 8 possible flavan-3-ols (Ribereau-Gayon et al. 1998).

Proanthocyanidins and condensed tannins are formed by the polymerisation of flavan-3-ols (Dixon et al, 2005). The oligomers and polymers form stable bonds with proteins and polysaccharides, including the proteins in the mouth, causing wine astringency (Gambuti et al., 2006). The stability of these complexes depend on the tannin's dimension and on the number of free phenolic groups. Monomeric flavan-3-ols are too small to form stable complexes with proteins.

Proanthocyanidins are classified on the basis of the kind of chemical bond:

- Proanthocyanidins A ($C_{30}H_{24}O_{12}$): dimer proanthocyanidins with two flavan-3-ols condensed with a C4-C6 or a C4-C8 bond (interflavanic bond) and forming an ether between the C2 of the first unit and the C5 and C7 of the terminal unit.

- Proanthocyanidins B ($C_{30}H_{26}O_{12}$): dimer proanthocyanidins with only a C4-C6 or C4-C8 interflavanic bond.

- Proanthocyanidins C: trimer proanthocyanidins with only a C4-C6 or C4-C8 interflavanic bond.

- Proanthocyanidins D: trimer proanthocyanidins. In this case the first two monomers have the interflavanic bond only, but the central and the terminal monomer have both the interflavanic and the ether bond.

Oligomer proanthocyanidins, condensed proanthocyanidins or, more simply, condensed tannins are polymers with more than three units. The molecular mass of these tannins can go over 3000 Da. There is a great number of possible isomers, and studying these molecules deeply can be a difficult task (Heiderich and Smith, 2005).

In a hot and acid medium The interflavanic bond breaks down releasing an unstable carbocation producing, eventually, an anthocyanin. For this reason flavan-3-ol polymers are called proanthocyanidin (Bate-Smith, 1975). More specifically, procyanidins produce cyanidin from catechin and epicatechin, while prodelphinidins produce delphinidin from gallocatechin and epigallocatechin. (Monagas et al., 2005).

Condensed tannins are the typical tannins of grapes. Their concentration in wine ranges between 100 mg/l in white wine and 4.000 mg/l in red wines. It varies depending on the grape cultivar, farming practices and the season. They are present in all the solid part of grapes: in the skin, in the seeds and in the stalk. During wine ageing the can precipitate, they may undergo many structural changes and they may form stable complexes with other organic compounds. Some reaction positively influence the sensorial quality of wine, others may be negative (i.e. proteinic colour break) (Riberau-Gayon et al., 1998).

Flavonoids and grape ripening

A dynamic view.

Total polyphenol content increases during grape ripening reaching the highest level at full ripeness. After this peak, the concentration of polyphenols may decrease during senescence. The accumulation kinetics of each flavonoid class is not the same: for example anthocyanin synthesis begins at *veraison*, while tannins are synthesised also in earlier stages of the berry's development.

Anthocyanins' kinetics: synthesis and degradation

Anthocyanin synthesis in red grape berry skins starts at veraison. In the first phase synthesis is very rapid and there is a massive accumulation of pigments. In this stage anthocyanin accumulation kinetics is parallel to sugar accumulation, and up to 90% of total pigments can be synthesised in this first phase. Anthocyanin kinetics stops following sugar accumulation and accumulation slows down towards full maturity (Coombe & McCarthy 2000; Guidoni et al., 2004; Braidot et al., 2008).

Anthocyanin degradation occurs in parallel to the synthesis, but the molecular mechanisms and the importance of its contribution to anthocyanin kinetics is still to be clarified. The catabolic activity may be due either to the chemical instability of the pigments (particularly to high temperature) or to specific enzymatic activity, or even to transport issues. Anthocyanin degradation was reported in many fruits and flowers (Borovsky et al, 2004; Steyn et al. 2005; Vaknin et al., 2005; Zhang et al., 2005). Anthocyanin turnover under high temperature growing conditions has been shown also in grapes (Mori et al 2007). Polyphenoloxydases (PPOs) might be involved in the enzymatic degradation of anthocyanins, but PPOs are mostly found in the chloroplast, and they could hardly reach

anthocyanins stored in the vacuole. In 2006, Ono and his co-worker first found a flavonoid biosynthetic PPO (Aureusidin Synthase, AS) involved in the enzymatic oxydation of chalcone for the production of aurones in yellow snapdragon petal cells. They showed that AS is localised in the vacuole (Ono et al., 2006). So, the presence in the vacuole of other PPOs that may have a role in anthocyanin degradation can't be excluded.

Tannins' kinetics

Tannin accumulation in the seeds and in the skins begins at fruit set and it continues until veraison. At this stage there is peak in the concentration of tannins. After the onset of ripening the concentration of tannins normally remains constant, or it may diminish for dilution, because the synthesis of flavan-3-ols is not significative and it does not follow berry growth and sugar accumulation after veraison (Harbertson et al., 2002; Bogs et al., 2005; Fournand et al., 2006). The composition of tannins changes in different organs.

At veraison grapes seeds contain mostly low molecular weight flavans. After the onset of ripening there is a dramatic decrease in flavan-3-ols and proanthocyanidins (90% and 60% respectively). This results in the change of the seed coat's colour. Nevertheless, the average degree of polymerisation of the seeds tannins is low throughout the whole ripening period. Thus, seed tannins are the major responsible for the excessive astringency of wine. The contribution of seeds tannins to wine depends not only on the absolute concentration in the seed, but also on the average number of seeds per berry (Kennedy et al., 2000; Harbertson et al., 2002).

Grape berry skins have different tannin structure. The molecular weight of tannins increases during berry development and the proportion of flavan-3-ols and proanthocyanidin diminishes accordingly. For this reason the high-molecular-weight skin's tannins contribution to wine's astringency is smaller than the seed's. (Kennedy et al., 2001; Kennedy et al., 2002; Harbertson et al., 2002; Fournand et al., 2006).

Flavonols' kinetics

Flavonol kinetics are interesting. The maximum concentration per fresh weight is about 9 weeks prior to *veraison*. Flavonol concentration decreases dramatically in 4-5 weeks and then slowly diminishes until harvest. Analysing flavonol quantity per berry (or per berry skin area), the situation is opposite: flavonols accumulate until veraison. After the onset of ripening, flavonol accumulation slows down or stops and flavonol content remains constant. This indicates that flavonol synthesis is active throughout the whole berry development . Furthermore, flavonol synthesis is very much influenced by the environment, especially by light. (Downey et al., 2003; Fujita et al., 2006; Matus et al., 2009).

Molecular aspects of grape's flavonoids

Flavonoid Biosynthesis in grapes

Polyphenols are synthesised in grape berries following the same multi-branched phenylpropanoid pathway described in many plant species, although with some peculiarities (e.g. the absence of monosubstituted anthocyanins) (Sparvoli et al., 1994; Boss et al., 1996, Mol et al., 1998; Winkel-shirley, 2001; Shubert et al., 2003; Matus et al., 2009). The early steps of the pathway are the transformation of *phenilalanine* to *cinnamate* first, and then to *p-coumarate* by the phenilalanine-ammonia-lyase (PAL) and cinnamate-4-hydroxylase (C4H) respectively. The *p-coumarate* may either produce cinnamic esters ending the synthesis, or produce the *coumaroil*-CoA through the action of the 4-coumarate-CoA-ligase (4CL). This compound is the substrate of two alternative enzymes: the chalcone sinthase (CHS) or the stilbene sinthase (StSy) producing the chalcone (2,4,6,4'-tetrahydroxychalcone) or the stilbenes respectively.

The chalcone is the first flavonoid synthesised in this pathway. The action of the chalcone isomerase (CHI) (producing the 5,7,4'-trihydroxyflavone) and of the flavonoid-3-hydroxylase (F3H) produce the dihydrokaempferol. This flavononol is the substrate of two alternative enzymes: the flavonoid-3'-hydroxylase (F3'H) and the flavonoid-3'5'-hydroxylase (F3'5'H) obtaining dihydroquercitin and dihydromyricetin respectively. The flavononls are the substrate of the flavonol-synthase (FLS) producing the corresponding flavonol: quercetin, myricetin and kaempferol. This is a very important part of the pathway: F3'H leads to cyanidin-based disubstituted anthocyanins and procyanidins, while F3'5'H leads to delphinidin-based trisubstituted anthocyanins and prodelphinidins. In grapevine monosubstituded anthocyanins (pelargonidin) are absent: this is due to the selectivity of grapevine's dihydroflavonol reductase (DFR) that does not accept dihydrokaempferol,

while dihydroquercetin and dihydromyricetin are accepted to produce the leucoanthocyanidins (leucyanidin and leucodelphinidin respectively). Leucoanthocyanidin dioxygenase (LDOX) synthesise anthocyanidins that are eventually glycosylated by the UDP-glucose-flavonoid-3-glucosyltransferase (UFGT). The cyanidin-3-glucoside and the delphinidin-3-glucoside are the first stable anthocyanin to be synthesised (Sparvoli et al., 1994; Boss et al., 1996, Mol et al., 1998; Winkel-shirley, 2001; Shubert et al., 2003;). Methoxylated anthocyanins are produced through the action of the anthocyanin O-methyltransferase (OMT) from cyanidin-3-glucoside (peonidin-3-glucoside) and delphinidin-3-glucoside (malvidin-3-glucoside and petunidin-3-glucoside) (Hugueney et al., 2009). Acylation may occur after anthocyanin biosynthesis. Anthocyanin acyltransferase have been identified in other species but not yet in the grapevine (Fujiwara et al., 1998; Luo et al. 2007).

Tannins are synthesised starting from leucoanthocyanindins and anthocyanidins. The *trans* flavan-3-ols (catechins) are formed from the reduction of leucoanthocyanidins by the leucoanthocyanidin reductase (LAR), while the *cis* isomers (epicatechins) are synthesised from the anthocyanidins by the anthocyanidin reductase (ANR). (Shubert et al., 2003; Xie et al., 2003; Tanner et al., 2003; Dixon et al., 2005). Polymerisation of flavan-3-ols and the synthesis of proanthocyanidin are still to be clarified. Some evidence in *Arabidopsis thaliana* suggest the enzymatic activity of a polymerase (TT10), but they are not conclusive, thus, other possible mechanisms, such as non enzymatic polymerisation and acid catalysis, can't yet be excluded (He et al. 2008; Kleindt et al., 2010; Zhao et al., 2010).

Flavonoid regulation and transport in plants

The regulatory genes of the flavonoid pathway

Flavonoids synthesis is tissue, organ and time specific. There is a wide range of colour patterns in several flowers, fruits and seeds. In grape vine, flavonoid composition changes in different parts of the berries (pulp, skin, seeds) and in different physiological stages(fruit set, veraison, ripeness), for example: the structure of tannins is different in the seeds and in the skins; the synthesis of anthocyanins starts at veraison while other flavonoids are synthesised earlier. Thus, the flavonoid pathway must have a very refined spatial and temporal regulation.

Numerous studies show that most of the regulation of this pathway is due to coordinated transcriptional control of the structural genes (Mol et al., 1998; Winkel-Shirley, 2001; Koes et al., 2005; Lepiniec 2006; Dixon and Pasinetti, 2001). Also post-transcriptional control was reported for some genes (Pairoba and Walbot, 2003; Johansen and Wilson, 2008). Several regulators controlling the flavonoid pathway were identified for the first time mostly in *Arabidopsis thaliana, Zea mays*, and *Petunia hybrida* mutants*.*

In all species the regulation of the flavonoid pathway involves three kinds of transcription factors: MYB, basic helix-loop-helix (bHLH or MYC) and WDR (or WD40) repeat proteins. The interaction among these factor indicates that they are part of a transcription activation pathway that acts directly on the structural genes, without intermediate regulators (Koes et al., 2005; He et al., 2008).

In maize, the *ZmC1* and the *ZmR* (and *ZmB*) belong to the MYB and bHLH transcription factor families respectively. Each family has several paralog genes and their different expression patterns are able to explain the distribution of anthocyanin-related pigmentation in maize. The ectopic expression of *ZmC1* and *ZmB* triggers anthocyanin

synthesis in otherwise colourless tissues (Mol et al., 1998). *ZmC1* binds directly to the promoter region of the structural gene, but this is not sufficient to trigger transcription. The presence of its partner *ZmR* is essential. bHLH proteins don't seem to bind DNA, so the transcription activation probably follows different mechanisms (Koes et al. 2005).

WDR are highly conserved regulators and they are found also in algae, fungi and animals (even human) that do not synthesise flavonoids (Koes et al., 2005). Although they play a central role in numerous biological pathways, how exactly these proteins actually regulate other genes from a molecular point of view is not completely clear. No WDR domain has been reported to have intrinsic enzymatic activity. Recent interactome studies suggest that they work as scaffolds interacting with other protein, peptides and nucleic acid, using a different interaction modes (Stirnimann et al., 2010).

Simple models have been proposed for the activation of the flavonoid pathway's structural genes on the basis of this knowledge. These models involve the formation of a MYB-bHLH-WDR (MBW) complex activating the transcription of the target gene: e.g., in *Arabidopsis* TT2, TT8 and TTG1 (a MYB, a bHLH and a WDR factor respectively) form a complex that directly activates BAN (ANR) transcription (Koes et al., 2005; He et al., 2008). WDR genes are virtually ubiquitous, while MYBs and bHLHs are expressed only in the tissues where flavonoids are synthesised. WDR domains are so highly conserved during evolution, that some of these regulators are actually older than the pathway they regulate. High-throughput studies show that they are probably involved in more interaction pair s than any other domain (Stirnimann et al., 2010).

Possibly, a given WDR protein may be involved in the regulation of a number of different pathways: for example, in *A. thaliana ,* the WDR factor *TTG1* activating the flavonoid biosynthesis is also involved in the formation of hair. bHLH factors are also pleitropic, even though to a lesser extent. Many studies have shown that bHLH are

involved in several processes that are apparently not so closely related to the flavonoid pathway, In *Petunia*, PhAN1 (a bHLH) is involved, in the acidification of the vacuole in petals, in the formation of the seed coat as well as in the pigmentation. In contrast, MYB factors show more specificity to a single pathway or a single gene. Nevertheless, many studies show that at least some of them have have a dual function: they directly activate the structural genes, but they also activate the genes encoding for they bHLH partner. (Koes et al., 2005; Stirnimann et al., 2010).

It is likely that WDR, bHLH and their complexes, that co-regulate numerous processes, interact with specific MYB proteins to trigger specific branches of a pathway. However, also the competition of alternative enzymes for a common substrate may play a role, for example the inactivation of ANR leads to a higher anthocyanin synthesis (Xie et al., 2003).

Furthermore, other transcription factors are associated with the regulation of the flavonoid pathway. The gene families involved include WRKY domains, MADS box and TFIIIA-like proteins (WIP). WRKY factors act downstream of the WDR protein. WRKY seem to be directly regulated by MYB transcription factors. MADS gene directly control the expression of BAN, while WIP proteins seem involved in proanthocyanindin polymerisation (Koes et al., 2005; He et al., 2008).

Despite the identification of a great number of regulators, the question of the "regulation of regulators" still needs for a comprehensive answer.

The flavonoid transport network

The flavonoid biosynthetic enzymes are found in the cytosol. Immunolocalization experiments suggest that they are localised around the endoplasmic reticulum associated cytochrome P450 proteins and that they are possibly organised as multi-enzyme

complexes. (Koes et al., 2005; Zhao and Dixon, 2010). Some flavonoid biosynthetic enzymes have also been found in the tonoplast, in the chloroplast, in the cell wall and in the nucleus (Saslowsky et al., 2005). Plastidial localisation of CHS has been reported also in grapevine (Tian et al., 2008).

Anthocyanins and proanthocyanidins are found mostly in the vacuole. The pH conditions of the vacuole, and the presence of co-pigments (flavonols, metals) allows the formation of colour in the cells of the fruit skins or of flower petals. Flavonoids are found even in the plastids, in the tonoplast, in the cell wall and the nucleus. (Hernandez et al, 2009; Zhao et al., 2010).

Thus, a complex transport system is required to store flavonoids into the right cell compartment. The association of the multi-enzyme complexes to the endoplasmic reticulum may facilitate flavonoid transport, while the co-localisation of the enzymes in different parts of the cell could help meeting specific biosynthetic requirements in particular conditions. (Koes et al., 2005; Zhao and Dixon, 2010).

Two different kinds of flavonoid transport mechanisms have been proposed, one mediated by membrane vesicles formed from the endoplasmic reticulum and the Golgi apparatus, the other mediated by transporters (including GSTs, ABCs and MATEs). It is likely that both these mechanisms play a role inside the cell's flavonoid transport network.

Anthocyanoplasts (ACP) are cytoplasmic membrane-bound vecicles containing anthocyanins and they are involved in the synthesis and transport of these flavonoids. ACPs are exclusively found in grapevine cells and in red radish seedlings protoplasts (Braidot et al., 2008; Zhao and Dixon 2010) and they originate from the fusion of a large number of small vesicles. Inside the vacuole there are similar structures called anthocyanic vacuolar inclusions (AVI). AVIs have been found in many species. AVIs are anthocianic complexes containing proteins but, in contrast to anthocyanoplasts, they don't

have a proper membrane AVIs. It is likely that ACPs transport anthocyanins to vacuole, while the AVIs represent the storage unit within the vacuole. However direct evidence supporting vesicle transport are still to be found (Braidot et al., 2008; Zhao and Dixon 2010).

Gluthathione-S-transferases (GST) seem somehow involved in the flavonoid transport network as well, but their exact role is not very clear. Infact, no natural occurring gluthation-anthocyanin conjugates have yet been reported, but the GST protein itself can bind the anthocyanins. Many studies support the hypothesis that it GST is a transport related protein inside the flavonoid-protein complexes (Koes et al., 2005; He et al., 2008; Kleindt et al., 2010; Zhao and Dixon, 2010).

ATP binding cassettes (ABC) are a broad, ubiquitous family of secondary metabolite transporters. ABCs draw energy from ATP hydrolysis to transport metabolites across membranes. There are indications of the involvement of ABCs also in flavonoid transport in maize, barley and soybean but, to date, there is very little knowledge about their role (Zhao and Dixon, 2010; Dixon and Passinetti, 2010).

Multi drug and toxic compound extrusion (MATE) proteins are a widespread, large family of transporters using electrochemical gradient to transport secondary metabolites, and they have been associated in anthocyanin and proanthocyanidin transport in many species, including grapevine (He et al., 2008; Gomez et al., 2009). Many flavonoid transporter show a strong substrate specificity (Zhao and Dixon, 2010). MATE transporters need proton pumps to power transport, so they depend on the activity of different sorts of H^+ATPases mantaining the H^+ gradient across the tonoplast. A mutation of the pump may cause differences in the vacuole pH and in anthocyanin transport, resulting ultimately in a colour shift (Zhao and Dixon, 2010).

The transport of flavonoids into the vacuole is not one-way only. Experiments in legumes cell cultures showed the efflux of flavonoids from the vacuole to other parts of the cell. It has also been proposed the involvement of the transport of flavonoids from the vacuole to the apoplast (Buer 2010; Dixon and Pasinetti, 2010; Zhao and Dixon, 2010).

Many studies are needed to give a full understanding of the flavonoid transport network both at cellular and long distance level.

The regulation of the flavonoid pathway in the grapevine

Structural Genes

Most of the flavonoid synthesis regulation in grape vine occurs at the transcriptional level (Sparvoli et al., 1994; Boss et al., 1996). Furthermore, the transcriptional patterns of structural genes explain most of the inter-variety differential phenotypic expression in the berry colour (Kobayashi et al., 2001) and hue (Castellarin et Di Gaspero, 2007).

In 1994 PAL, CHS, CHI, F3H, DFR, LDOX, UFGT and StSy were first isolated and characterised in *Vitis vinifera* (Sparvoli et al., 1994).

Several expression studies in grape flower and berries showed the expression of all genes, except for UFGT, follow the same pattern: a peak in the first 4 weeks after flowering, very low expression for about 6-8 weeks, another peak at veraison and stable expression up to harvest. UFGT was never expressed until veraison, in accordance with the appearance of anthocyanins (Boss et al, 1996). The same pattern is also shown in white varieties, but UFGT is expressed in pigmented skin variety only (Kobayashi et al., 2001; Ageorges et al., 2006; Castellarin and Di Gaspero, 2007). This suggests that all the early genes of the pathway participate in the synthesis of all flavonoids, while UFGT is the key for anthocyanin synthesis.

Goto-Yamamoto and co-workers isolated and characterised in 2002 CHS1, CHS2 and CHS3. RT-PCR showed these isogenes co-expressed with other genes of the pathway, including UFGT. Later Q-PCR experiments confirmed that CHS1, CHS2 and CH3, as well as CHI1, CHI2, F3H1, F3H2, DFR and LDOX, followed the same pattern as UFGT in colouring Cabernet Sauvignon grape berry skins, showing a peak around 2 weeks post veraison. CHS2, CHS3, CH1 and F3H2 showed to be the predominant isogenes in grapes among their family (Jeong et al, 2004).

F3'H and F3'5'H were first isolated in grapevine in 2006 (Jeong et al., 2006; Bogs et al., 2006). F3'H and F3'5'H compete for the hydroxylation of the flavonoids' B-ring. Recent studies showed that F3'H is more expressed than F3'5'H in the flower, the stem, the tendril in the seed and in young berries. This is consistent with quercetin/myricetin and the procyanidin/prodelphinidin ratio in these tissues. In ripening berries, F3'H is highly expressed in both white and red cultivars before and after veraison. To the opposite, F3'5'H is activated at veraison in the cultivars that synthesise more trisubstituted anthocyanins (Jeong et al., 2006;Bogs et al., 2006;Castellarin and Di Gaspero, 2007). These findings suggest a strong transcriptional control in the determination of the B-ring hydroxylation degree of anthocyanins, flavonols and proanthocyanidins. However, young leaves showed a higher proportion of disubstituted anthocyanins despite a higher expression of F3'5'H (Jeong et al., 2006). Furthermore, other studies reported higher accumulation of quercetin despite higher expression of F3'5'H (Fujita et al.,2006). Hence, the role of F3'H and F3'5'H transcription in the determination of the composition of flavonoids is not completely clear. Post-transcriptional regulation mechanisms or different enzyme specificity might be involved as well.

High-throughput transcriptomic and gene expression studies highlighted the co-expression of a OMT with the flavonoid biosynthetic genes (Ageorges et al., 2006;

Castellarin and Di Gaspero, 2007; Pilati et al., 2007; Cutanda-Perez et al., 2009; Hugueney et al., 2009). A cation dependent anthocyanin-OMT was fully characterised in grapevine in 2009. *In vitro* experiments showed that OMT (or AOMT) accepts cyanidin-3-O-glucoside as well as delphinidin-3-O-glucoside and that it is able to yield all kinds of methylated anthocyanin found in grapevine (malvidinin-3-O-glucoside, penonidin-3-O-glucoside and petuidin-3-O-glucoside); OMT is active *in vitro* also with the aglycone anthocyanidins and with flavonols, but not with flavan-3-ols. Tobacco transformation experiments confirmed the ability to produce methylated anthocyanin (Hugueney et al., 2009). The expression patterns confirm that OMT regulates the B-ring methoxylation degree of anthocyanins in all cultivars. Cultivars with higher levels of methylated anthocyanins express more OMT. However, its role in the methoxylation of flavonols is still unclear (Castellarin and Di Gaspero, 2007; Hugueney et al., 2009).

