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Chapter 4

Anthocyanin acyltransferases involved in flower pigmentation and coloration of berries

With Kees Spelt, Filippa Brugliera, Yoshikazu Tanaka, Andrea Schubert, Alessandra Ferrandino, Ronald Koes and Francesca Quattrocchio.

Abstract

In grapevine (Vitis vinifera) and petunia (Petunia hybrida), anthocyanins are the main pigments responsible for the coloration of the berry and the flower petals respectively. These compounds are coloured flavonoids synthesized from the condensation of phenylalanine and malonyl CoA through a series of enzymatic steps. The very last part of this biosynthetic pathway consists of a series of modifications of the anthocyanin molecule that fine tune the colour and the stability of the pigment. Acylation of anthocyanins is one such modification and is one of the least understood steps in anthocyanin biosynthesis. The enzyme anthocyanin acyltransferase catalyses the transfer of cinnamic acids from their CoA esters to the glycosyl group of the anthocyanin. Here, we describe the cloning and characterization of a petunia acyltransferase encoded by the GLUCOSYLATION AT FIVE (GF) locus. We identified a gene, DIFc, which is primarily expressed in the limb and tube of petals and is regulated by transcription activators of the anthocyanin pathway. Recombinant DIFc protein is able to acylate anthocyanins in vitro and the constitutive expression of DIFc in petunia gf mutants results in complementation of the mutation and in the accumulation of acylated anthocyanins in flower petals. Moreover, we found that gf lines contain frameshift mutations in DIFc, indicating that DIFc is encoded by the GF locus. We used the GF/PhDIFc sequence to identify by homology two putative acyltransferases from grape Vvacyltransferase1 (VvAAT1) and Vvacyltransferase2 (VvAAT2) and show that the expression of VvAAT2 in grape correlates perfectly with the accumulation of anthocyanins, suggesting that this might be the best candidate for a grape anthocyanin acyltransferase gene. The functional analysis of the two grape candidate genes by complementation of the petunia gf mutant is under way. Interestingly, GF, VvAAT1 and VvAAT2 have a phylogenetic origin distinct from that of all other anthocyanin acyltransferases identified so far.
Anthocyanin acyltransferases

Introduction

Anthocyanins are coloured flavonoids responsible for the orange, red, purple and blue colours of many flowers, leaves and fruits. They play an important role in attracting pollinators and protecting plants against various stresses. The interest in these compounds is intensified because of their health properties as antioxidants. In the last years several reports have been published in which the activity of anthocyanins as anti-inflammatory, anti-mutagenic and protectants against cancer has been shown (Rice-Evans et al., 1997; Jang et al., 1998; Butelli et al., 2008).

In many plant species anthocyanins are modified by glycosylation, methylation and acylation which determines the final colour of the tissue where the pigment accumulates, increases the stability of the compounds and the interaction with other molecules (e.g. copigmentation) (Tanaka et al., 2009).

Acylation is one of the most common types of modification in plant secondary metabolism and, consequently, plant acyltransferases constitute a large family of enzymes. It has been estimated that some 88 genes, encoding members of acyltransferase family, are present in the Arabidopsis genome (Gang, 2005). These enzymes are called BAHD, according to the first letter of the first four characterized enzymes: benzylalcohol O-acyltransferase (BEAT), anthocyanin O-hydroxycinnamoyltransferase (AHCT), anthranilate N-hydroxycinnamoyl/benzoyltransferase (HCBT), and deacetilvindoline 4-O-acetyltransferase (DAT). They catalyze a reaction that utilizes CoA thioesters to produce a large spectrum of compounds ranging from small volatile esters to larger molecules like modified anthocyanins and phytoalexins (St.-Pierre and De Luca, 2000).

The anthocyanin acyltransferases (AATs) represent a subfamily of BAHD enzymes that catalyze the transfer of acyl groups from an acyl donor to an anthocyanin molecule. AATs often display a strong preference to acylate one specific position on the anthocyanin substrate. The consequence of this strict “regio-specificity” is that the acylated product cannot be further acylated by the same enzyme (Gould et al., 2008). AATs are divided in aromatic and aliphatic acyltransferases based on the acyl donor that is used. In the first case the donor is an aromatic acyl-CoA ester, such as coumaroyl-CoA and caffeoyl-CoA and the acylation makes the anthocyanin more stable and bluer, instead, aliphatic acylation, (e.g. malonylation), results in an increase of solubility in water (Heller and Forkmann, 1994).