The ratio between the expression of F3'5'H/F3'H and of OMT/UFGT is explains most of the phenotypic variability in berry colour among grape cultivars (Castellarin and Di Gaspero, 2007).

Based on the expression pattern shown in many studies, it is possible to distinguish between two groups of genes. Early flavonoid synthesis genes (EGs) are those showing two peaks during berry development: the first one around fruit set and the second one around veraison; late flavonoid synthesis genes (LGs) show only one peak at veraison and they are not detected in the early stages of berry development. CHS, F3H, F3'H, F3'5'H, DFR and LDOX are EGs, while UFGT and OMT are LGs. However, some isoforms of EGs behave as LGs, showing a differential regulation.

The flavan-3-ol genes ANR and LAR were isolated and characterised in 2005 (Bogs et al., 2005; Fujita et al., 2005). Their expression is temporal and tissue specific. Similarly to the EGs, they show a peak around fruit set. At veraison they have a modest activation.

This is consistent with the flavan-3-ol, proanthocyanidin and tannin accumulation kinetics. Furthermore, the expression of LAR1 and LAR2 in the grape berry skins follow almost the same pattern. The expression of LAR1 in the seeds doesn't change, but LAR2 is completely different, showing a peak at veraison (Bogs et al., 2005; Fujita et al., 2005; Fujita et al., 2007; Gagné et al., 2009; Lacampagne et al, 2010). This indicates that the regulation system occurring in the seeds is different from that occurring in the berry skin. Further studies are needed to elucidate the regulation of flavan-3-ol synthesis.

In 2003 and 2006, respectively two and five grapevine FLSs were cloned and characterised. FLSs are expressed in several tissues and organs. FLS1(VvFLS2) showed a constitutive, but significantly low, expression pattern. FLS2, FLS3, and FLS5 transcripts were found in small and medium leaves, in flowers and buds, while FLS4 was the most ubiquitous as it was detected in all leaves. In developing grape berries, FLS4 (VvFLS1) and FLS5 have a "EG like" expression pattern, showing one peak around flowering and one around veraison. This consistently with flavonol accumulation, while FLS2 was detected only around flowering (Downey et al., 2003; Fujita et al., 2006).

Transcription factors

In grapevine the transcript activation of the flavonoid pathway is very likely to involve an interaction of MYB, bHLH (MYC) and WDR-like factors, as described in other plant species.

MYB factors

In grapes MYB transcription factors were first found and characterised in 2002 in Kyoho, a tetraploid *Vitis labruscana* (*Vitis labrusca* X *Vitis vinifera*) variety. The authors demonstrated that VlMYBA1 controls the expression of UFGT in Kyoho grapes (Kobayashy et al., 2002). VlMYBA1 is a homolog of the VvMYBA1 gene, controlling UFGT in *Vitis vinifera*. The absence of anthocyanins in white grape varieties is linked to the lack

of VvMYBA1 and UFGT transcrips in these cultivars. This is due to the loss of function of VvMYBA1, caused by the insertion of a Gret1 retrotransposon in the promoter region of VvMYBA1 (Kobayashi et al., 2004; Kobayashi et al., 2005; Lijavetzky et al., 2006). The deletion of the functional VvMYBA1 allele causes the loss of pigmentation in the berry, this caused the appearance of the new white variety Pinot Blanc from Pinot Noir (Yakushiji et al, 2006). Bronze and white cabernet sauvignon sports have a similar deletion of functional VvMYBA1 alleles (Walker et al, 2006). Furthermore, it has been shown that the generation of red sports from white cultivars is associated with with a mutational function recovery of VvMYBA1 (Azuma et al., 2009). Red grapes accumulate anthocyanin in the skin, and VvMYBA1 shows specificity for this tissue. Some varieties (*teinturier*) accumulate anthocyanin also in the pulp. Recent studies suggest that this is associated with the loss of tissue specificity of VvMYBA1 (Jeong et al., 2006b). The VvMYBA1 Gret1 mutation is widespread in white grape cultivars, while pigmented cultivars have at least one functional copy of the gene. The allelic variation of VvMYBA1 is strongly associated with the different fruit colour phenotype found in *Vitis vinifera*'s cultivars (This et al., 2007; Azuma et al., 2008). It has recently demonstrated that VvMYBA1-2 not only UFGT, but also GST and AnthoMATE transporters (Cutanda-Perez et al., 2009).

Various MYB-factors are involved in the activation of the other structural genes of the flavonoid pathway. VvMYB5a and VvMYB5b (Deluc et al., 2006; Deluc et al., 2008) are involved in the expression of other genes of the flavonoid pathway such as CHS, CHI, F3H, DFR, LDOX, LAR and ANR, but not to UFGT or FLS. For this reason they are regarded as putative general regulator of the pathway.

VvMYBPA1 and VvMYBPA2 are putative regulators of proanthocyanidin biosynstesis. They are particularly active on LAR and ANR, the key enzymes leading to the flavan-3-ols, but also on other structural genes of the flavonoid pathway, but not UFGT or FLS.

VvMYBPA1 is more expressed in the seed, while VvMYBPA2 is more expressed in the berry skin, indicating a tissue specific activity of these factors (Bogs et al., 2007; Terrier et al., 2009).

VvMYB12 and VvMYBF1 are MYB-factors putatively associated to the expression of FLS in grapevine reported in 2008 and 2009 (Matus et al., 2008; Czemmel et al., 2009). Their sequence is almost identical and they are homologous to AtMYB12. The expression of these genes is correlated to the accumulation of flavonols in the berry. Czemmel and coworkers showed that it lacks of a bHLH binding site, so it is probably independent from a bHLH factor, similarly to other FLS regulators in *Arabidopsis* and maize.

These studies indicate a very complex regulation system, but it seems that there are some regulators involved in the general activation of the genes of the pathway, such as the MYB5s, while other genes activate specific branches of the pathway, leading to the synthesis of the target metabolites. With the knowledge available to date, the regulation scheme of the flavonoid pathway can be temptatively summarised as follows:

- MYB5s --> general activation --> flavonoid intermediates
- MYBAs --> UFGT /OMT --> Anthocyanins;
- MYB12 --> FLS --> Flavonols;
- MYBPAs --> LAR/ANR --> Proanthocyanidins.

However, recent Rna-seq high-throughput expression studies indicated 36 MYB genes involved in grape ripening (Zenoni et al., 2010). Thus, other unknown MYB factors could still be involved. The role of MYBA is still controversial, as some authors propose that it could co-regulate directly or indirectly other genes of the pathway (Jeong et al., 2004; Matus et al., 2009; Cutanda Perez et al., 2010). Furthermore, many question (e.g. anthocyanin acylation, proanthocyanidin condensation) still need an answer, so this model is still incomplete.

<u>**bHLH and WDR factors**</u>

bHLH and WDR factors related to the flavonoid pathway were found in grapevine only in 2010. The first studies suggest that some of these genes (VvMYC1, VvMYCA1, VvWDR1) are implied in the transcriptional cascade that leads to flavonoid synthesis, and that they are associated to MYBAs (Hicri et al., 2010; Matus et al., 2010). However, the complex MYB-bHLH-WDR interactions regulating the flavonoid pathway in grapevine are still far from being understood.

Flavonoid transport in the grapevine

In grape berries anthocyanins are stored in the vacuole of the cells of the first external layer of the hypoderm. The other flavonoids are not only present in the vacuole, but also in the tonoplast and in the cell wall of berries and seeds.

Vesicle transport is likely to be active as anthocyanins were found in ACPs and AVIs, but this mechanism is still controversial (Braidot et al., 2008; Zhao and Dixon 2010).

High throughput and ectopic expression studies showed that a grapevine MATE-type transporters (Antho-MATE) are co-expressed with the transcription factor VvMYBA1-2 (Ageorges et al., 2006) and VvMYBPA2 (Terrier et al., 2009) and therefore whit anthocyanin ad proanthocyanidin synthesis respectively. Recently two antho-MATE transporters (AM1 and AM3) were isolated and characterised in the grapevine (Gomez et al., 2009). The authors demonstrated that these MATE proteins are acylation-dependent anthocyanin transporters in grape berries. Interestingly, AM1 and AM3 seem to under the control of different regulators. A GST was associated with fruit pigmentation in grapevine for the first time in 2006 (Ageorges et al., 2006). Later, it was demonstrated that VvGST1 and VvGST4 are involved in anthocyanin transport into the vacuole (Conn et al., 2008). VvGST4 expression pattern shows a particularly interesting expression peak at veraison.

Furthermore, it has been proposed that a homolog of the mammalian bilitranslocase (BTL) may be involved in flavonoid translocation in the grapevine. Recent studies suggest that a BLT-like translocator could be responsible for anthocyanin accumulation in the skin and for intermediate metabolite translocation during grape berry development (Braidot et al., 2008). Despite the number of transporters directly or indirectly associated to the flavonoid pathway, little is known about the flavonoid transport network in grapevine, and more studies are required to fully elucidate this system.

Light and flavonoids

Factors influencing the flavonoid pathway.

Flavonoid synthesis, transport and accumulation are strongly influenced by a number of endogenous (e.g. physiological state of the plant, plant vigour, sink/source balance, plant hormones) and environmental factors (e.g. water supply, soil fertility, viticultural practices, temperature, sunlight, biotic adversities). Abscissic acid, auxins and ethylen increase flavonoid synthesis, while giberellic acid inibites it (Jeong et al., 2004; Braidot et al., 2008; Lacampagne et al., 2009). Wounding and pathogenesis have been identified as negative factors (Braidot et al., 2008). Moderate water stress consistently enhances anthocyanin synthesis (Castellarin et al., 2007) High vine vigour (and, consequently, all farming practices leading to high vigour) has been identified as responsible for a lower flavonoid content in the berries (Cortell et al., 2007). This may have a number of physiological reasons (i.e. source\sink balance), but it also may be connected to a worse exposure of grapes to sunlight due to the excessive vegetation. The anthocyanin profiling is strongly cultivar dependent and it is successfully used for chemotaxonomy studies (Mattivi et al., 2006; Ortega Regules, 2006), however cluster microclimate influences the relative proportion of anthocyanins. Extreme high and low temperatures have a negative effect on anthocyanin synthesis (Rustioni et al., 2006), (Mori et al, 2007). Light is generally recognised as a positive factor for flavonoid synthesis, but in spite of many studies, its role is not fully elucidated.

The role of light and shading.

Light is the energy supply for plants. This alone makes it one of the most important environmental factor for plant life. Plants are able to sense several parameters (fluence,

wavelength, direction, duration) of ambient light. Light is a key factor to a great number of physiological processes in plants, including seed germination, chloroplast movement, flower induction and circadian rhythms. Four families of photoreceptors are known in plants: phytochromes, cryptochromes, phototropines and presumably some kind of UV-photoreceptors. The light signal perceived by the photoreceptors is mediated by a transcriptional regulatory network that up- and down-regulates specific downstream genes, activating or repressing entire metabolic pathways in response. The role of light in the activation of the main metabolic pathways in plants was reviewed by Jiao et al. in 2007. In higher plants, anthocyanin synthesis seems to be regulated by the phytochrome A and by the UV-A and UV-B photoreceptors (Gollop et al., 2002)

Light have several roles in in the photoprotection, UV- screening and antioxidant activity in plants (Hernandez et al., 2008; Agati and Tattini, 2010). In some species (e.g. apple and petunia) light exclusion prevents fruits and flowers from accumulating red pigments, but in the grapevine light is not essential for flavonoid biosynthesis, although it is generally recognised as a positive factor (Downey et al., 2004). UV-A and UV-B radiation influence many processes connected to plant development, morphology and physiology. Synthesis of flavonoids is most effective plant response to UV stress, so it is not surprising that the expression patterns of several genes of the flavonoid pathway showed a positive correlation with UV radiation exposure (Guo et al., 2008).

In the past, many studies reported a higher quantity of anthocyanins and other flavonoids in bunches exposed to higher light levels. However, some studies reported no changes of anthocyanin levels of shaded and exposed bunches, and in some cases even the opposite effect. Possible explanation for these contradictory effects are connected not only to the cultivar, to the location and to the season, but also on the micro-climatic effect of shading treatments. In particular, the negative effect of high temperature may have not

been taken into account accurately enough. The use of appropriate shading screens (Downey et al., 2004; Rustioni et al., 2006) allow to minimise the effect of temperature, allowing for a better understanding of the role of light.

The effect of light on anthocyanins

Sunlight exposition significantly increased the total anthocyanin content in Merlot grapes (Spayd et al., 2002; Pereira et al., 2006). However, low light incidence alone showed no significant effect on the total anthocyanin content of Merlot berries (Tarara et al., 2008). Regardless the effect on the absolute concentration, shading caused a proportional increase of acylated forms (Pereira et al., 2006; Tarara et al., 2008). The influence of light on the anthocyanin profiling of Merlot is controversial: Pereira et al. (2006) reported the relative increase of cyanidin-3-glucoside and peonidin-3-glucoside in the shaded bunches, while Tarara et al., (2008) reported the opposite effect.

The anthocyanin content in in Pinot Noir was not significantly affected by sun exposure (Price et al., 1995; Cortell and Kennedy 2006). Nevertheless, shading caused a proportional increase of peonidin-3-glucoside in the anthocyanin profiling (Cortell and Kennedy, 2006).

In Cabernet Sauvingon and Grenache grapes, cluster exposure to sunlight showed two opposite effects: on the northern side of the canopy total anthocyanins and phenolics increased, on the southern side of the of the canopy they were reduced. (Bergqvist et al., 2001). In other works, shading reduced Cabernet Sauvignon anthocyanin content (Jeong et al., 2004; Matus et al., 2009). The anthocyanin profiling of Cabernet Sauvingnon was also affected by light (Matus et al., 2009).

In Shiraz grapes grown under artificial conditions, anthocyanin accumulation was faster in exposed berries at the beginning of ripening. However, the anthocyanin content at

harvest were the same in both exposed and shaded berries. Conversely, shaded berries accumulated more anthocyanins under open field conditions. Shaded berries accumulated also a larger proportion of malvidin-3-glucoside (Haselgrove et al, 2000).

In another experiment, cluster shading in Shiraz grapes caused no changes (in two seasons) or a a reduction in total anthocyanin (in one season). However, the authors highlighted a change in the anthocyanin profiling in all years: shading resulted in a decrease of delphinidin-based anthocyanins both on a relative and absolute basis, (Downey et al., 2004). Accordingly, cluster shading in Shiraz grape caused no changes in Shiraz grape berries total anthocyanin content, but the combined proportion of cyanidin-3-glucoside and peonidin-3-glucoside increased from 18% in the control to 27% in the shaded grapes (Ristic et al., 2007).

Cluster shading in Nebbiolo caused a lower accumulation of anthocyanins in the early stages of berry ripening, while the concentration at harvest was the same of control grapes. However, there was a shift in the anthocyanin composition, as the proportion of methoxylated anthocyanins (malvidin particularly) was higher in exposed bunches (Rustioni et al., 2006).

UV radiation showed a positive effect on anthocyanin accumulation in Gros Colman grapes (Kataoka et al., 2003), while it showed no effect in Merlot, Cabernet Sauvignon and Grenache grapes (Bergqvist et al., 2001; Spayd et al., 2002).

Many studies investigated the effect of bunch exposure on anthocyanin accumulation in grapes, but the results are sometimes in contrast. The role of light may be difficult to interpret because of the interference of temperature. The positive effect of cluster exposure on total anthocyanin accumulation is evident when temperature is optimal for synthesis and light is the only limiting factor. In warm weather, the positive effect of light exposure can be contrasted by the high temperature reached during the day, hence bunch

exposure to sunlight show no or, in some cases, negative effects. Taken together, these studies suggest that light has a larger effect slowing down or delaying anthocyanin synthesis close to veraison rather than towards end of ripening, as the shaded and exposed bunches often reach similar anthocyanin levels at full maturity. These findings also indicate that light has an effect on the anthocyanin profiling, most of the times causing a shift in the proportion of the disubstituted and trisubstituted anthocyanins and in the ratio of acylated/non acylated anthocyanins.

The effect of light on flavonols

The role of light on flavonol synthesis in grape berries seems well established and it is consistent with the UV-protection function exerted by these compounds.

Flavonols showed high concentration in exposed Pinot Noir berry skins (Price et al., 1995; Cortell and Kennedy, 2006). Merlot berries exposed to sunlight showed up to 10 fold more flavonols compared to shaded berries. (Spayd et al., 2002; Pereira et al., 2006; Tarara et al., 2008). Cluster shading caused a significant reduction in the synthesis of flavonols in Shiraz (Haselgrove et al., 2000; Downey et al., 2004; Ristic et al., 2007) and Cabernet Sauvignon grapes (Matus et al., 2009). UV radiation also had a positive effect on flavonoid accumulation (Spayd et al., 2002). Moreover, sunlight exposure specifically increased the quantity of quercetin-glucoside in Merlot grapes, while the kaempferol-glucoside remained unchanged. However, the authors did not detect the levels of myricetin-glucoside (Tarara et al., 2008).

Taken together, these results point out that light is the most important factor determining the levels of flavonols in grape berries.

The effect of light on tannins

Cluster shading in Shiraz grapes caused a reduction of condensed tannins in the skins, while no effect was observed in the seeds. Furthermore, a significant reduction in the proportion of epicatechin based flavan-3-ols was observed in shaded berries (Downey et al., 2004). In 2007, cluster shading caused Shiraz grapes to accumulate more tannins in the seeds and less tannins in the skins compared to control (Ristic et al., 2007).

Accordingly, cluster shading in Pinot Noir reduced the accumulation of skin tannins, but in the seeds differences were very small (Cortell and Kennedy, 2006).

The accumulation pattern in Cabernet Sauvignon is very similar: shading caused a lower accumulation of flavan-3-ols during berry development, however the differences were smaller in both in the berry skin and in the seeds at harvest (Fujita et al., 2007).

In Shiraz and Pinot Noir, shading caused a shift in the flavan-3-ol composition. The proportion of the cyanidin-based (catechin and epicatechin) sub-units was reduced by shading Conversely, the proportion of the delphinidin-based (gallocatechin and epicatechin) sub-units was increased (Downey et al., 2004; Cortell and Kennedy, 2006). The shift in the flavan-3-ol composition resembles the anthocyanin profiling shift reported for some cultivars.

The effect of light on the flavonoid pathway's genes transcription

The effect of light on the transcription of the genes of the flavonoid pathway in the grapevine was first described by Sparvoli et al., in 1994. Anthocyanin synthesis in cultivar Lambrusco f.f. seedlings was triggered by 12 hours of continuous light. Six downstream structural genes of the flavonoid pathway showed to be up-regulated by light: CHS, CHI, F3H, DFR, LDOX and UFGT. The same genes where expressed at a very low level in the

seedlings grown in the dark. White light induces the expression of DFR in Gamay red cell suspension cultures (Gollop et al., 2002).

In recent years some experiments attempted to elucidate the effect of shading on the expression of the genes of the flavonoid pathway under open field conditions. CHS, CHI, F3H, DFR, LDOX and UFGT were down-regulated in Cabernet Sauvignon shaded berries (Jeong et al., 2004). Similarly, CHS2, LDOX, OMT and UFGT in Cabernet Sauvignon berries were down-regulated by the shading treatment (Matus et al, 2009). These findings seem to confirm the previous studies. However, Shading showed little or no effect on the transcription of UFGT in Shiraz berry skins (Downey et al., 2004).

VvFLS1 is significantly more expressed in exposed Shiraz berry skins (Downey et al., 2004). FLS4 (corresponding to VvFLS1) is more expressed in sunlight exposed Cabernet Sauvignon and Merlot ripening grapes (Fujita et al., 2006; Matus et al., 2009). These results are in accordance with the higher accumulation of flavonols in exposed berries reported in several works. Surprisingly, the expression pattern of FLS5 in Merlot and Cabernet Sauvignon seemed to be light-independent (Fujita et al., 2006), however the enzymatic activity of the FLS5 protein still needs to be studied, in order to asses its role in flavonol synthesis.

Shading affects the transcription of ANR and LAR to a lesser extent than FLS4 (Fujita et al, 2007). During the early stages of development the expression of VvANR in shaded berry skins was down-regulated, but cluster exposure showed no significant effect in the later stages. VvLAR2 followed a similar pattern in the skins, while the expression of VvLAR1 was unaffected by the shading treatment. In the seeds, both exposed and shaded VvANR and VvLAR1 showed little or no difference during the whole berry development, while VvLAR2 was surprisingly more expressed in the seeds of shaded berries (Fujita et al., 2007). The differential control of light on the genes of this branch of the pathway

seems in accordance with some of the shifts in the flavan-3-ol composition caused by shading.

In Cabernet Sauvignon, MYB12 was dramatically down-regulated by the shading treatment, the expression of MYBA1 and MYB5a was also reduced, even though to a lesser extent, while MYB5b and MYBPA was apparently unaffected (Matus et al., 2009). Similarly, VvMYBA1 was down-regulated also in a previous experiment (Jeong et al., 2004).

To date, information about the effect of shading on the key enzymes of the flavonoid pathway is far from being complete. The elucidation of the effect of light on the regulation network of the flavonoid pathway is only at the beginning, and it needs more knowledge to be better understood. F3'H and F3'5'H are key enzymes determining the composition of the anthocyanin and flavan-3-ol profiling, and the shifts caused by shading suggest that these genes may also be affected, but to date there is no data about the effect of light on the expression of F3'H and F3'5'H. Furthermore, to our knowledge, no data is available yet on the effect of light on the expression of the genes encoding for anthocyanin transport-related proteins. In this work the effect of cluster shading on the expression pattern of several structural genes, transcription factors and transporters of the flavonoid pathway is investigated.

Aglianico

Origin and main characteristics

Aglianico is a red grape cultivar widespread in Southern Italy renowned for the quality of its wines. Aglianico is grown in several Italian Regions, but it is mainly cultivated in Campania and Basilicata, and particularly in the provinces of Benevento, Avellino and Potenza. Aglianico has several synonyms: Aglianica, Aglianichella, Aglianico del Vulture, Aglianico Femminile, Aglianico Mascolino, Aglianico Nero, Aglianico Tringarulo, Aglianico Zerpoluso, Aglianicuccia, Agliano, Agnanico, Agnanico di Castellaneta, Cascavoglia, Cerasole, Ellanico, Ellenico, Fresella, Gagliano, Ghiandara, Ghianna, Ghiannara, Glianica, Gnanico, Olivella di San Cosmo, Ruopolo, Sprierna, Tringarulo, Uva dei Cani, Uva di Castellaneta.

The origin of this variety is very ancient. The cultivation of Aglianico is Southern Italy is traditionally is dated back to the Greek colonization. Following this hypothesis, the name "Aglianico" could derive from the word "Hellenica" (Boselli., 2003). However, the first written evidence of the cultivation of Aglianico dates back to the 16th century. Anyway, Aglianico has been grown in Southern Italy for many centuries.

Aglianico is well defined from the ampelographic point of view:

• medium-small, pentagonal or orbicular, three or five lobed, dark green leaves;

• V shaped petiolar sinus and U shaped lateral sinus;

• conic or cylindric, medium size, rather compact clusters;

• medium-small, spherical, blue-black berries.

The vegetative cycle is long: around 180 days from bud burst to full maturity. Bud burst is early while veraison and ripening are late. It is a vigourous variety and yield is

high. Fruit-bering shoots are produced from the 3rd or the 4th node, with one (rarely two) clusters per shoot. Lateral shoots are scarcely fertile.

The main traditional growing areas of Aglianico are Taburno, Taurasi (in Campania) and Vulture (in Basilicata). A recent study investigated the differences in the grape wine-making potential among the traditional Aglianico growing areas (Simone Di Lorenzo, 2009)

Vulture vineyards showed a larger homogeneity and a lower yield: Aglianico grapes in this area generally accumulated high sugars although keeping a good acidity. The grapes in Vulture showed also high levels of anthocyanin and tannins.In Taburno vineyards were rather homogeneous, but grapes were generally low in sugars and phenolic compounds. Taurasi showed intermediate results.

The results of this work indicate that the different wine-making potential of the grapes are mainly due to the different environmental conditions in the three areas. However, over time, different clonal lines have been selected in the different area. So, the genetic component may play a role in the determination of the differences in Aglianico grapes.

Flavonoid composition of Aglianico

Aglianico is rich in phenolic compounds compared to other widespread cultivars like Cabernet Sauvignon and Merlot (Moio et al., 2004). Aglianico shows a high quantity of extractable polyphenols (between 3,6 and 3,9 g/kg grapes), a medium-high content of anthocyanins (between 0.7 and 0.9 g/kg grapes) and a high content of proanthocyanidins (between 3,4 and 3,7 g/kg grapes) (Mattivi et al., 2002; La Gatta et al., 2007)

Aglianico's anthocyanin profiling is mainly composed of malvidin-3-glucoside (around 60% of total anthocyanins) followed by petunidin-3-glucoside and delphinidin-3-glucoside (between 5% and 10% each) while peonidin-3-glucoside cyanidin-3-glucoside are very low

(less than 5% and 1% respectively); acylated anthocyanin range between 20% and 25% of total anthocyanins. . Trisubstituted anthocyanins are over 90% of anthocyanin glucosides, while p-coumarate-anthocyanindins are the main acylated form (Lovino et al., 2005; Suriano et al., 2005; Mattivi et al., 2006).

Aglianico grapes have a high content of tannins. A high proportion of extractable proanthocyanidins (between 40 and 45%) and flavans reactive to vanillin (between 70% and 75%) are localised in the seeds (Mattivi et al., 2002).

Aglianico has a medium content of flavonols, and myricetin and quercetin derivatives are the main flavonol (Mattivi et al., 2006; Tamborra and Esti, 2010).

The expression of the flavonoid pathway in Aglianico

As opposed to the well-known Pinot Noir, Cabernet Sauvignon, Merlot and Shiraz grapes, very little work has been devoted to the study of the expression of the genes of the flavonoid pathway in this particular variety. Castellarin and Di Gaspero, in 2007, studied the transcriptional control on grape berry pigmentation. The authors analysed the transcript levels of F3H, UFGT, GST, F3'H, F3'5'H, MYBA, MYBB, MYBC and MYBD in several ripening grape cultivars shoving different berry pigmentation patterns, including Aglianico. The cumulative level of transcripts of all genes were high in Aglianico, compared to other cultivars, meaning that this pathway is active in Aglianico ripening berries. This is consistent with the high content of flavonoids shown by this cultivar. The expression of UFGT and other anthocyanin genes was delayed compared to all cultivars. Aglianico showed a high F3'5'H/F3'H ratio, in accordance to the abundance of trisubstituted anthocyanins, and a medium high OMT/UFGT ratio.

A model for grape intra-variety variability

Aglianico is characterised by a great intra-variety variability originating from the conscious or unconscious selection operated over the centuries by farmers in the differen (Caputo et al., 2009). The molecular basis of grape intra-variety variability are still largely unknown, however it is connected to the occurrence of structural and epigenetic mutation, normally at bud level, that are fixated through vegetative propagation (Shneider, 2006). Among several "Aglianicos", three main biotypes were selected in the the corresponding main cultivation areas: Taurasi (from the province of Avellino), Taburno (from the province of Benevento) and Vulture (from the province of Potenza) (Boselli, 2003). The monophyletic origin of these biotypes was confirmed by DNA fingerprinting (Costantini et al., 2005). The different behaviour shown by the Aglianico biotypes is a good example of intra-variety variability, and it is the base for the genetic improvement of this grape variety.

The differences among the biotypes regard mainly the morphology of the bunches and the timing of veraison and ripening. In particular, from a recent ampelographic survey of Region Campania an the University of Naples:

• Taurasi has a conic-pyramidal cluster, often with a lateral cluster. Berries are round, the skin is blue-black and the pulp is colourless. The average weight of clusters is low, the weight of the berries is very low. Sugar accumulation is high and titrable acidity is medium-high.It is not very vigourous, bud fertility is discrete and yield is consistent. It may show a moderate millerandage (hen and chicken), depending on the season.

• Taburno has a conic-pyramidal cluster, often with a lateral cluster. Berries are round, berry skin is blue-black and the pulp is colourless. The average weight of clusters is low, the weight of the berries is low. Sugar accumulation is high and titrable acidity is medium-high. Taburno is vigourous, with a discrete fertility and a high yield. It shows moderate millerandage.

- Vulture has a medium compact, medium-small, medium-short, conic-cylindric cluster, sometimes with a lateral cluster and with a short stem. Berries are round, the skin is blue with bloom, the thickness of the skin is medium, and the pulp is colourless. Vigour is medium, and the yield per hectar ranges from 4 to 10 tons, depending on the soil fertility.

Taburno has a lower bud average bud fertility, a later onset of ripening, a earlier ripening and a higher cluster weight (SeSirca, 2001).

The three main Aglianico biotypes show differences also in the flavonoid accumulation kinetics. In 2000 and 2001 the phenolic accumulation pattern Taburno, Taurasi and Vulture biotypes was compared. Vulture presented high levels of anthocyanins and low levels of tannins both in skins and seeds. Taburno had the lowest anthocyanins, but the highest levels of seed tannins. Taurasi had an average behaviour (Moio et al., 2004).

The differences in the transcription of the genes of the flavonoid pathway in Aglianico's biotypes has not been studied yet. The range of phenotypic expression shown by Aglianico makes it a good model for studying the basis of intra-variety variability in grape cultivars. For this reason in this work the effects of shading on the genes of the flavonoid pathway in the three main biotypes of Aglianico will be studied.

Materials and Methods

Plant material and experimental design

Grape samples were collected in the experimental vineyard located in Galluccio (CE) during the 2008 season. Samples were collected from 3 biotypes of *Vitis vinifera* L. cv Aglianico: "Taurasi", "Taburno" and "Vulture". On the 27th of July, a shading screen was applied to grape bunches of 12 plants from each biotype. The shading screens were designed on the basis of the World Meteorological Organization's standards defined for screen boxes of meteorological stations (W.M.O. 1996). The boxes were made of white reflective laminated paper measuring 200 x 200 x 250 mm (L x W x H) , and a set of double parallel angled slats were positioned on all sides of the box, except for the top. The boxes had vents in order to maximize airflow and, therefore, minimize temperature and relative humidity differences between the bunches inside the boxes and the air conditions in a meteorological station. Previous works demonstrated that air temperature and humidity conditions inside and outside the box are similar (Rustioni et al., 2006). Control bunches were fully exposed to sun light trough leaf removal. Veraison occurred in the second decade of September.

Triplicate samples for each biotype and condition where collected for the metabolite profiling along four sampling times for Taurasi and Vulture (256, 279, 276 and 289 GG) and, due to a different timing, three for Taburno (279, 276 and 289 GG). Whole berries were collected and stored at -20°C.

Samples for the gene expression analysis were collected from veraison to full maturity at three ripening stages for Taurasi and Vulture (256, 270 and 276 GG) and, due to a different timing, in the last two stages of ripening (270 and 276 GG) for Taburno. Berry

skins for the gene expression analysis were manually separated from the mesocarp and instantly frozen in liquid nitrogen and stored at -80°C until use.

The different sampling for Taburno are due to the peculiar ripening timing shown by this biotype.

Determination of the metabolite profiling

In order to assess the progress of grape berry ripening and to associate the physiological phases to the observed gene expression, we measured soluble solids, pH and titrable acidity, total polyphenols, total flavonoids, non-anthocyanin flavonoids, tannins, total anthocyanins and the anthocyanin profiling.

Grape juice was obtained by manual crushing of the grapes to determinate total soluble solids (°Brix), pH and titrable acidity using a hand held refractometer (ATAGO CO., Ltd), a pH meter (Hanna) and an automatic titrator (Crison Compact Titrator).

Polyphenol extraction was performed from the skins of 20 randomly selected frozen berries. Berry skins where added with 100 ml of Methanol/HCl 1%. Samples were kept in the dark for 24 h and then filtered and stored in the dark until analysis.

An aliquot of the extraction mixture was diluted 1:32 with Ethanol:H_2O:HCl 70:29:1 and the total anthocyanin content was evaluated by measuring the absorbance at wavelength 535 nm, referring the values to a malvidin 3-glucoside calibration curve, while total flavonoids and non anthocyanic flavonoids by registering the absorbance spectrum between wavelength 230 nm and 700 nm.

50 μl of the extraction mixture were added with 0,5 ml of the Folin-Ciocalteu reagent and 4ml of water. After 3 minutes the samples were added with 2 ml of Na_2CO_3 and water to 10ml. After 90 minutes, total polyphenols were determined by measuring the

absorbance at wavelength 700 nm, referring the values to a (+) cathechin calibration curve. In a 2ml eppendorf tube, 750 μl of extraction mixture diluted 1:1 were added with 200 mg of PVPP and thoroughly mixed. After 15 minutes at 4°C, samples were centrifuged at 13000 rpm for 5 minutes. The supernatant was treated and analysed as previously described for total polyphenols determination, and tannin concentration was calculated by difference between total polyphenols and polyphenols after PVPP.

The anthocyanin profiling was analysd with a Shimadzu LC-10ADvp, SIL10ADvp HPLC equipment. Chromatographic analysis was performed with a mobile phase linear gradient of HClO3 0,3% in water as Solvent A and MeOH as solvent B at a constant flow-rate of 0,45 ml/min. The gradient elution profile was the following: 0 min, 27% B, 73% A; 1-32 min, 43% B, 57% A; 32- 45 min, 68,5% B, 31,5% A; 45-47 min, 100% B; 3 min constant 100% B. A wavelength of 520 nm was used for the absorbance detector.

Gene expression analysis

Total RNA from berry skins was exctracted with a protocol similar to the one described by Moser et al. (2004). Grape berry skins were crushed to a powder with a electric grinder in presence of N_2 and stored at -80°C An extraction buffer (XT) was prepared as follows: Na borate decahydrate 0,2 M; EDTA pH 8 0,03 M; SDS 1%(v/v); Na deoxycholate 1% (w/v). Prior to use, the XT buffer buffer was warmed up to 50°C and added with: β-Mercapto Ethanol 2%; Spermidin 0,05 M; Nonidet p-40 1%; PVP-40 2%.

The buffer was then warmed up to 80°C. 400 μg of crushed skins were added with 1400 μl of XT buffer 80°C in a 2 ml Eppendorf tube. Samples were kept at 80°C for 5 minutes and then 42°C for 1 hour; samples were then added with 120 μl 2M KCl and kept for 45 minutes at 4°C. After centrifugation for 15 minutes at 8°C at 13000 RPM, the supernatant was transfered into a new 2 ml Eppendorf tube and added with 1/3 v/v LiCL

8M and 1% β-ME and it was stored at 4°C overnight. Samples were centrifuged for 25 minutes at 8°C at 13000 RPM, the supernatant was discarded and added with 300 μl of LiCl and 600 μl of H2O. Samples were again centrifuged for 15 minutes at 8°C at 13000 RPM; washing with LiCl was repeated until supernatant became colourless. Samples were added with 600μl tris-HCl 10m pH 7,5 and 1/10 v/v K-acetate 2M pH 5,5 and kept for 10' minutes on ice. Samples were centrifuged for 15 minutes at 8°C for 13000 RPM, Added with 0,9 volumes of IPA and stored for 1 hour at -20°C. Samples were centrifuged for 25 minutes at 8°C at 13000 RPM, supernatant was discarded and pellet was added with 1 ml EtOH 80%. Samples were centrifuged for 15 minutes at 8°C at 13000 RPM, supernatant was discarded and pellet was kept in vacuum until ethanol completely evaporated and pellet was dry. Pellet was suspended in 100 μL RNase-free H2O.

Samples were treated with 60 μl of LiCl 8M (3M final) and thoroughly mixed, kept for 3 hours at 4°C and then centrifuged for 20 minutes at 4°C at 13000 RPM. supernatant was discarded and pellet was added with 1 ml EtOH 80% and thoroughly mixed. Samples were centrifuged for 15 minutes at 8°C at 13000 RPM, supernatant was discarded and pellet was kept in vacuum until ethanol completely evaporated and pellet was dry. Pellet was suspended in 100 μL RNase-free H2O.

RNA was subsequently purified and concentrated using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. The purified total RNA was treated with the DNAse I AMP GRADE (Invitrogen). Total RNA quantity and quality was controlled with a nanodrop spectrophotometer. cDNA synthesis was performed using the SUPERSCRIP III 1st strand kit (Invitrogen) following the manufacturer's handbook. Relative transcript quantification of target genes was performed through Real Time PCR using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) following the manufacturer's protocol on an Applied Biosystem 7300 Real Time PCR system (Applied Biosystem). Thermal cycling

conditions were: 50°C for 2 min, 95°C for 10 min followed by 95°C for 15 s, 60°C for 30 s for 40 cycles, followed by a melting cycle from 60°C to 95°C. Each cDNA sample was analysed in quadruplicate. Gene transcripts were quantified upon normalization with GADPH (CB973647) comparing the cycle threshold (Ct) of the target gene with that of GADPH (Reid et al, 2006). GADPH efficiency was tested in all samples at different dilutions (10^{-1}, 10^{-2}, 10^{-3}) and the locus proved to be suitable as housekeeping gene (Figure I*)* Primers were newly designed for this work are shown in Table I. Statistical analysis was performed with the the SPSS statistical software and the R statistical package.

Results

Metabolite kinetics

The effect of shading on ripening kinetics

Physiological and technological variables

Berry weight increased from veraison (256th day) to the end of ripening. Berry weight
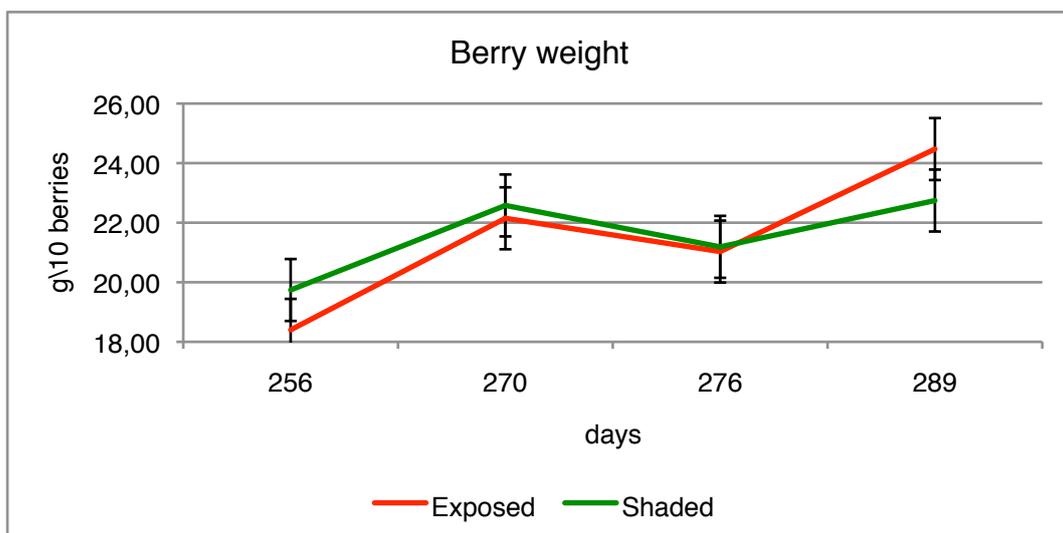
Figure 1. The evolution of berry weight in exposed (red) and shaded (green) clusters during Aglianico grape ripening.

was unaffected by the shading treatment (figure 1).

Skin weight was relatively constant in the first stages of ripening, but it grew towards harvest. Skin weight was unaffected during the early stages of ripening but, towards the end of ripening,shaded bunches skin weight grew significantly faster (figure 2). Thus, shading generally caused a significative progressive increase of the skin/berry ratio, while exposed bunches showed a decrease during grape ripening (figure 3).
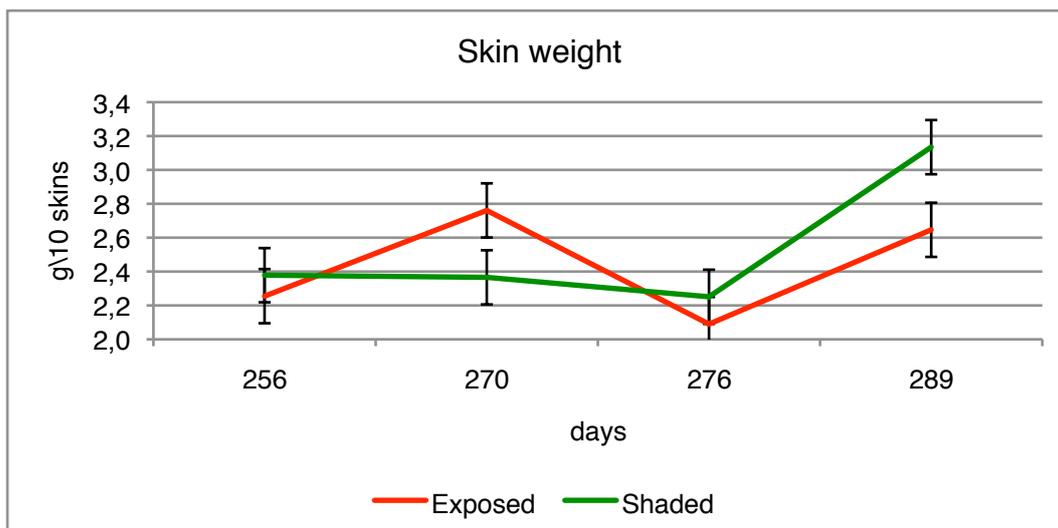
Figure 2. The evolution of skin weight in exposed (red) and shaded (green) clusters during Aglianico grape ripening.
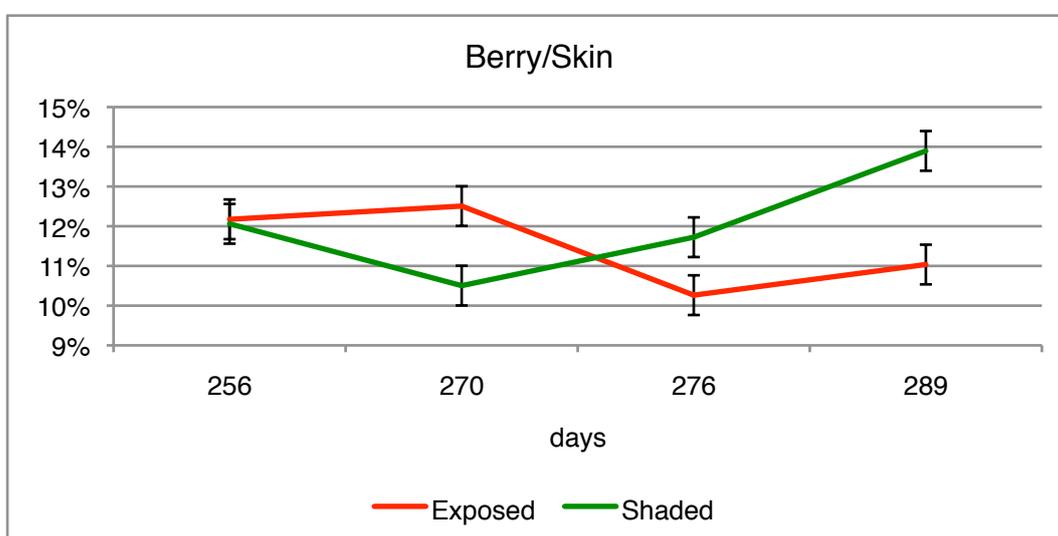
Figure 3. The evolution of berry/skin weight ratio in exposed (red) and shaded (green) clusters during Aglianico grape ripening.
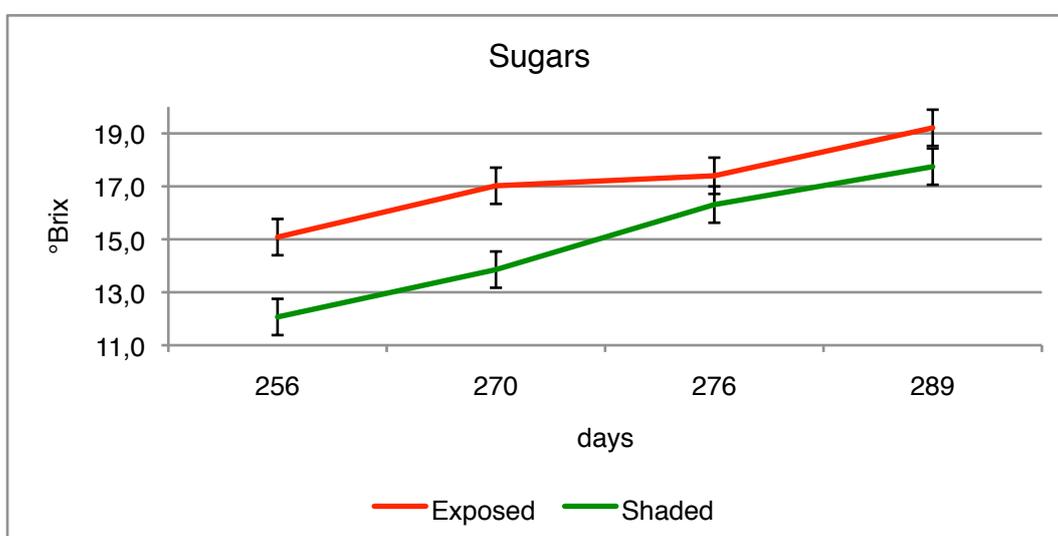
Figure 4. The evolution of sugar concentration in exposed (red) and shaded (green) clusters during Aglianico grape ripening.

Sugar concentration grew constantly all-throughout the ripening peridod. Shading caused a delay in the accumulation of sugars in the shaded bunches, however, towards the end of ripening, exposed and shaded bunches showed no significant difference in sugar concentration (Figure 4). Also pH showed a regular increase during ripening. Shaded bunches showed a significantly lower pH at veraison, but no difference in pH was shown in ripe exposed and shaded bunches (Figure 5). Consequently, titrable acidity decreased steadily from veraison to ripening. Tirable acidity was significantly higher in shaded berries all-throughout berry ripening, but differences got smaller towards the harvest date (Figure 6).
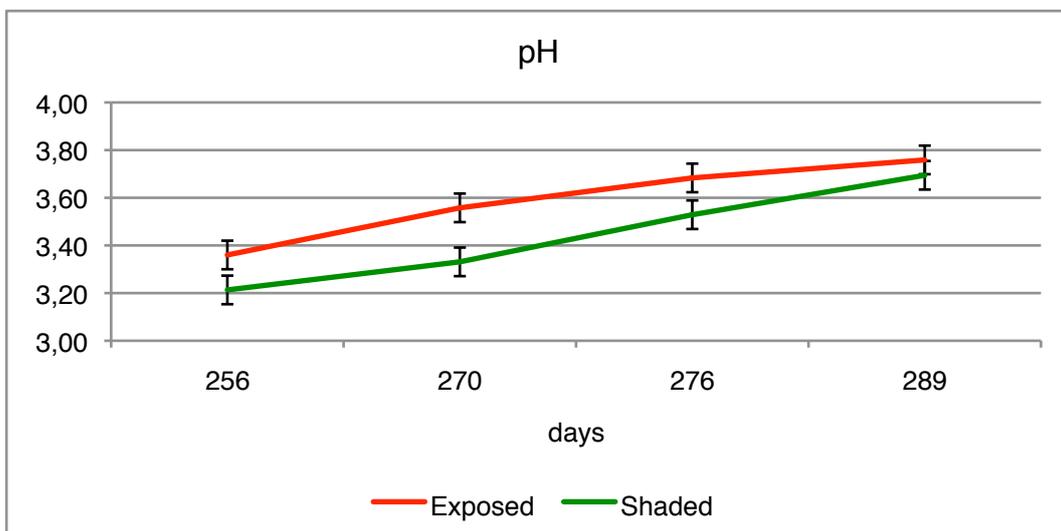
Figure 5. The evolution of pH in exposed (red) and shaded (green) clusters during Aglianico grape ripening.
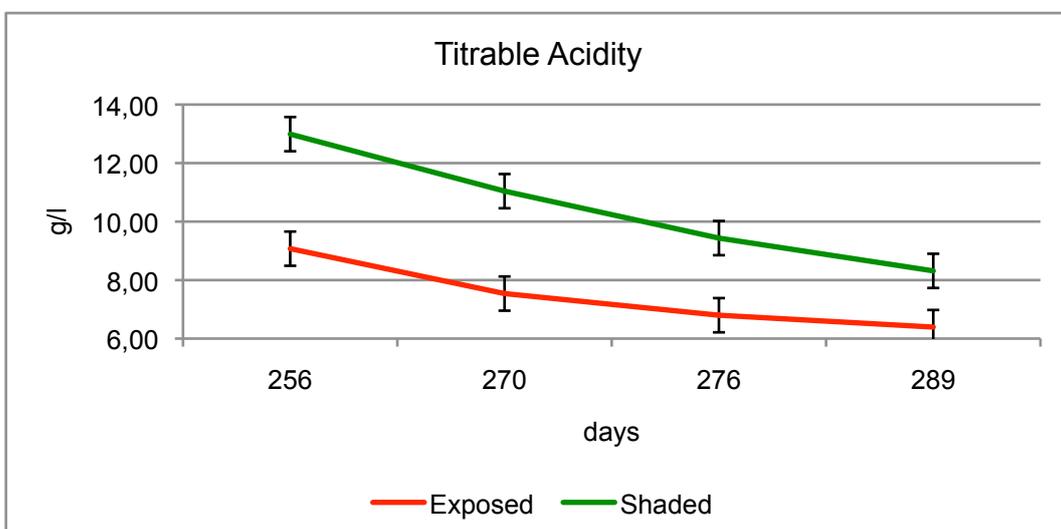
Figure 6. The evolution of titrable acidity in exposed (red) and shaded (green) clusters during Aglianico grape ripening.

Phenolic compounds

Total polyphenols generally showed a slow decrease in the first part of ripening and a significant increase in the final stages. Shaded bunches decreased more rapidly, so at harvest the content in total polyphenols was significantly lower than in exposed bunches (Figure 7).

Total flavonoids remained constant in the first stages and showed a general increase towards the end of ripening. Shaded bunches had a significant lower level of total
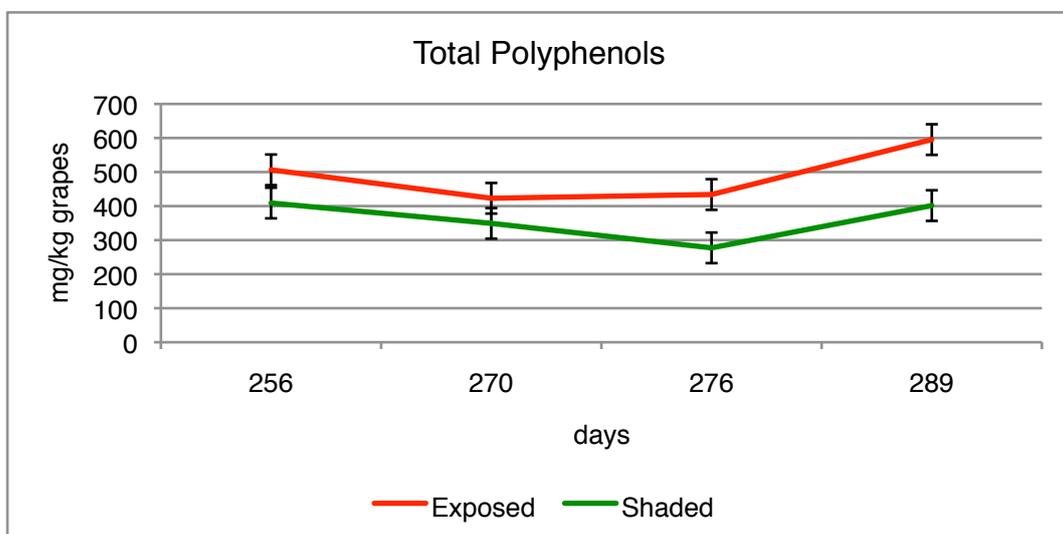
Figure 7. The evolution of total polyphenols in exposed (red) and shaded (green) clusters during Aglianico grape ripening.
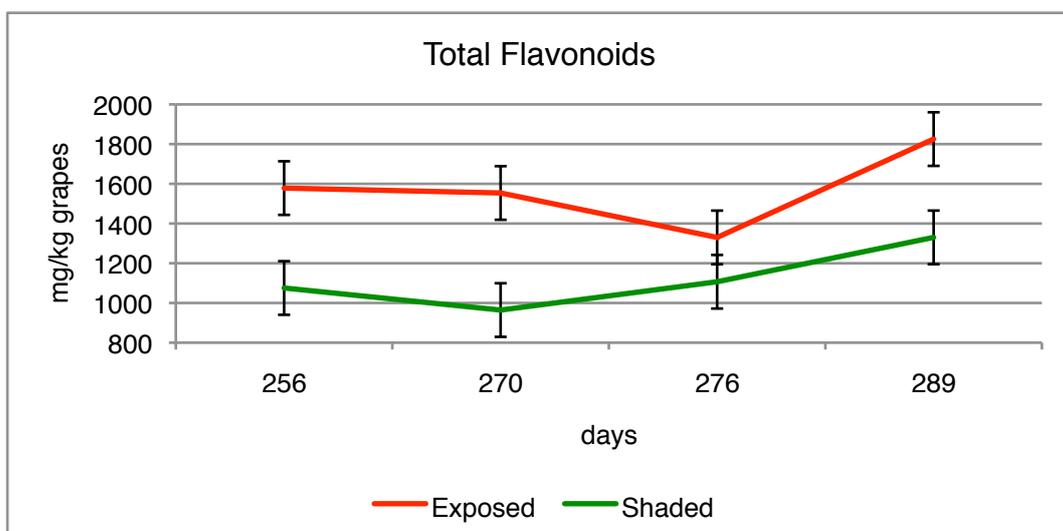
Figure 8. The evolution of total flavonoids in exposed (red) and shaded (green) clusters during Aglianico grape ripening.

flavonoids all-throughout the course of ripening, but differences got smaller towards harvest (Figure 8). Non anthocyanin flavonoids showed a slight decrease during ripening. Shading no effect non anthocyanin flavonoids in any given sampling point (Figure 9).

At veraison, exposed and shaded bunches showed no significant difference in the total tannins' level. However, toward full ripening , total tannins increased in exposed bunches, while remained almost constant in the shaded bunches, thus resulting in a lower accumulation of total tannins in the ripe shaded grapes (Figure 10).
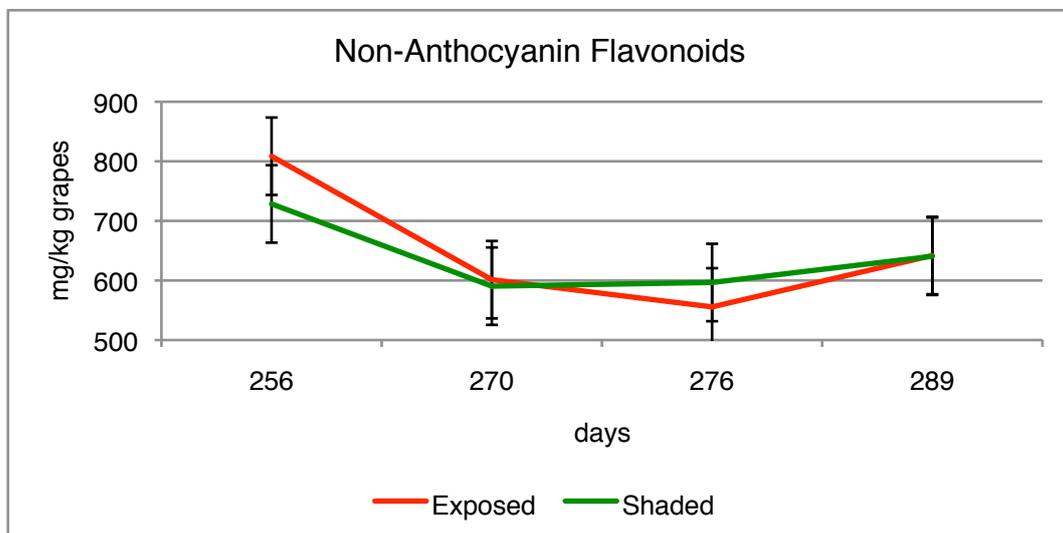
Figure 9. The evolution of non anthocyanin flavonoids in exposed (red) and shaded (green) clusters during Aglianico grape ripening.
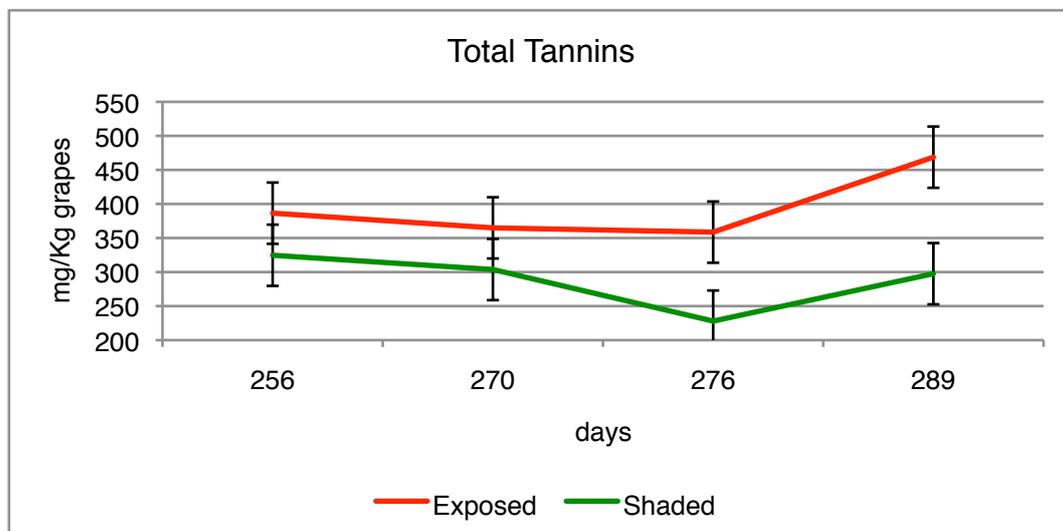
Figure 10. The evolution of total tannins in exposed (red) and shaded (green) clusters during Aglianico grape ripening.

Total anthocyanins were higher in the exposed clusters all-throughout berry ripening, but differences were higher towards veraison, rather than towards harvest (Figure 11).
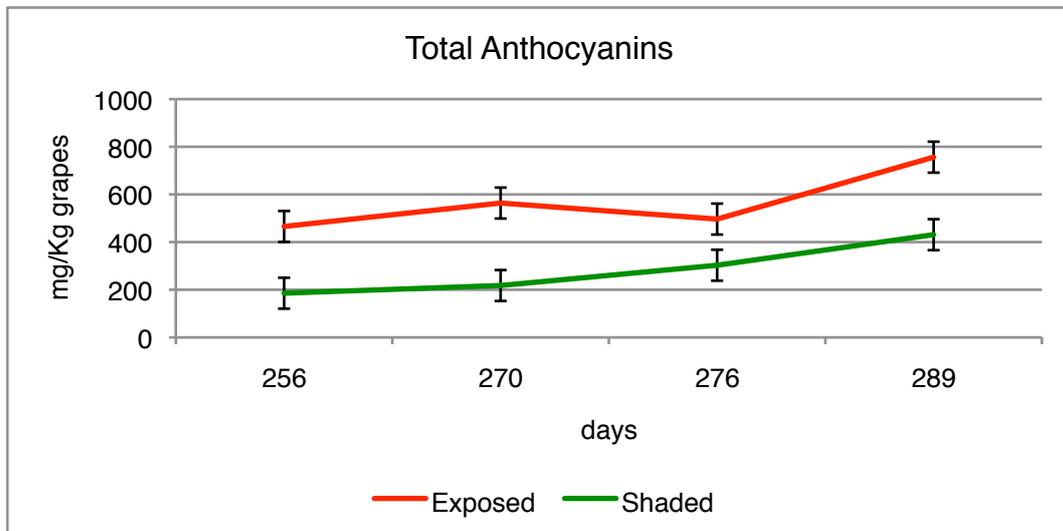
Figure 11. The evolution of total anthocyanins in exposed (red) and shaded (green)
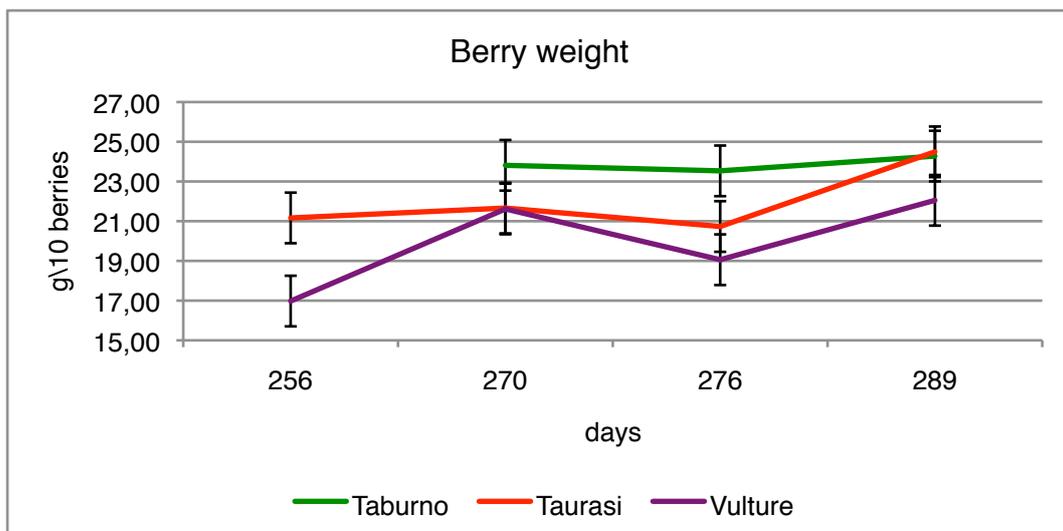
Figure 12. The evolution of berry weight in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.

Ripening kinetics in the three Aglianico biotypes

Physiological and technological variables

Taurasi and Vulture showed a significant difference in berry weight at veraison, vulture berries being smaller. At harvest, Vulture berries were the smallest, while no significant difference was shown between Taburno and Taurasi (Figure 12). Skin weight
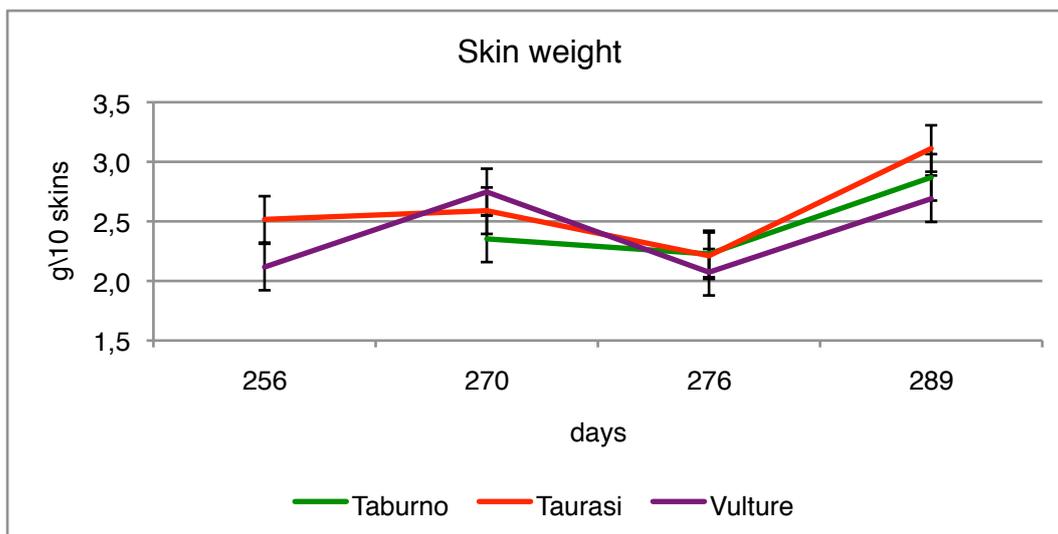
Figure 13. The evolution of skin weight in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.
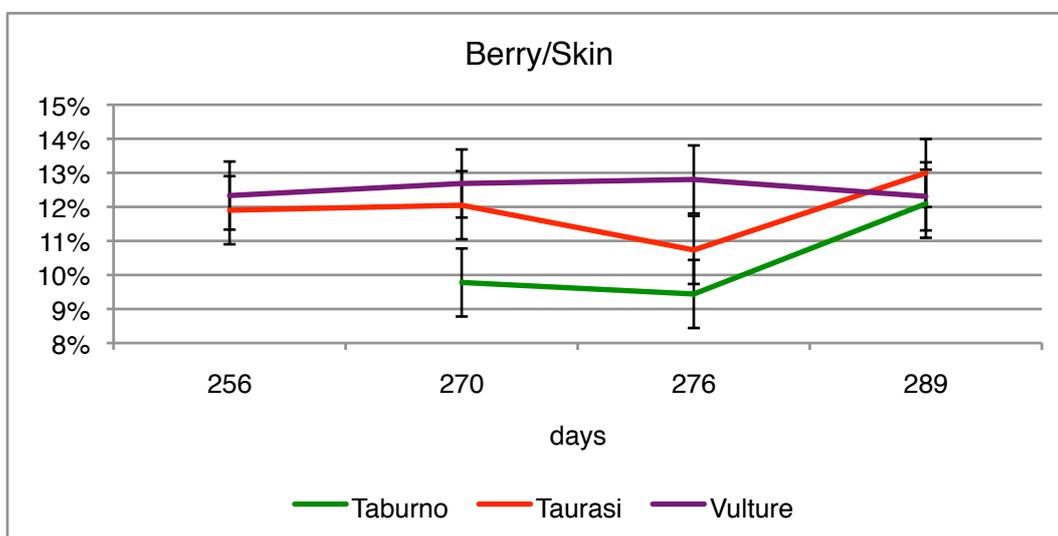
Figure 14. The evolution of the berry/skin weight ratio in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.
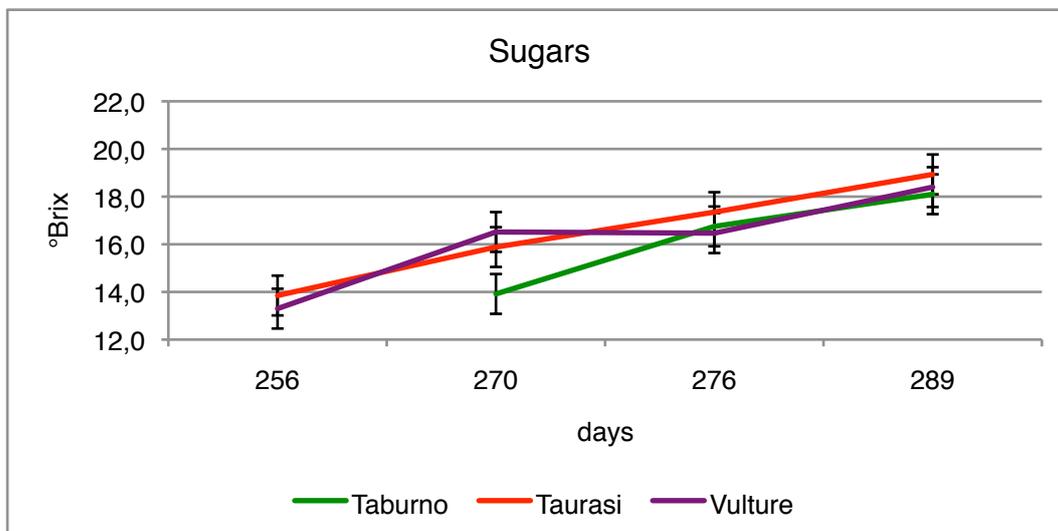
*Figure 15. The evolution of sugars in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.*

showed no significant difference among biotypes all-throughout the ripening process (Figure 13). However, Taburno showed a lower skin/berry ratio in the early stages of ripening, but it reached the same final level as Vulture and Taurasi (Figure 14).

Sugar content increased normally from veraison to harvest. Taurasi and Vulture showed no significant difference in sugar concentration during the whole ripening period. Taburno had very low levels of sugars in the beginning, but at the end of the season it reached the same sugar concentration as Taurasi and Vulture (Figure 15).

pH increased in all biotypes during grape ripening. Taburno had a significantly lower pH in the first part of ripening, while Taurasi and Vulture showed no differences between each other during the whole ripening period. However, Taburno reached the same pH level as Taurasi and Vulture at the end of ripening (Figure 16).

Titrable acidity in berries decreased during ripening in all biotypes. At veraison, Taburno had the highest acid concentration, Vulture had the lowest, while Taurasi had an intermediate level. However, at the end of the ripening period the three biotypes showed no significant difference (Figure 17).
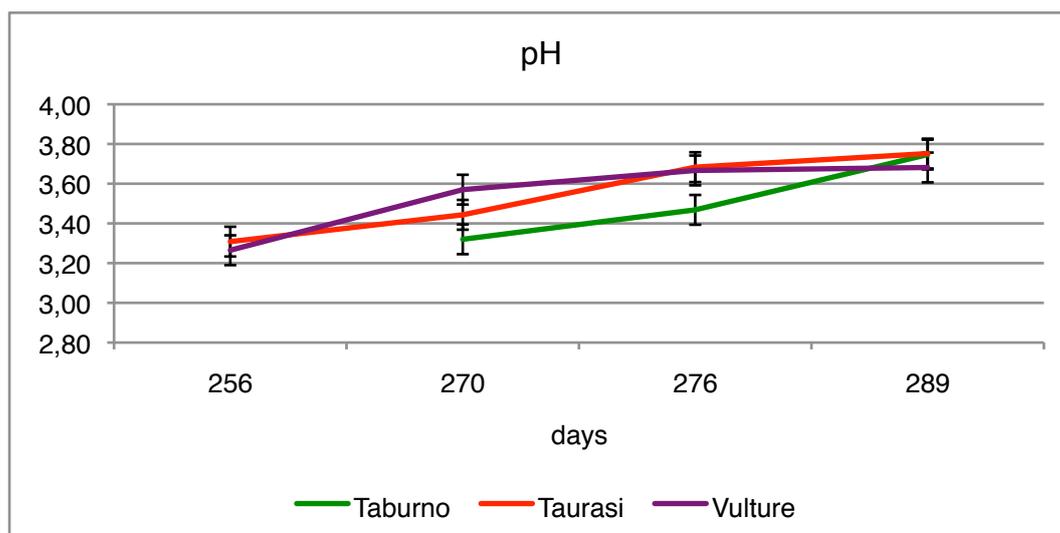
Figure 16. The evolution of pH in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.
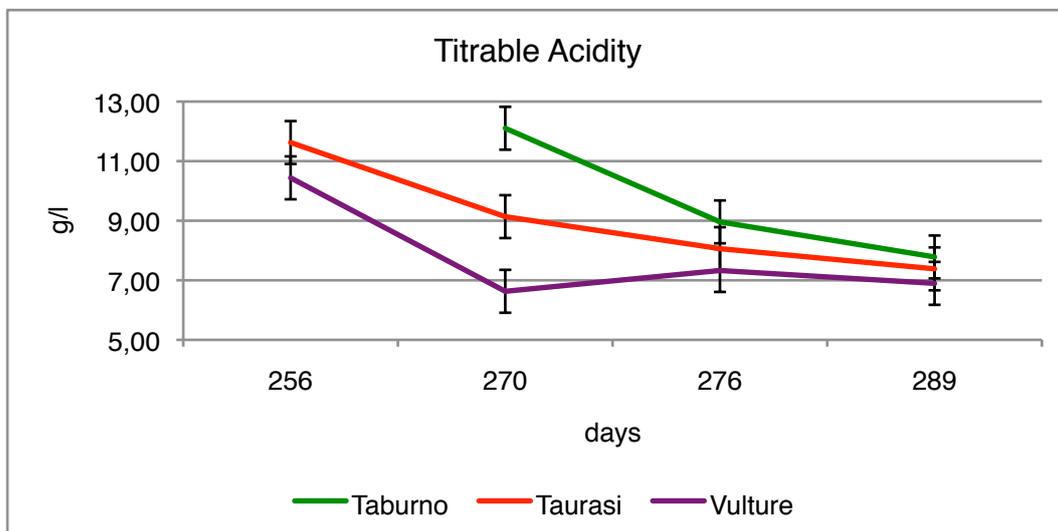
*Figure 17. The evolution of titrable acidity in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.*
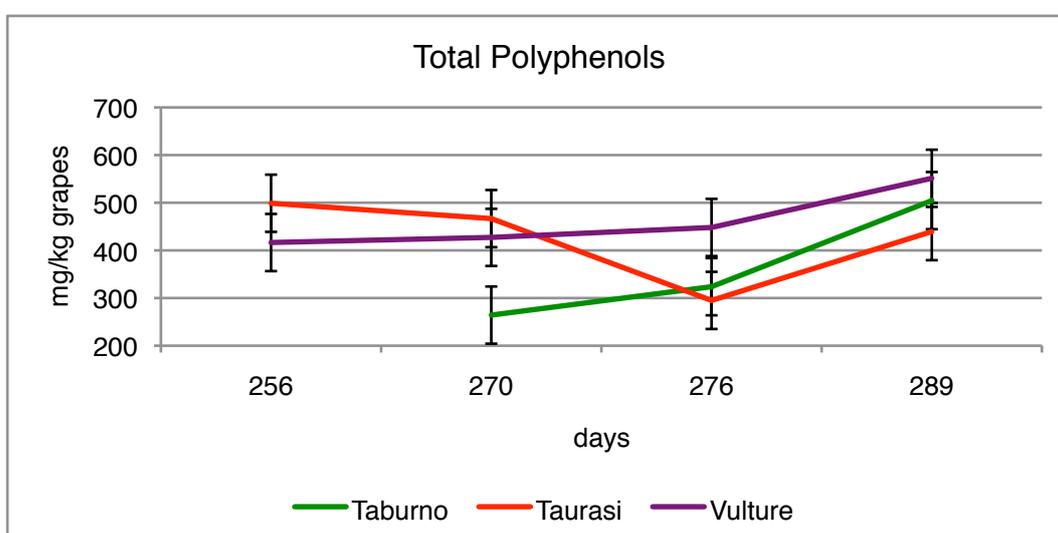
Figure 18. The evolution of total polyphenols in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.

Phenolic compounds

Total polyphenols remained generally constant throughout grape ripening. Taurasi and Vulture showed no significant difference during the whole period, while Taburno showed lower total polyphenols in the beginning of ripening. At harvest there was no significant difference in total polyphenols accumulation among biotypes (Figure 18).

Total flavonoids concentration remained constant from veraison to harvest. Taurasi and Vulture showed the same pattern, while Taburno had lower total flavonoids at veraison, but then it reached the same levels as Vulture and Taurasi towards the end of ripening (Figure 19).
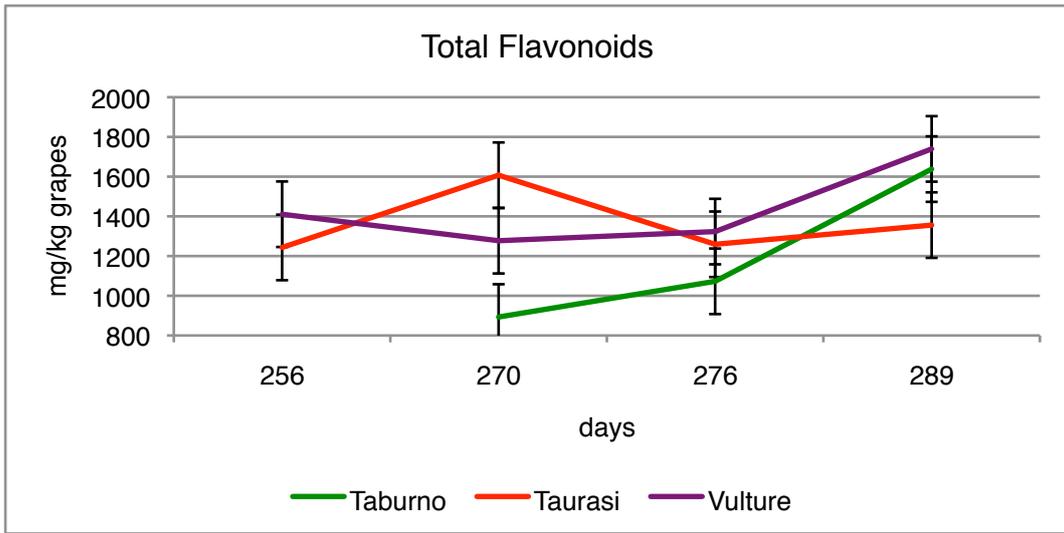
Figure 19. The evolution of total flavonoids in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.
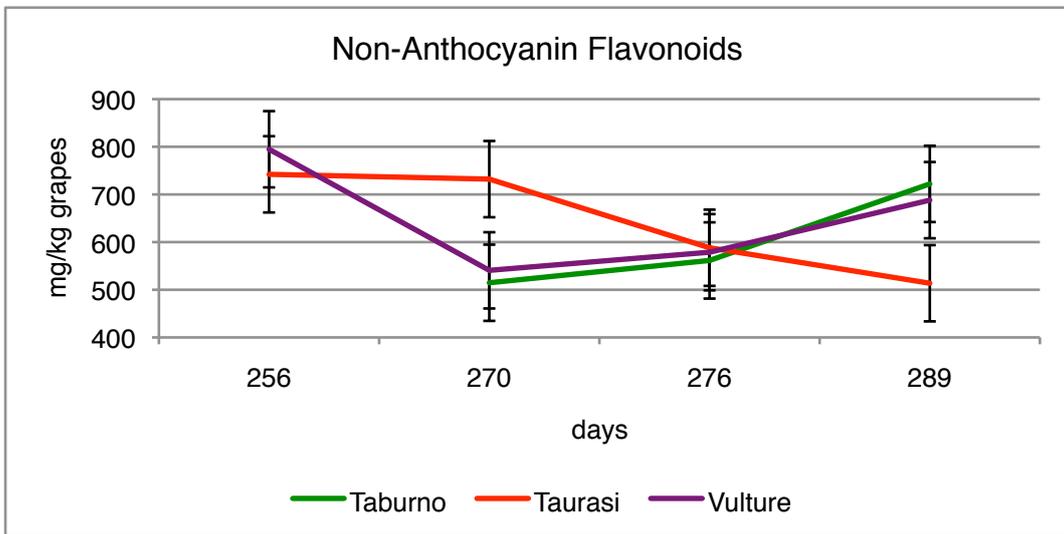
Figure 20. The evolution of non anthocyanin flavonoids in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.
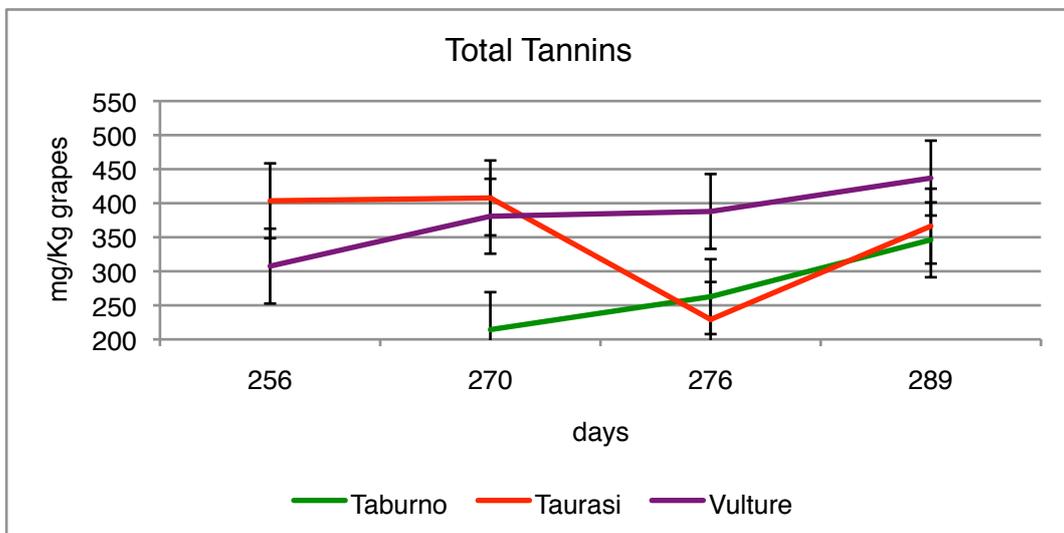
Figure 21. The evolution of total tannins in Taburno (green), Taurasi (red) and Vulture

Non anthocyanin Flavonoids showed little significative difference during the ripening period among the three biotypes (Figure 20).

Total tannins showed different patterns in the three biotypes. Taurasi showed a high concentration of tannins in the skins at veraison, but then it dropped half-way through ripening, eventually rising again towards the end. Vulture had slightly less tannins than Taurasi at veraison, but Vulture slowly accumulated tannins until ripening. Taburno had the least tannins at veraison, however taburno showed a faster tannin accumulation compared to the other biotypes. At harvest there was no significant difference in the concentration of total tannins in the skins among the three biotypes (Figure 21).

Total anthocyanin concentration grew during grape ripening in all the biotypes. Taurasi and Vulture showed the same pattern and concentration during the whole ripening period. Taburno had less anthocyanins at veraison, but it showed a faster accumulation during ripening, reaching eventually the same levels of total anthocyanins as the other biotypes (Figure 22).
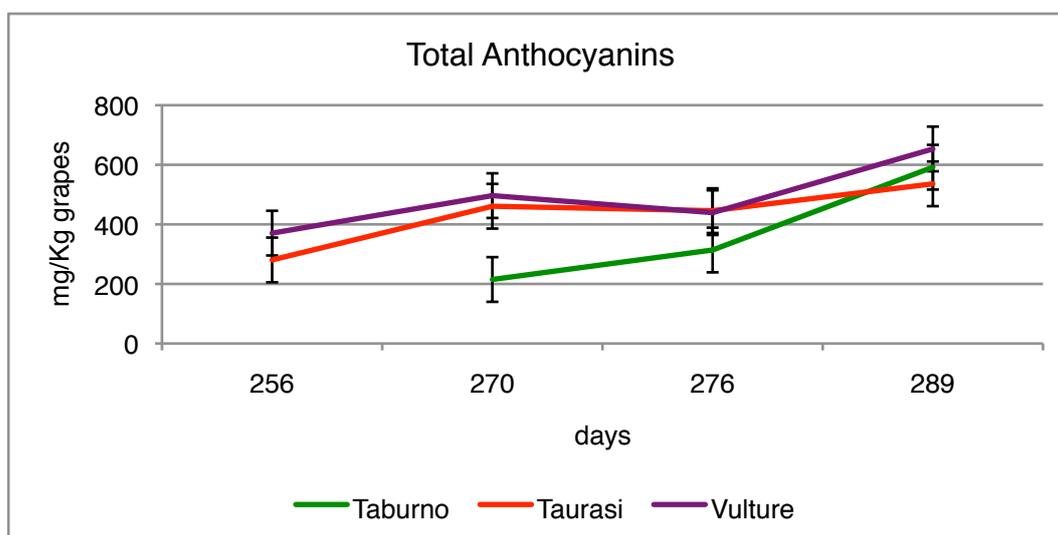
Figure 22. The evolution of total anthocyanins in Taburno (green), Taurasi (red) and Vulture (purple) grapes during ripening.

The anthocyanin profiling

The effect of shading on the anthocyanin profiling
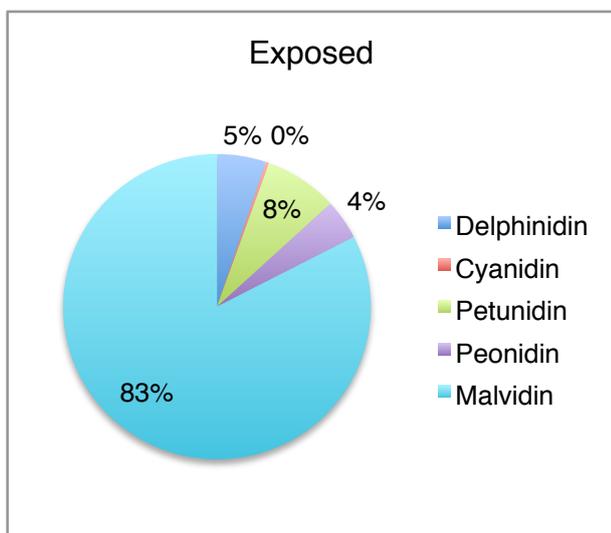
Figure 24. The relative proportion of delphinidin-3-glucoside (dark blue), cyanidin -3-glucoside (red), petunidin -3-glucoside (green), peonidin -3-glucoside (purple) and malvidin -3-glucoside (light blue) in exposed Aglianico mature grapes.
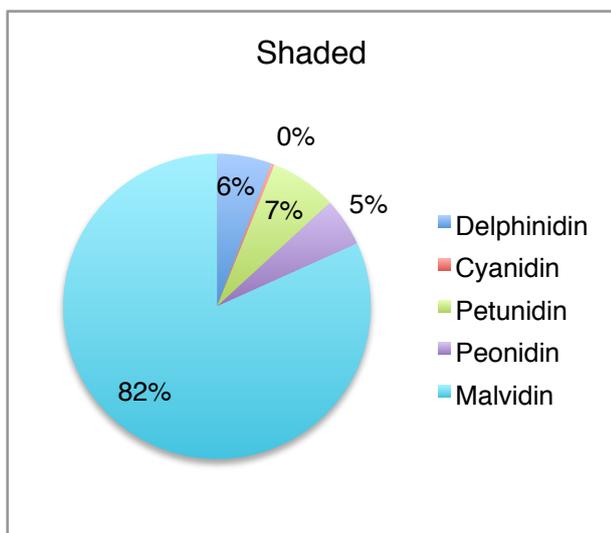

Figure 23. The relative proportion of delphinidin-3-glucoside (dark blue), cyanidin -3-glucoside (red), petunidin -3-glucoside (green), peonidin -3-glucoside (purple) and malvidin -3-glucoside (light blue) in shaded Aglianico mature grapes.

Among the five anthocyanidins, shaded aglianico grapes showed a very high proportion of Malvidin (82%), a low proportion of delphinidin, petunidin and peonidin (6%, 7% and 5% respectively) and a very low proportion of cyanidin (less than 1%) (Figure 23). Exposed bunches also showed a very high proportion of Malvidin (83%), a low proportion of delphinidin, petunidin and peonidin (5%, 8% and 8% respectively) and a very low proportion of cyanidin (less than 1%) (Figure 24). Free anthocyanidin-glucosides, anthocyanidin-acetates and anthocyanidin-p-coumarates were respectively 76%, 1% and 23% of total anthocyanins (Figure 25). In exposed berries 79% were free anthocyanidin-glucosides, 1% were anthocyanidin-acetates and 20% were anthocyanidin-p-coumarates (Figure 26). The anthocyanin profiling showed no statistically significant difference between shaded and

exposed berries.
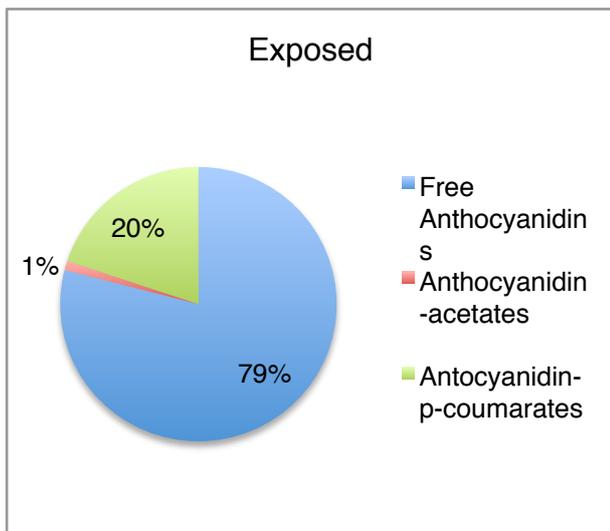
Figure 26. The relative proportion of free anthocyanins (dark blue), anthocyanidin acetates (red) and anthocyanidin p-coumarates (green), in exposed Aglianico mature grapes.
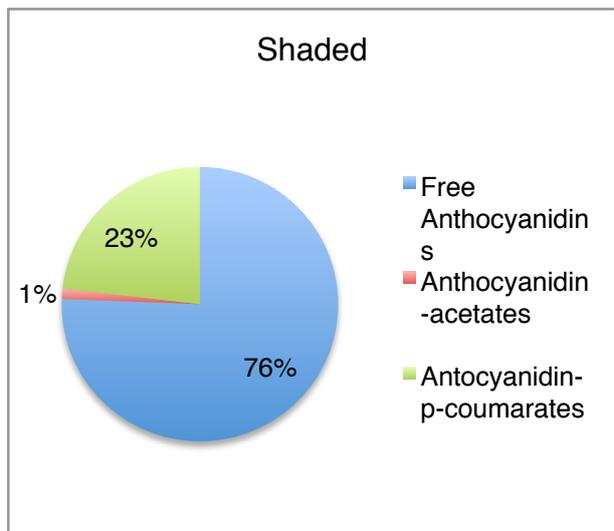
Figure 25. The relative proportion of free anthocyanins (dark blue), anthocyanidin acetates (red) and anthocyanidin p-coumarates (green), in shaded Aglianico mature grapes.

The anthocyanin profiling of the three Aglianico biotypes

Taburno showed a very high proportion of Malvidin (77%), a low proportion of delphinidin, petunidin and peonidin (8%, 9% and 5% respectively) and a very low proportion of cyanidin (1%) (Figure 27). Similarly Taurasi grapes had a very high proportion of Malvidin (84%), a low proportion of delphinidin, petunidin and peonidin (4%, 6% and 6% respectively) and a very low proportion of cyanidin (less than 1%) (Figure 28). Also the Vulture anthocyanin profiling showed a very high proportion of Malvidin (85%), a low proportion of delphinidin, petunidin and peonidin (5%, 7% and 3% respectively) and a less than 1% of cyanidin (Figure 29). Taburno showed 80% of free anthocyanin-glucosides, 19% of anthocyanin-p-coumarates and 1% of anthocyanin-acetates (Figure 30); similarly, Taurasi had 75% of free anthocyanin-glucosides, 24% of anthocyanin-p-coumarates and 1% of anthocyanin-acetates (Figure 31); Vulture showed a similar pattern 78% of free anthocyanin-glucosides, 21% of anthocyanin-p-coumarates and 1% of anthocyanin-acetates (Figure 32). Aglianico's biotypes showed no significant difference in the anthocyanin profiling.
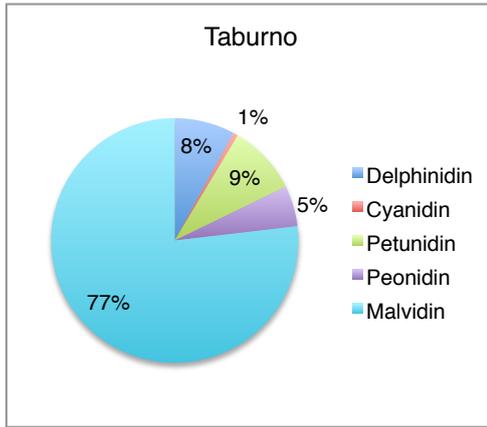
Figure 27. The relative proportion of delphinidin-3-glucoside (dark blue), cyanidin -3-glucoside (red), petunidin -3-glucoside (green), peonidin -3-glucoside (purple) and malvidin -3-glucoside (light blue) in Taburno mature grapes.
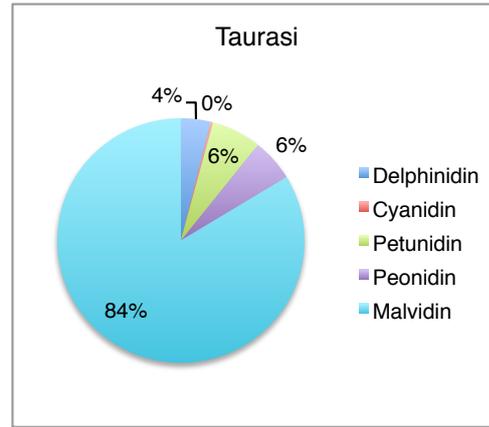

Figure 28. The relative proportion of delphinidin-3-glucoside (dark blue), cyanidin -3-glucoside (red), petunidin -3-glucoside (green), peonidin -3-glucoside (purple) and malvidin -3-glucoside (light blue) in Taurasi mature grapes.
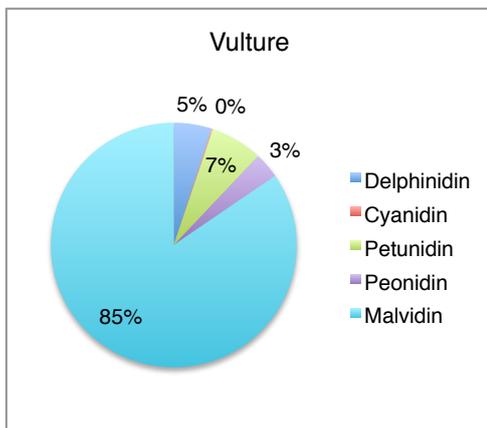
Figure 29. The relative proportion of delphinidin-3-glucoside (dark blue), cyanidin -3-glucoside (red), petunidin -3-glucoside (green), peonidin -3-glucoside (purple) and malvidin -3-glucoside (light blue) in Vulture mature grapes.
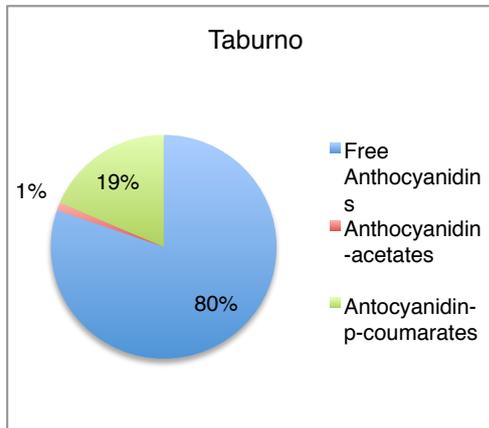
Figure 30. The relative proportion of free (dark blue), acetate (red) and p-coumarate (green), anthocyanins in Taburno mature grapes.
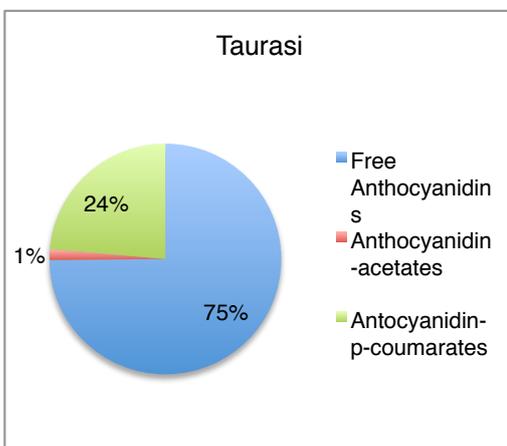
Figure 31. The relative proportion of free (dark blue), acetate (red) and p-coumarate (green), anthocyanins in Taurasi mature grapes.
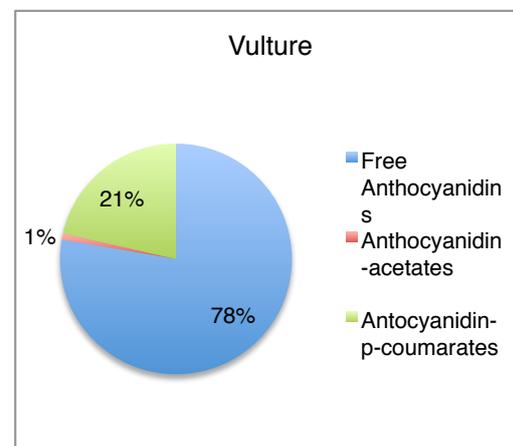

Figure 32. The relative proportion of free (dark blue), acetate (red) and p-coumarate (green), anthocyanins in Taburno mature grapes.

The average anthocyanin profiling of Aglianico resulted to be composed by 82% of malvidin, 6% of delphinidin, 7% of petunidin, 5% of peonidin and less than 1% of cyanidin (Figure 33), 78% of anthocyanin are as free glucosides, 21% are acetates and 1% are p-coumarates (Figure 34).

	Taburno			
	Exposed		Shaded	
gene\date	270	276	270	276
CHS2	0,3782	0,3046	0,1981	0,2758
F3'5'H	0,5143	0,3408	0,2667	0,2173
F3'H	0,0396	0,0308	0,0258	0,0396
F3H	0,3636	0,2322	0,1761	0,1414
FLS4	0,0158	0,0147	0,0006	0,0008
DFR1	0,0808	0,0292	0,0428	0,0222
LAR2	0,0026	0,0018	0,0013	0,001
LDOX1	0,7189	0,1916	0,5045	0,1217
UFGT	0,1051	0,0355	0,0112	0,069
OMT	0,1796	0,3165	0,0474	0,2427
AM1	0,009	0,0026	0,0075	0,0022
AM3	0,2153	0,1061	0,1761	0,0773
GST4	0,156	0,1378	0,0784	0,1014
MYB12	0,0012	0,0043	0,0002	0,0001
MYB5a	0,0059	0,14	0,0053	0,0379
MYB5b	0,0222	0,0307	0,0115	0,0355
MYBA1	0,0829	0,238	0,0465	0,1828

Table 1. The mean expression relative to GADPH of the CHS2, F3'5'H, F3'H, F3H, FLS4, DFR1, LAR2, LDOX1, UFGT, OMT, AM1, AM3, GST4, MYB12, MYB5a, MYB5b, MYBA1 genes in exposed and shaded grape berry skins during Aglianico biotype Taburno ripening.
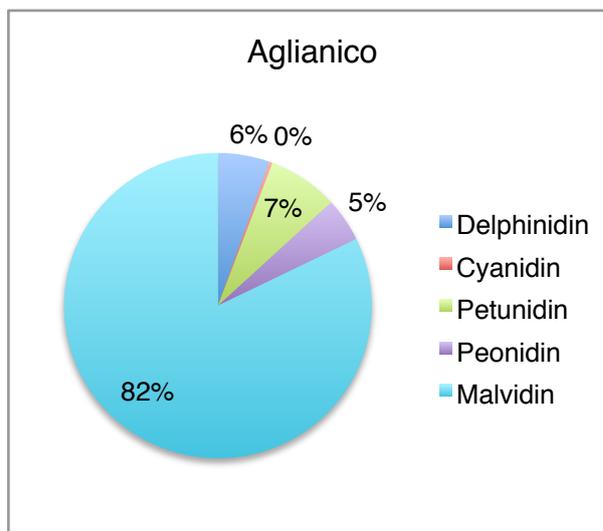

Figure 33. The relative proportion of delphinidin-3-glucoside (dark blue), cyanidin -3-glucoside (red), petunidin -3-glucoside (green), peonidin -3-glucoside (purple) and malvidin -3-glucoside (light blue) in Aglianico mature grapes.
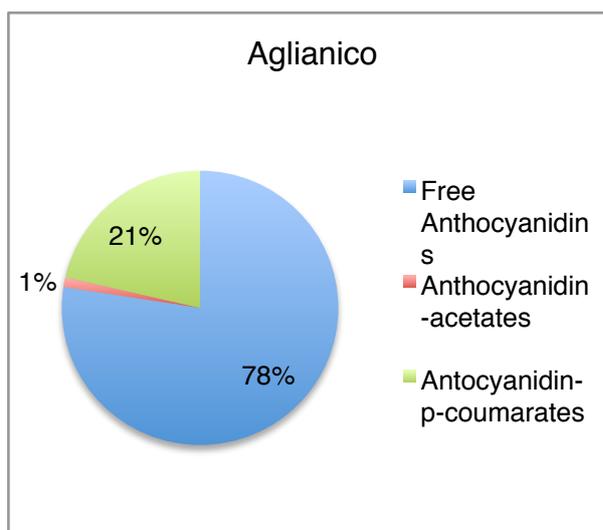

Figure 34. The relative proportion of free anthocyanins (dark blue), anthocyanidin acetates (red) and anthocyanidin p-coumarates (green), in Aglianico mature grapes.

| gene\date | Taurasi | | | | | |
| | Exposed | | | Shaded | | |
	256	270	276	256	270	276
CHS2	0,2562	0,7588	0,271	0,1575	0,4338	0,2655
F3'5'H	0,1671	0,3263	0,2495	0,3232	0,1693	0,3525
F3'H	0,0183	0,0331	0,0206	0,0081	0,0174	0,0206
F3H	0,2839	0,3801	0,1973	0,1526	0,0863	0,1432
FLS4	0,0034	0,0089	0,0013	0,0003	0,0001	0,0014
DFR1	0,0259	0,0276	0,0313	0,0125	0,0178	0,0221
LAR2	0,0035	0,0019	0,0022	0,0013	0,001	0,0018
LDOX1	0,3622	0,2921	0,2414	0,1924	0,1146	0,2121
UFGT	0,2136	0,0438	0,01	0,0147	0,0374	0,024
OMT	0,6051	0,2478	0,0264	0,0544	0,0932	0,0912
AM1	0,0031	0,0033	0,0017	0,0023	0,0024	0,0016
AM3	0,1213	0,1245	0,0608	0,0737	0,0688	0,059
GST4	0,1425	0,2163	0,0632	0,0675	0,0837	0,0591
MYB12	0,0002	0,0002	0,0001	0,0001	0,0001	0,0001
MYB5a	0,0023	0,0034	0,0131	0,0047	0,013	0,0113
MYB5b	0,007	0,0128	0,0074	0,0048	0,0164	0,0102
MYBA1	0,061	0,0744	0,0976	0,0254	0,0599	0,0955

Table 2. The mean expression relative to GADPH of the CHS2, F3'5'H, F3'H, F3H, FLS4, DFR1, LAR2, LDOX1, UFGT, OMT, AM1, AM3, GST4, MYB12, MYB5a, MYB5b, MYBA1 genes in exposed and shaded grape berry skins during Aglianico biotype Taurasi ripening.

| gene\date | Vulture | | | | | |
| | Exposed | | | Shaded | | |
	256	270	276	256	270	276
CHS2	0,583	1,6989	0,7777	0,2825	0,99	0,2879
F3'5'H	1,8006	0,4756	0,5613	0,435	0,6325	0,9983
F3'H	0,0231	0,022	0,0449	0,0325	0,1072	0,0332
F3H	0,8489	0,301	0,3093	0,2266	0,4562	0,6049
FLS4	0,0044	0,0184	0,0036	0	0,0003	0,0271
DFR1	0,0592	0,0728	0,0705	0,0401	0,0439	0,0643
LAR2	0,0057	0,0026	0,0034	0,0018	0,0021	0,0029
LDOX1	0,709	0,2568	0,4311	0,4289	0,3778	0,5533
UFGT	0,092	0,0372	0,0206	0,244	0,0472	0,0128
OMT	0,1811	0,1321	0,0653	0,1045	0,4596	0,0082
AM1	0,0051	0,0028	0,0067	0,0069	0,0066	0,0038
AM3	0,1204	0,057	0,1398	0,2682	0,246	0,0815
GST4	0,2025	0,0699	0,1424	0,1944	0,3462	0,2005
MYB12	0,0002	0,0005	0,0001	0,0001	0,0002	0,0193
MYB5a	0,0079	0,0499	0,0101	0,0077	0,1121	0,0146
MYB5b	0,0096	0,0319	0,0092	0,0187	0,0188	0,0442
MYBA1	0,1789	0,3234	0,141	0,155	1,9986	0,1115

Table 3. The mean expression relative to GADPH of the CHS2, F3'5'H, F3'H, F3H, FLS4, DFR1, LAR2, LDOX1, UFGT, OMT, AM1, AM3, GST4, MYB12, MYB5a, MYB5b, MYBA1 genes in exposed and shaded grape berry skins during Aglianico biotype Vulture ripening.

Gene expression

In this work the expression level of the genes CHS2, F3'5'H, F3'H, F3H, FLS4, DFR1, LAR2, LDOX1, UFGT, OMT, AM1, AM3, GST4, MYB12, MYB5a, MYB5b, MYBA1 in the skins of shaded and exposed berries of three Aglianico biotypes (Taburno, Taurasi, Vulture) were assessed by mean of Real-Time PCR. In general, all genes were expressed, to different extents, in all biotypes and in all conditions. The expression levels of each gene in each condition are summarised for Taburno (Table 1), Taurasi (Table 2) and Vulture (Table 3).

In general, the sampling date significantly affected the relative expression of CHS2, UFGT and AM3. The relative expression of CHS2 was significantly higher on the second sampling date (Figure 35), UFGT's expression level was significantly higher in the first sampling date (veraison) than in the second and third (Figure 36). The relative expression levels of AM3 were similar in the first two sampling dates, but it was significantly lower on
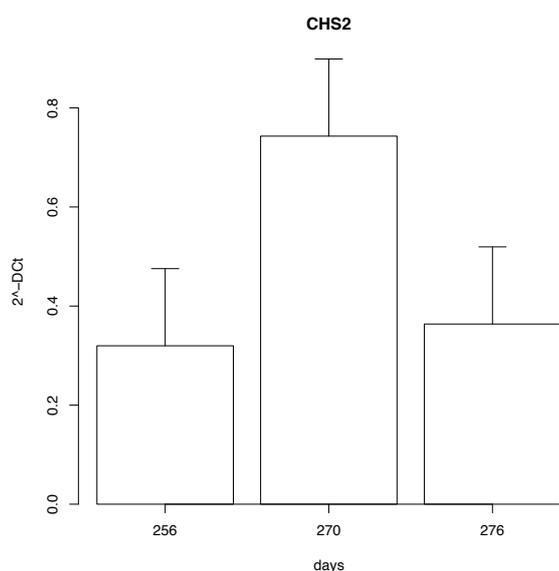
Figure 35. The mean expression relative to GADPH of the CHS2 gene during Aglianico ripening. Error bars indicate MDS.
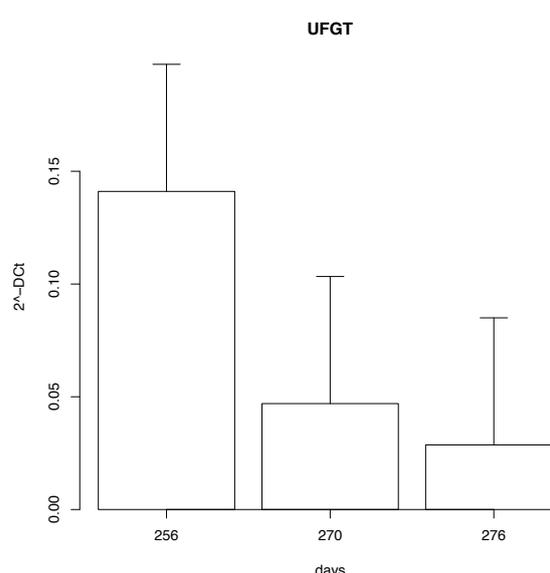
Figure 36. The mean expression relative to GADPH of the UFGT gene during Aglianico ripening. Error bars indicate MDS.

the last date (ripeness) (Figure 37). In general the sampling date ha no significant effect on
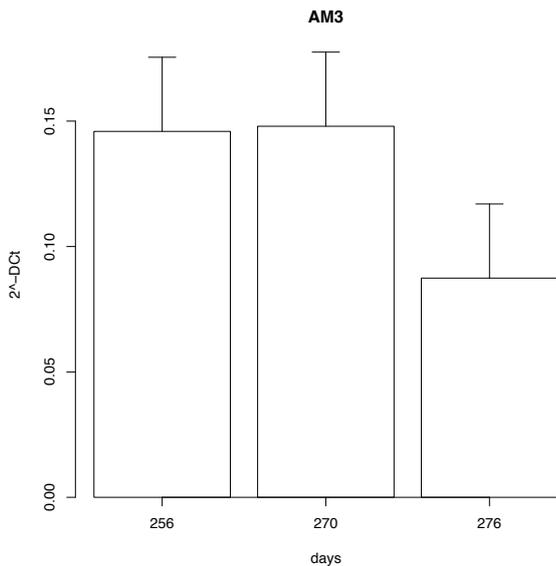
Figure 37. The mean expression relative to GADPH of the AM3 gene during Aglianico ripening. Error bars indicate MDS.

the expression levels of the other genes of the flavonoid pathway analysed in this work.

In general, shading significantly affected the expression level of CHS2, F3H and LAR2. The expression levels of these genes were significantly lower in the berry skin of shaded cluster (Figures 38, 39 and 40). The other genes analysed in this work showed no significant difference related in general to the treatment.
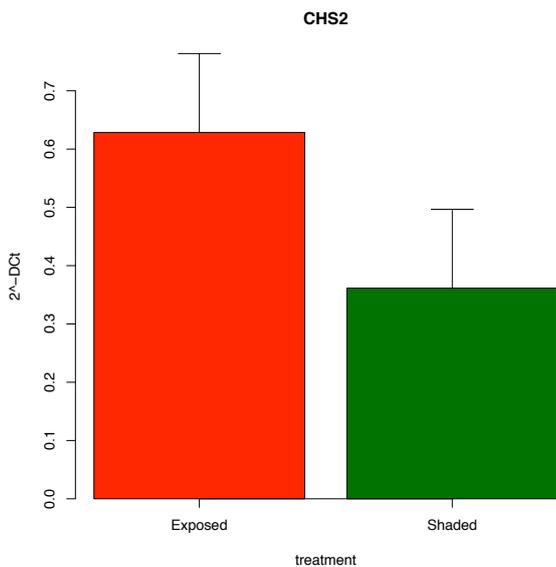
Figure 38. The mean expression relative to GADPH of the CHS2 gene in exposed (red) and shaded (green) Aglianico grapes. Error bars indicate MDS.
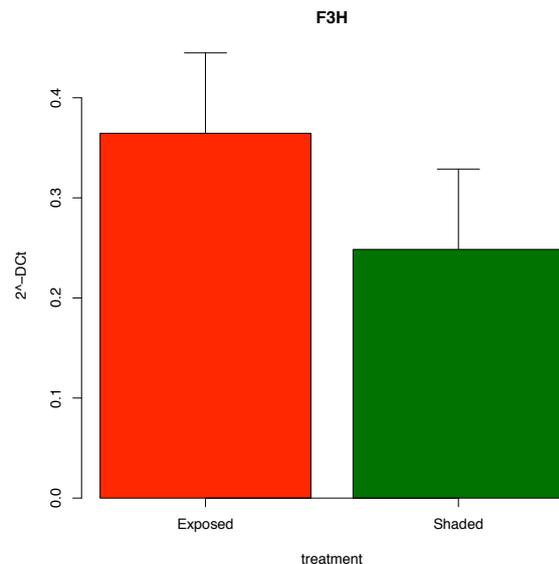
Figure 39. The mean expression relative to GADPH of the F3H gene in exposed (red) and shaded (green) Aglianico grapes

Generally, biotype significantly influenced the relative expression of a few of the genes analysed in this work. CHS2 was significantly more expressed in Vulture grapes, while Taburno and Taurasi showed similar and lower expression levels (Figure 41); F3'5'H showed the same expression pattern: higher expression in Vulture and lower expression in Taburno and Taurasi (Figure 42); F3'H was
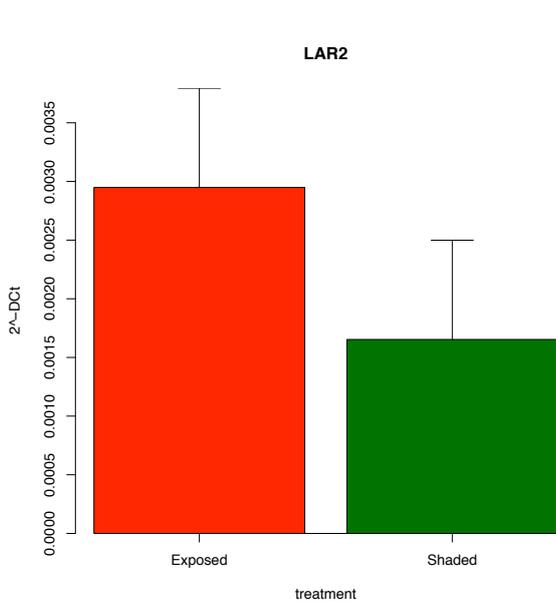
Figure 40. The mean expression relative to GADPH of the LAR2 gene in exposed (red) and shaded (green) Aglianico grapes
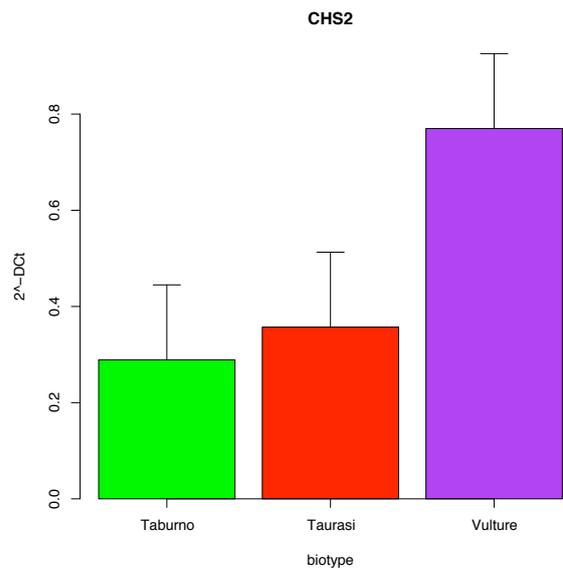

Figure 41. The mean expression relative to GADPH of the CHS2 gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
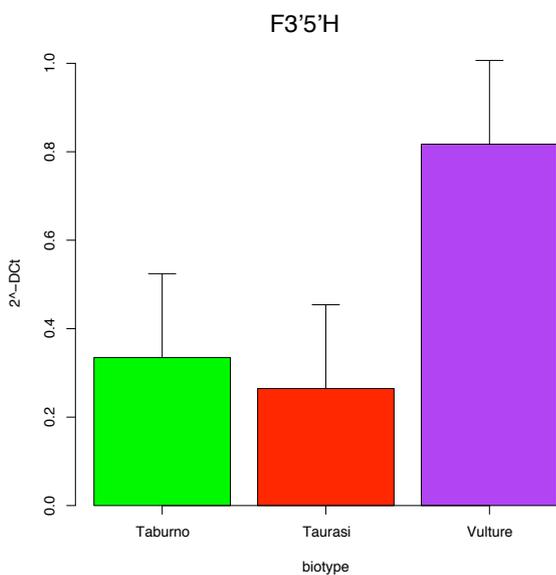

Figure 42. The mean expression relative to GADPH of the F3'5'H gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
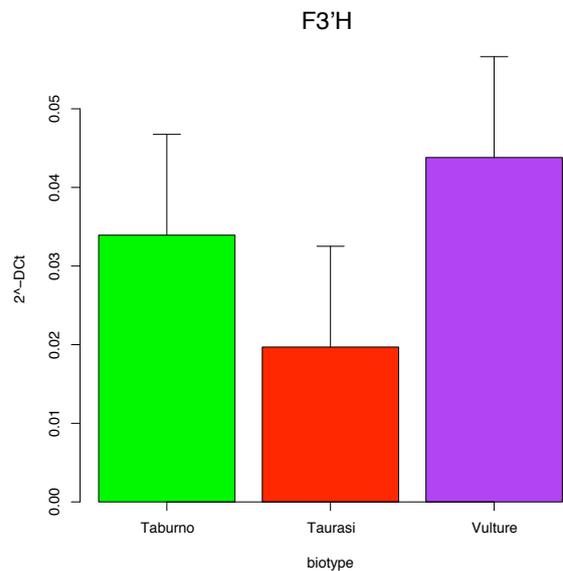

Figure 43. The mean expression relative to GADPH of the F3'H gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.

significantly more expressed in Taburno and Vulture and it was lower in Taurasi (Figure 43); F3H was significantly more expressed in Vulture, and it was lower in Taburno and Taurasi (Figure 44); DFR1's expression was significantly higher in Taburno and Vulture
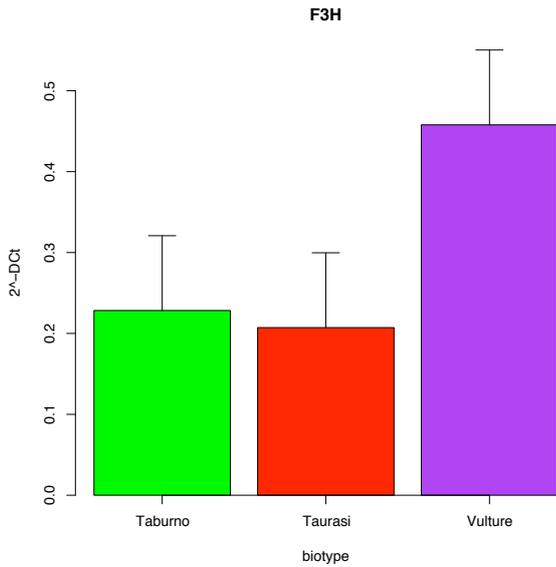
Figure 44. The mean expression relative to GADPH of the F3H gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
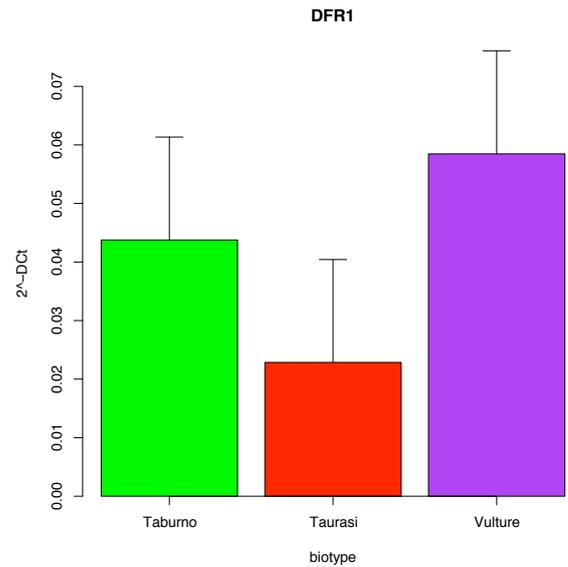

Figure 45. The mean expression relative to GADPH of the DFR1 gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
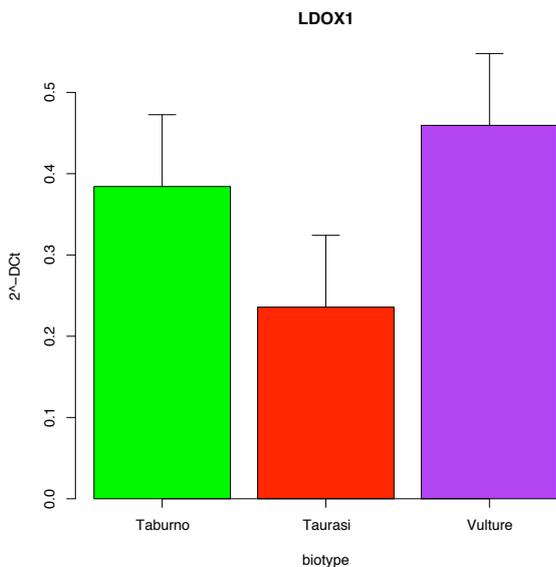

Figure 46. The mean expression relative to GADPH of the LDOX1 gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
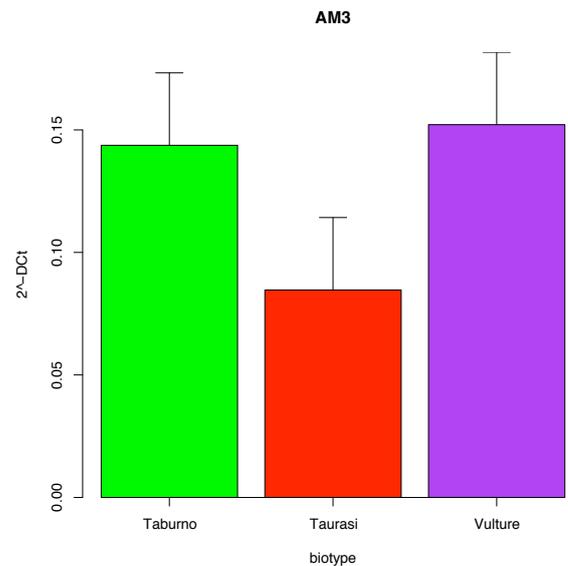

Figure 47. The mean expression relative to GADPH of the AM3 gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.

and lower in Taurasi (Figure 45); LDOX1 showed significantly lower expression levels in Taurasi and higher expression in Taburno and Vulture (Figure 46); AM3 and AM1 were significantly more expressed in Taburno and Vulture than Taurasi (Figures 47 and 48); the relative expression levels of GST4 were higher in Vulture and lower in Taurasi and Vulture (Figure 49). In general, FLS4, LAR2, UFGT, OMT, MYB12, MYB5a, MYB5b and MYBA1

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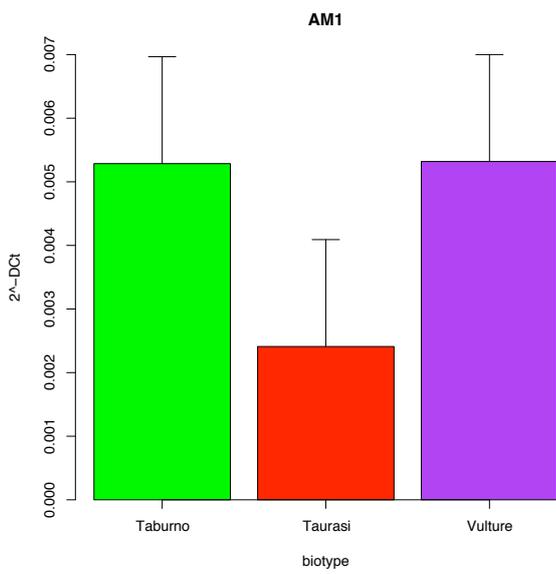
Figure 48. The mean expression relative to GADPH of the AM1 gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
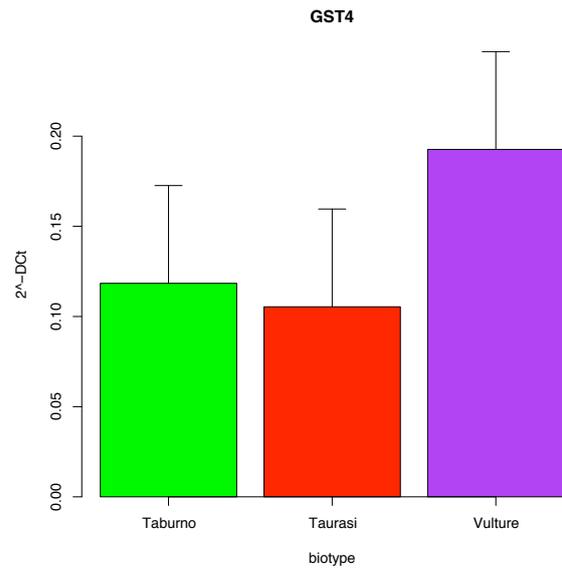

Figure 49. The mean expression relative to GADPH of the GST4 gene in Taburno (green), Taurasi (red) and Vulture (purple) grapes. Error bars indicate MDS.
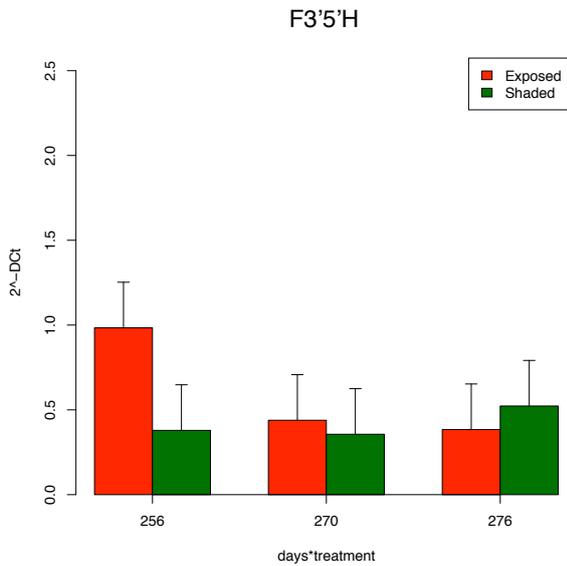
Figure 50. The mean expression relative to GADPH of the F3'5'H gene in shaded (green) and exposed (red) ripening grapes. Error bars indicate MDS.
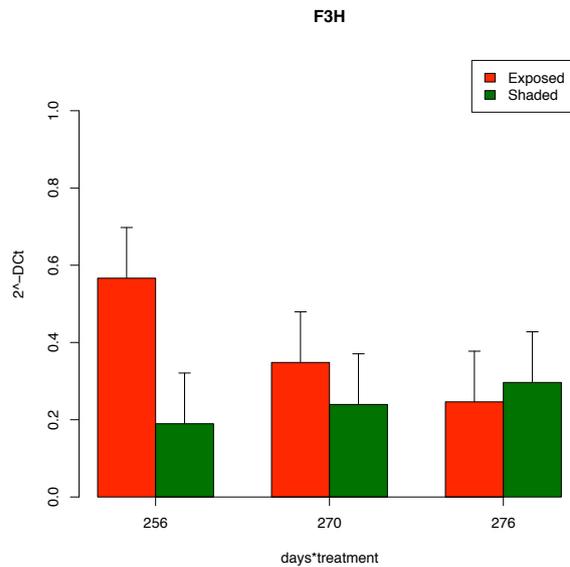
Figure 51. The mean expression relative to GADPH of the F3H gene in shaded (green) and exposed (red) ripening grapes. Error bars indicate MDS.

showed no significant difference in the average relative expression levels in Taburno,

Taurasi and Vulture.
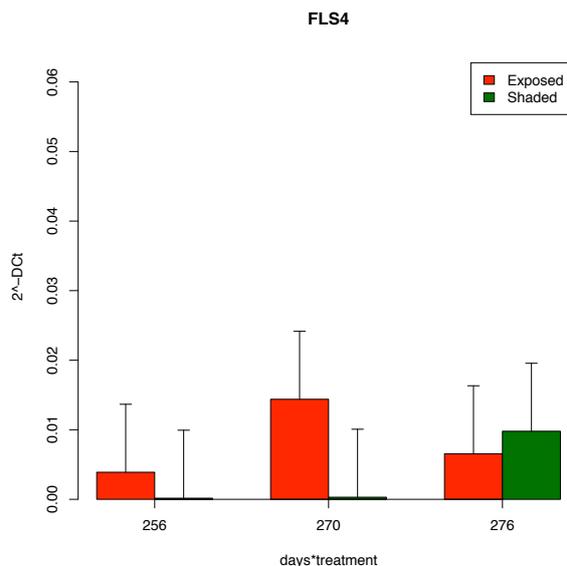
FLS4

Figure 52. The mean expression relative to GADPH of the FLS4 gene in shaded (green) and exposed (red) ripening grapes. Error bars indicate MDS.

The sampling date and the treatment significantly affected the expression pattern of F3'5'H: in exposed berries, the relative expression of this gene was significantly higher at veraison and then it was lower towards ripening, in the shaded berries the expression was constant during the whole ripening period, and it was lower than in the exposed berries in the first sampling date (Figure 50). F3H showed the same expression pattern: higher expression at veraison in the exposed berries while constant expression in the shaded grapes (Figure 51). FLS4 was more expressed in exposed berries in the first two sampling dates, showing a significantly higher expression in the second date, while the expression in shaded and exposed bunches was the same in the last sampling date (Figure 52). in The sampling date and treatment interaction did not significantly affected the expression pattern of the other genes analysed in the present work.

The sampling date and the biotype influenced the expression pattern of CHS2, DFR1, LDOX1, AM1, AM3 and MYB5a. On the first sampling date, Vulture and Taurasi showed no significant difference for CHS2; on the second sampling date the relative expression of CHS2 where highest in Vulture, lowest in Taburno and intermediate in Taurasi; on the last sampling date Taurasi and Taburno had similar levels and Vulture was slightly higher. In Taurasi and Vulture the expression levels of CHS2 were significantly higher on the second sampling date, while no significant difference in the sampling dates was shown by Taburno (Figure 53). The expression of DFR1 was constant in Taurasi and
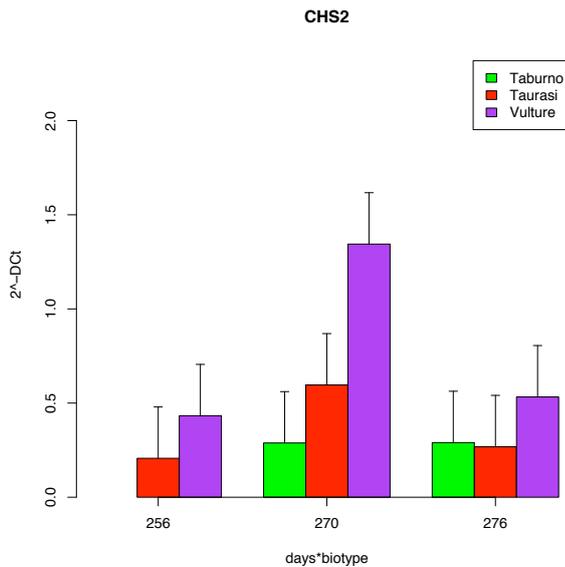
CHS2

Figure 53. The mean expression relative to GADPH of the CHS2 gene in Taburno (green), Taurasi (red) and Vulture (purple) ripening grapes. Error bars indicate MDS.
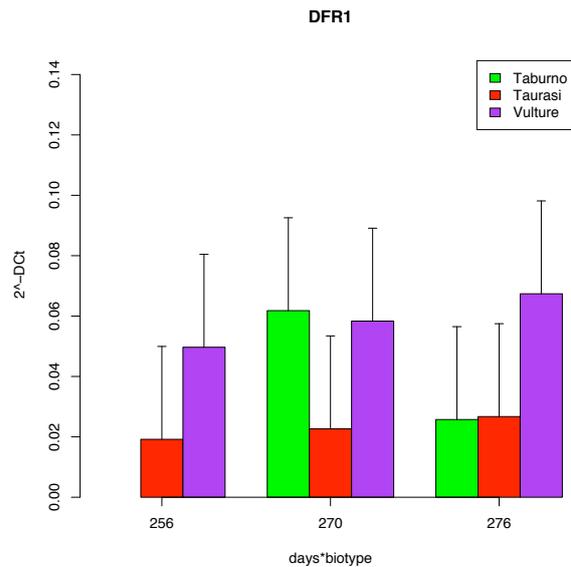
DFR1

Figure 54. The mean expression relative to GADPH of the DFR1 gene in Taburno (green), Taurasi (red) and Vulture (purple) ripening grapes. Error bars indicate MDS.
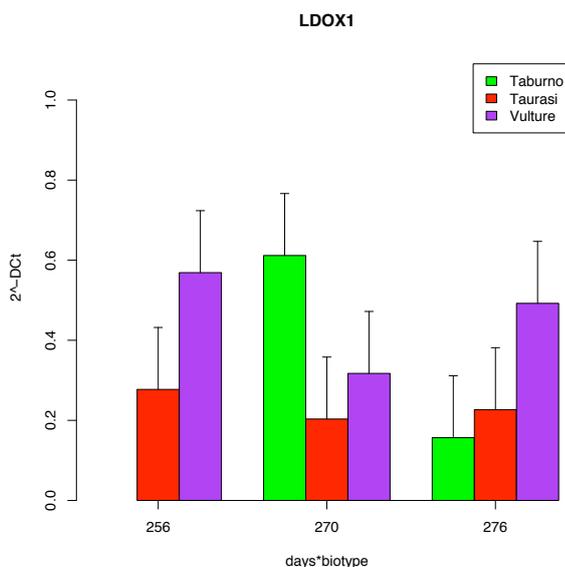
LDOX1

Figure 55. The mean expression relative to GADPH of the LDOX1 gene in Taburno (green), Taurasi (red) and Vulture (purple) ripening grapes. Error bars indicate MDS.

AM1
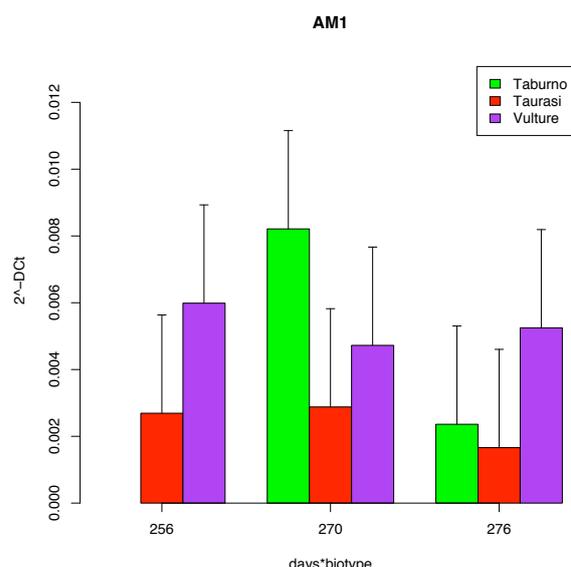
Figure 56. The mean expression relative to GADPH of the AM1 gene in Taburno (green), Taurasi (red) and Vulture (purple) ripening grapes. Error bars indicate MDS.

Vulture, while it showed a decrease in Taburno grapes (Figure 54). The expression pattern of LDOX1 was constant in Taurasi during the whole ripening period; In Vulture, showed a U-shaped pattern, being significantly higher relative expression levels at veraison and harvest, and a lower level on the second date; In Taburno, LDOX1 showed higher

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AM3
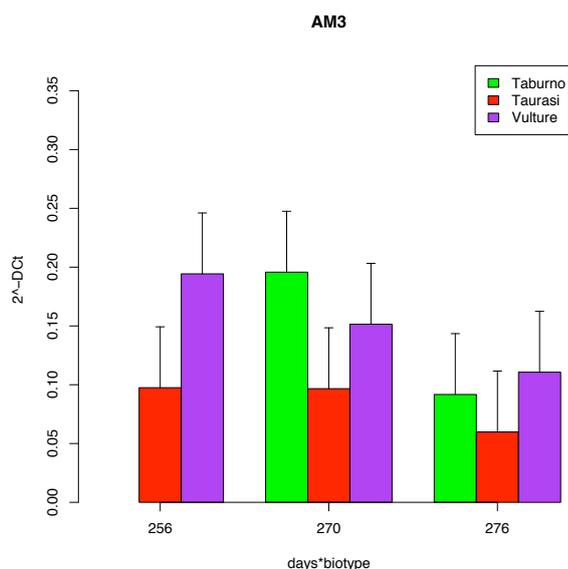
Figure 57. The mean expression relative to GADPH of the AM3 gene in Taburno (green), Taurasi (red) and Vulture (purple) ripening grapes. Error bars indicate MDS.
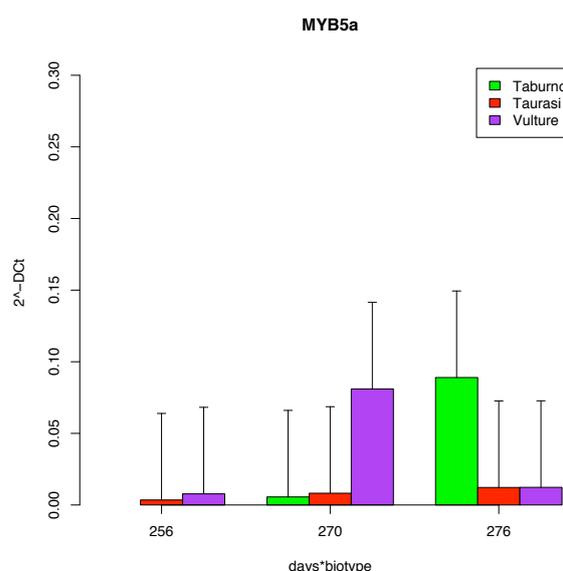

MYB5a

Figure 58. The mean expression relative to GADPH of the MYB5a gene in Taburno (green), Taurasi (red) and Vulture (purple) ripening grapes. Error bars indicate MDS.

expression levels on the second date and decreased towards ripening (Figure 55). The expression pattern of AM1 was significantly different in Taburno, showing a decrease, while it was constant in Vulture and Taurasi (Figure 56). The same pattern was shown by AM2 (Figure 57). MYB5a relative expression was significantly higher in Vulture on the second sampling date and in Taburno on the final sampling date (Figure 58).

The three-way interaction (day*biotype*treatment) showed significant diffeences in the expression patterns of four genes. Vulture shaded grapes showed low expression levels of F3'5'H at veraison, but the expression of this gene was higher towards veraison; conversely, the expression of F3'5'H showed a significant peak at veraison and then decreased in exposed Vulture berries; similarly, but less significantly, there was a decrease in exposed and shaded Taburno; exposed and shaded Taurasi resulted similar and constant during ripening (Figure 59). F3'H expression was constant throughout the whole ripening period, in all conditions, except for a peak in the expression level in shaded Vulture on the second sampling date (Figure 60). F3H showed a pattern very similar to the one shown by F3'5'H (Figure 61). The expression levels of AM3 were significantly
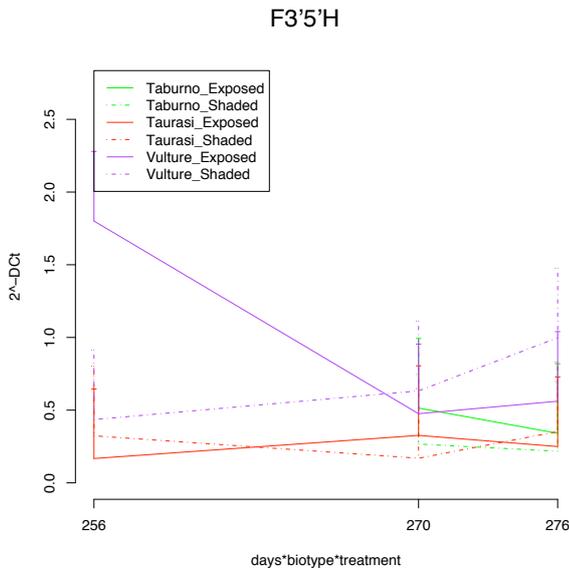
F3'5'H

Figure 59. The expression relative to GADPH of the F3'5'H gene in exposed Taburno (green), shaded Taburno (dotted green), exposed Taurasi (red), shaded Taurasi (dotted red), exposed Vulture (purple) and shaded Vulture (purple) during grape ripening. Error bars indicate MDS.

F3'H
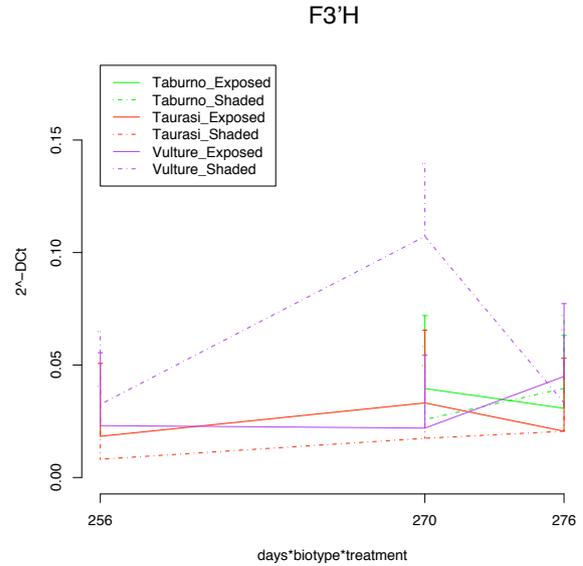
Figure 60. The expression relative to GADPH of the F3'H gene in exposed Taburno (green), shaded Taburno (dotted green), exposed Taurasi (red), shaded Taurasi (dotted red), exposed Vulture (purple) and shaded Vulture (purple) during grape ripening. Error bars indicate MDS.

AM3
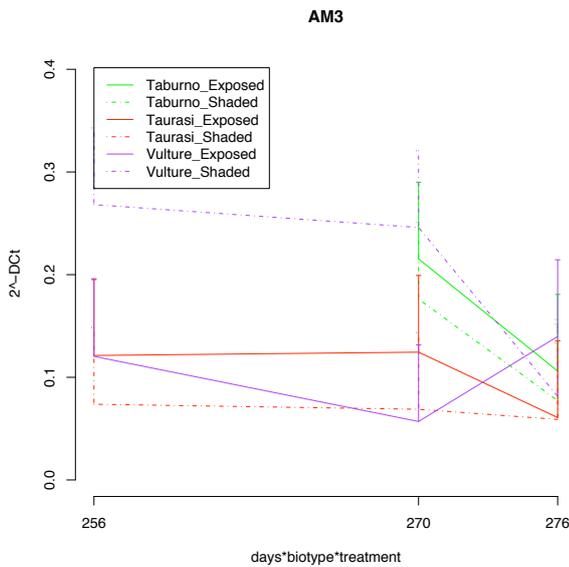
Figure 62. The expression relative to GADPH of the AM3 gene in exposed Taburno (green), shaded Taburno (dotted green), exposed Taurasi (red), shaded Taurasi (dotted red), exposed Vulture (purple) and shaded Vulture (purple) during grape ripening. Error bars indicate MDS.

F3H
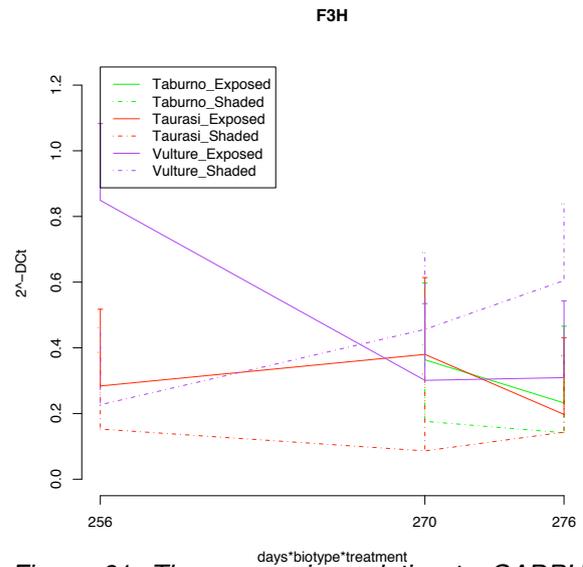
*Figure 61. The expression relative to GADPH of the F3H gene in exposed Taburno (green), shaded Taburno (dotted green), exposed Taurasi (red), shaded Taurasi (dotted red), exposed Vulture (purple) and shaded Vulture (purple) during grape ripening. Error bars indicate MDS.*

higher in shaded Vulture and shaded and exposed Taburno grapes on the first and second

date, and decreased towards the third date (Figure 62).

The ratio of the cumulative relative expression of F3'5'H and F3'H and of F3'5'H and UFGT were calculated (Castellarin and Di Gaspero, 2007). The F3'5'H/F3'H ratio was significantly higher in exposed berries towards veraison and it decreased during ripening; shaded berries showed a constant pattern for this index (Figure 63). The F3'5'H/UFGT ratio increased during ripening showing no difference between shaded and exposed
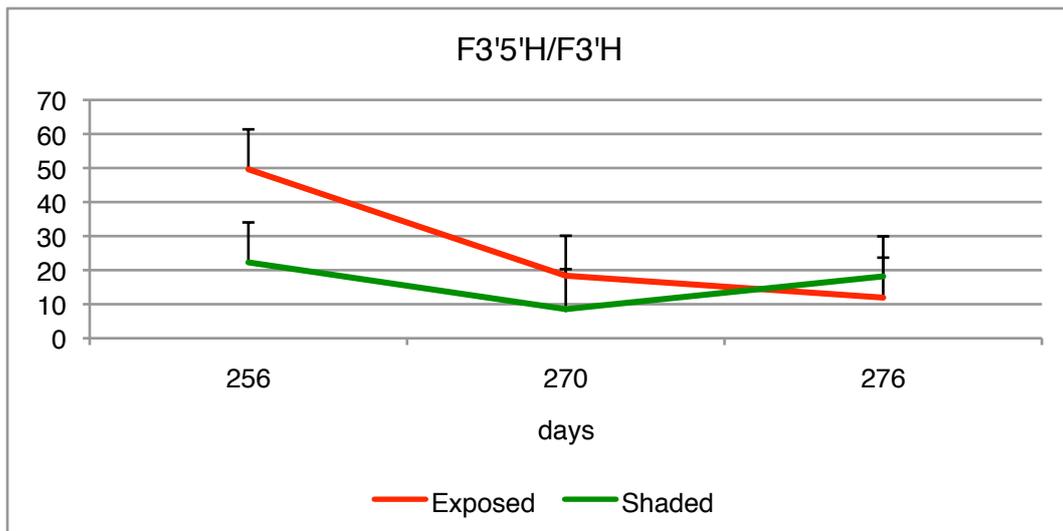
Figure 63. The evolution of the ratio between the relative expression of the F3'5'H and F3'H loci. Error bars indicating MDS.
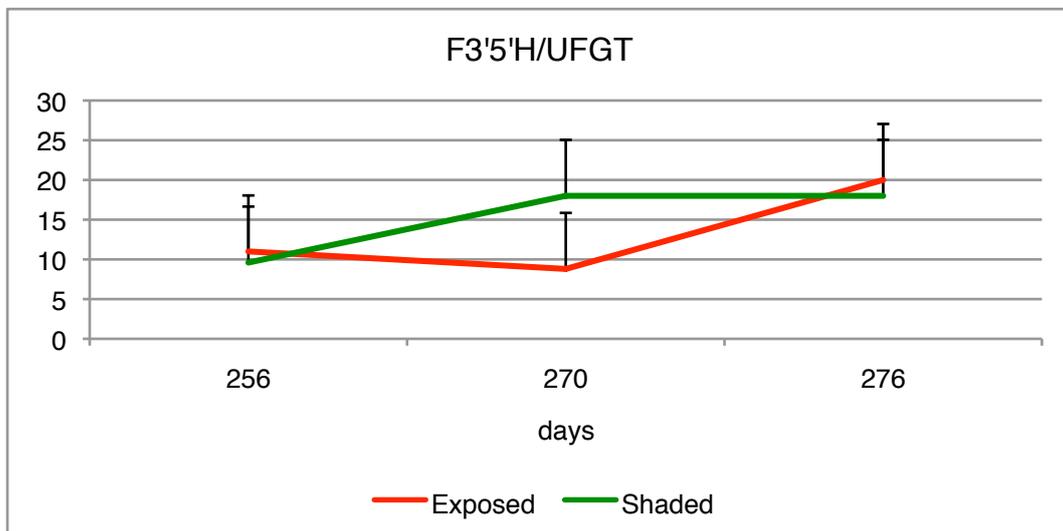
Figure 64. The evolution of the ratio between the relative expression of the F3'5'H and UFGT loci. Error bars indicating MDS.

bunches (Figure 64).

Discussion

The effect of cluster shading on the flavonoid pathway

Light is the energy supply of plants, thus one of the most important environmental factors. It influences a great number of plants' primary physiological processes, including photosynthesis, flower induction and seed germination. (Jiao et al., 2007). Several works investigated the effect of bunch exposure on the accumulation of flavonoid in grape berries (Sparvoli et al., 1994; Price et al., 1995; Haselgrove et al., 2000); Bergqvist et al., 2001; Gollop et al., 2002; Spayd et al., 2002; Downey et al., 2004; Jeong et al., 2004; Cortell and Kennedy, 2006; Fujita et al., 2006; Pereira et al., 2006; Rustioni et al., 2006; Fujita et al., 2007; Ristic et al., 2007; Guo et a., 2008; Tarara et al., 2008; Matus et al., 2009). However results have been in some cases contradictory. Moreover, the role of light on the expression of key genes of the pathway has not yet been established. The effect of light exclusion applied to the grape bunch was investigated in the present work. Grape clusters from three different *Vitis vinifera* Aglianico biotypes (Taburno, Taurasi and Vulture), cultivated in similar agronomic conditions in the same experimental vineyard, were treated with a shading screen before veraison. In the same time, control grapes were fully expose to sunlight with leaf removal.

The shading treatment showed little or no effect on berry weigh, but it positively affected berry skin weight and the skin/berry weight ratio. The accumulation pattern of sugars and the evolution pattern of pH and titrable acidity show that the shading treatment caused a initial delay in the onset of ripening. However, shaded bunches showed a faster rate of sugar accumulation and acid consumption, so the differences between shaded and exposed grapes were   reduced, or even cancelled, towards the end of ripening. This

indicates that shading generally induced a delay of the onset of ripening, but in the mean time shaded berries showed a sort of "recovery" phenomena in the second part of ripening.

The effect of shading on flavonoid kinetics.

In this experiment, exposed berries accumulated more polyphenols, and particularly more flavonoids, than shaded berries. However the differences between shaded and exposed bunches were larger at veraison and smaller towards harvest, suggesting that shading caused a delay in the start of flavonoid synthesis, but that, somehow, polyphenol accumulation was faster in shaded berries, thus showing a sort of recovery in the second part of ripening of ripening.

In this work, shading significantly reduced the accumulation of total anthocyanins in Aglianico grapes. A similar result was observed in other cultivar such as Merlot (Spayd et al., 2002; Pereira et al; 2006), Cabernet Sauvignon (Bergqvist et al., 2001; Jeong et al., 2004; Matus et al., 2009), Shiraz (Downey et al., 2004) and Grenache (Bergqvist et al., 2001). The differences in the anthocyanin accumulation indicate that in this experiment, the shading treatment induced a later onset of pigment accumulation. Towards maturity the differences in anthocyanin concentration of shaded and exposed bunches were smaller, indicating that accumulation was faster in shaded grapes. In other works it was observed a similar phenomena, and in some cases shaded bunches reached the same anthocyanin concentration as the exposed bunches (Downey et al., 2004; Rustioni et al., 2006; Ristic et al., 2007). Our results could confirm that the effect of shading is stronger towards the onset of ripening, delaying the pigment accumulation, rather than in the late stages of ripening. At the same time, shaded grapes show a higher efficiency towards the

end of ripening. The molecular basis of the "recovery" phenomena are not clear: it could due to a faster synthesis, as well as to a different rate in anthocyanin catabolism.

In this experiment, the anthocyanin profiling of Aglianico showed no significant shift due to bunch shading. The proportion of the single anthocyanidins, the trisubstituted \disubstituted anthocyanin ratio and the glucoside\acylate anthocyanin ratio showed no significant change in exposed and shaded berries. However, many works reported a shift in the anthocyanin profiling correlated to light exposure in several varieties. The shift often consisted in an increase of the proportion of disubstituted anthocyanin and of acylated anthocyanins (Downey et al., 2004; Rustioni et al., 2006; Cortell and Kennedy, 2006, Matus et al., 2009). This experiment suggests that the anthocyanin profile of Aglianico is very stable towards light.

The accumulation pattern of total tannins in in some way resulted opposed to that of anthocyanins: in this case the differences between exposed and shaded bunches was bigger towards the end of ripening, exposed bunches accumulating more tannins in the skin. A similar result was obtained in Pinot Noir (Cortell and Kennedy, 2006) and in Shiraz (Downey et al., 2004; Ristic et al., 2006) grapes. Taken together, these results suggest that there is a positive effect of light exposure on the accumulation of total tannins in grape berries.

The effect of shading on the transcription of the genes of the flavonoid pathway

Structural genes:

In this work all the genes of the pathway were steadily expressed during berry ripening. In particular UFGT and AM3 where more expressed at the beginning of ripening, and this is consistent with the higher speed of anthocyanin accumulation in the early stages of berry ripening.

CHS2 and F3H were generally up-regulated in exposed bunches; in particular F3H was more expressed in exposed berries at the beginning of ripening. This consistent with the with the higher accumulation of polyphenols, flavonoid, anthocyanins, tannins in exposed berries, as well as with the results shown in previous works (Sparvoli et al., 1994, Jeong et al., 2004 and Matus et al., 2009). However, these authors showed that light induced the expression also of the downstream genes of the pathway, namely DFR, LDOX, OMT and UFGT. In the experimental conditions of this work, DFR, LDOX1, LAR2 and OMT showed no significant change in the relative expression pattern in shaded and exposed berries. Surprisingly, in this work also the expression of UFGT was unaffected by the treatment. A similar result was reported in Shiraz grapes (Downey et al., 2004); t.

The highest concentration of anthocyanin in exposed berries, however, is hardly explained solely by the pattern of CHS2 and F3H. Several work pointed out that a higher expression of UFGT leads to a higher anthocyanin concentration (Jeong 2004; Matus 2009). Moreover, shaded bunches, although having an overall lower concentration of anthocyanins, showed a faster anthocyanin accumulation rate in the second part of ripening. However, no gene was up-regulated in the shaded bunches at any time. Hence, the "recovery" phenomena exhibited by shaded bunches finds no explanation in the

transcriptional pattern. Taking in due account these considerations, it is likely that the effect of light on the accumulation of flavonoids in grape berries might be explained also by other mechanisms. It can be reasonably speculated that one possiblity is a differential post-transcriptional regulation induced by light/shading, another one could be a different effect of light/shading on the catabolism of anthocyanins. More work is needed to verify these hypotheses.

Many works, shading induced a shift in the anthocyaninin profiling, particularly in the proportion of trisubstituted and disubstituted anthocyanins. The distribution of this ratio is associated to the expression of F3'5'H and F3'H, responsible for the hydroxylation of the flavonoid B-ring. However, to our knowledge, there is no report on the effect of shading on the transcriptional expression of the F'3'5'H and F3'H genes in grapes. Castellarin and Di Gaspero, in 2007, showed that the F3'5'H/UFGT and F3'5'H/F3'H ratios are strongly correlated to distribution of the proportion of trisubstituted and disubstituted anthocyanins in several grape varieties. High ratios correspond to higher percentage of trisubstituted anthocyanins in the berries. In this experiment the F3'5'H/UFGT ratio increased steadily during ripening with no significant change induced by treatment. This is consistent with the stability and the high proportion of malvidin, petunidin and delphinidin shown by Aglianico's anthocyanin profiling. The F3'5'H/F3'H ratio was significantly affected by the shading treatment, however only at the beginning of ripening, with no effect, though, on the final anthocyanin composition. A possible explanation for this is that the effect of light was lower in the second part of ripening.

These findings suggest that the F3'5'H/UFGT and F3'5'H/F3'H ratios might be important in determining the stability to light of Aglianico's anthocyanin profiling. However, this needs further investigation. Furthermore, post-transcriptional regulation and a

differential specificity and activity of the enzymes of the flavonoid synthesis of this cultivar also might be involved in the determination of the anthocyanin profiling.

In this work the expression pattern of FLS4 and MYB12 resulted higher in exposed berries, although not always significantly. Nevertheless, it is well established that light has a positive effect on flavonol synthesis (Price et al., 1995; Haselgrove et al., 2000; Spayd et al., 2002; Downey et al., 2004; Pereira et al., 2006; Cortell and Kennedy, 2006; Tarara et al., 2008). Moreover, the expression of FLS4 was positively influenced by light in Shiraz, Cabernet Sauvignon and Merlot (Downey et al., 2004; Fujita et al., 2006; Matus et al., 2009). The, the results obtained in this work are consistent with current literature, for what concerns the expression of FLS4 and its putative regulator MYB12, confirming a positive effect of light.

Shading showed a negative effect on the accumulation of total tannins. This is consistent with the expression of LAR2, that was significantly more expressed in the exposed bunches. A similar result was obtained also by Fujita et al., in 2007. This suggest that exposure to light positively influences the expression of the LAR2 gene in grape berries.

Transcription factors:

In this experiment the shading treatment showed no effect on the transcription factors MYB5a, MYB5b and MYBA1. This is consistent with the expression of DFR, LDOX1, UFGT and OMT. In a previous work MYBA1 and MYB5a were up-regulated by bunch exposure (Matus 2009). More work is needed to understand the effect of light on these transcription factors

Anthocyanin Transporters:

To date, this is the first report about the effect of shading on the expression of these transporters in the grapevine. The relative expression levels of AM1, AM2 and GST4 showed no significant difference between shaded and exposed clusters. This was consistent with the relative accumulation pattern of acylated anthocyanins. This work suggests that bunch exposure does not have a major role in the regulation of the expression level of these transporters. However, the anthocyanin profiling of Aglianico is particularly stable to light. AM1 and AM3 are involved in the transport of acylated anthocyanins (Gomez et al., 2009), so it is likely that AM1, AM3 may show a different behaviour in other cultivars, as shading often induces a shift in the proportion of acylated anthocyanins.

The expression of the flavonoid pathway in three monophyletic Aglianico biotypes.

Aglianico is one of the most important red grape varieties in Southern Italy. Three different biotypes are traditionally recognised for this variety. Each biotype is named after the historical growing region: "Aglianico di Taurasi", "Aglianico del Taburno" and "Aglianico del Vulture". These biotypes were differentiated through the patient selection work of farmers, choosing over the centuries the best plants in each territory that were more suitable for the environmental peculiarities of each region and more respondent to the farmers expectations. The genetic identity of these biotypes was confirmed by DNA fingerprinting (Costantini et al., 2005). The phenotypic differences shown by the biotypes are due to mutations and epigenetic modifications of the same original genotype. In order to asses possible differences in polyphenol kinetics and in the expression of the genes of

the flavonoid pathway, the three biotypes were studied in an experimental vineyard in the 2008 campaign.

Vulture showed a slightly smaller berry compared to Taurasi and Taburno, and this is consistent with current litterature (SeSirca, 2001, Simone Di Lorenzo 2009). However the skin weight and the skin/berry rate showed no significant differences among biotypes at harvest.

The patterns of sugar accumulation and of pH and titrable acidity evolution show clearly a different timing of the onset of ripening in Taburno. However, after the delay, the ripening kinetic seem faster as Taburno reaches the same final levels as Taburno and Vulture.

Taburno shows a different timing also in the total polyphenol, total flavonoids and in the non anthocyanin flavonoid accumulation kinetic.   The levels of these metabolites in Taburno are lower in the beginning of ripening, but there is no significant difference among Taburno, Taurasi and Vulture towards the end of ripening.

Taburno, Taurasi and Vulture showed no difference in the final concentration of total tannins in the skin at harvest. This is in contrast with previous reports (Moio et al., 2004). However, Taburno showed a different pattern, as it had very low tannins towards veraison, but it reached the same level as Vulture and Taurasi at harvest.

It was previously reported that Vulture accumulated the most anthocyanins, Taburno the least and that Taurasi had an intermediate behaviour (Moio et al., 2004). Indeed, Taburno showed a delay in the synthesis of pigments, but the accumulation rate of anthocyanins was higher in this biotype towards the end of ripening, thus, in the experimental conditions of this work, no significant difference was shown in the final accumulation of anthocyanins among the biotypes.

No significant difference was shown in the anthocyanin profiling of the three biotypes. In this work, the average anthocyanin profiling of Aglianico was composed by 82% of malvidin, 6% of delphinidin, 7% of petunidin, 5% of peonidin and less than 1% of cyanidin; 78% of free anthocyanins and 22% of acylated anthocyanins. This result is consistent with other previously published aglianico anthocyanin profiling (Lovino et al., 2005; Suriano et al., 2005; Mattivi et al., 2006). The results of this work indicate that aglianico anthocyanin profiling is very stable in different biotypes and in different conditions.

In this experiment, the average expression levels of CHS2, F3'5'H, F3'H, F3H, DFR, LDOX1, AM1, AM3 and GST4 were generally higher in Vulture and lower in Taurasi; in Taburno, the expression levels of F3'H, DFR1, LDOX1, AM1 and AM3 where higher, while those of CHS2, F3'5'H, F3H, and GST4 were lower. To the higher expression levels of these genes in Vulture it did not correspond a higher concentration of flavonoids in the berries, compared to the other biotypes. However, the downstream genes of the pathway (i.e. UFGT, OMT, FLS4) showed no significant difference among biotypes. Castellarin et al., 2007 showed a strong correlation between the relative expression levels of UFGT and the concentration of anthocyanins.Together with the result of this work, it suggest that they might have a more important role in determining the final concentration of anthocyanins.

DFR1, LDOX1, AM1 and AM3 showed a different trend in among biotypes: they were virtually constantly expressed in Taurasi and Vulture, while they showed significant peak in the second date in Taburno, and a lower level towards harvest. No significant change occurred among biotypes in the final concentration of flavonoids, however Taburno showed a significantly different timing in the ripening kinetics. The expression patterns of some of the genes show a different pattern in Taburno, this could be a symptom of a different temporal control of the pathway in this biotype.

Conclusions

The three biotypes of Aglianico showed no significant difference in the levels of primary and secondary at harvest, nor in the anthocyanin profiling. However they showed a different ripening kinetic. In particular, Taburno showed a delay in the onset of ripening, followed by a full recovery at the end of the ripening process. The expression pattern of the genes of the flavonoid pathway showed some differences in the three biotypes, however these differences might be due to the different timing shown by the biotypes.

The pattern shown by sugars, acids and phenolic compounds kinetics suggest that the shading treatment caused generally a delay in the onset of ripening. However, shaded bunches shower a faster accumulation of primary and secondary metabolites in the second part of ripening, partially or totally recovering the initial delay. The expression of CHS2, F3H, FLS4 and LAR2 are consistent with the higher accumulation of phenolics, and particularly of tannins, shown in the exposed berries. However, in the experimental conditions of this work, the shading treatment showed no significant effect in the transcription levels of of the genes of the structural and regulatory genes of the flavonoid pathway, and particularly particularly of UFGT. More over, no explanation of the recovery shown by the shaded berries was found in the analysed gene patterns. This suggests that the effect of light is not exerted only at the transcriptional level.

In many works, shading induced a shift in the anthocyanin profiling. However, Aglianico showed a very stable anthocyanin profiling and no significant shift was induced by light. This was supported by the similar F3'5'H/UFGT (and partially by the F3'5'H/F3'H ratio) ratio expressed by shaded and exposed bunches.

The effect of cluster shading on the expression of AM1, AM3 and GST4 was for time reported. The relative expression levels of these genes resulted unaffected by the treatment, however more work is needed to elucidate the role of light in the regulation of these transporters.

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