Until recently, cDNA encoding anthocyanin acyltransferases from few species had been cloned and characterized by in vitro studies (Gould et al., 2008). Here are some examples: Gentiana triflora,: Gt5AT, an acyltransferase involved in the biosynthesis of gentiodelphin, resulting from aromatic acylation with two caffeoyl residues (Fujiiwara et al., 1997; Fujiiwara et al., 1998), Perilla frutescens, Pf3AT, involved in the biosynthesis of malonylishisonin (Yonekura-Sakakibara et al., 2000), Salvia splendens, Ss5MaT1, and
Ss5MaT2, both malonyltransferases, (Suzuki et al., 2001; Suzuki et al., 2004), *Dahlia variabilis*, *Dv3MaT*, involved in the biosynthesis of anthocyanidin 3-O-glucoside-6-O-malonylglucoside in the red dahlia (Suzuki et al., 2002).

Recently, three AAT genes were identified in Arabidopsis (*At3AT1*, *At3AT2* and *At5MaT*) by the correlation of their expression patterns with anthocyanin synthesis (Luo et al., 2007). The capacity of these three enzymes to acylate anthocyanins has been shown in vivo by analysis of mutants (for the *At5MaT* gene) and ectopic expression in tobacco flowers, which contain a suitable substrate (Luo et al., 2007).

The activity of AATs is not dependent on hydroxylation of the B-ring of the anthocyanin substrate, as they are equally active on pelargonidin, cyanidin and delphinidin (Nakayama et al., 2003). Some AATs have been shown to accept also flavonol glycosides, such as quercetin 3-O-glucoside, as substrate (Suzuki et al., 2002). Considering the large variety of different acylated anthocyanidin molecules present in plants and the specificity of this class of enzymes for their substrates and acyl donor, we assume that a wide range of acyltransferases have not been identified yet. The two malonyl-CoA anthocyanin acyl transferases (Ss5MaT1, and Ss5MaT2), characterized in *Salvia splendens* (Suzuki et al., 2001; Suzuki et al., 2004), are a good example of the diversity of these enzymes. Although both enzymes transfer a malonyl group to a cyanidin 5-O-glucoside substrate, they are phylogenetically distinct, as they belong to separate clades of the BAHD tree (Figure 1). This means that, despite their substrate similarity, they have evolved from different ancestral proteins. Suzuki and colleagues (2004) proposed that the *Salvia* Ss5MAT2 enzyme, which groups outside the clade containing most other anthocyanin acyltransferases, might be an odd or ‘imperfect’ enzyme, because its catalytic activity is lower than Ss5MAT1. Examples like these underline the difficulties to predict the function of acyltransferases genes only on the basis of sequence similarity.

Acylation has been shown to affect the stability of anthocyanins in vitro and in vivo. In particular, coumaroylation is very effective in increasing the half life of anthocyanin molecules (Luo et al., 2007). Hence AATs may be useful to produce ornamentals with stronger and bluer flower colours or fruits with high anthocyanin and consequent positive effect on human and animal health. The accumulation of anthocyanins in grape (*Vitis vinifera*) is of large commercial interest because of its impact on the characteristics of wine. Acylated anthocyanins like delphinidin acetate, malvidin acetate, peonidin acetate, peonidin p-coumarate, malvidin p-coumarate are accumulated in some cultivars of grape (e.g. Barbera, see Figure 5A). However, despite the intense recent efforts to identify all the anthocyanin genes from *Vitis vinifera*, no AATs have been identified in this commercially important species.
Anthocyanin acyltransferases

The terminal part of the anthocyanin pathway, where modification like acylation occurs, is perhaps best studied in Petunia hybrida flowers, but, strikingly, also here the AAT genes had not been identified (Wiering and De Vlaming, 1977; Tornielli et al., 2009). The acylation, 5-glucosylation as well as methylation of anthocyanins in petals all require a functional GLUCOSYLATION AT FIVE locus. Because gf flowers contain a normal amount of 5-glucosyltransferase (Jonsson et al., 1984c) and because anthocyanin methyltransferase are encoded by distinct MT and MF loci (Chapter 3), we suspected that GF might encode or regulate the activity of an AAT.

We show here that the GF locus encodes an anthocyanin acyltransferase that has a strong effect on the final colour of the petal as the combination of acylation and methylation of the anthocyanin results in strong blueing. Interestingly, GF belongs to a different clade of BAHD acyltransferase than previously characterized AATs, suggesting that AATs evolved multiple times independently. Finally, we identified two GF homologs in grape. The expression of one of these closely mirrors the expression of anthocyanin genes, suggesting that it encodes the long sought AAT from grape.

Results

Isolation of a candidate anthocyanin acyltransferase from Petunia hybrida

Differential hybridization of a cDNA library from corolla limb of the petunia line V26 (AN1) with cDNA probes from AN1 and an1 petals resulted in the isolation of cDNA fragments of 7 distinct genes (DIFa, DIFc, DIFe, DIFf, DIFg, DIFh and DIFI) that are activated by the regulatory gene AN1 (Kroon et al., 1994). The DIFc cDNA measured about 1 Kb and encodes a protein with similarity to acyltransferases.

Several observations made us suspect that DIFc encodes an anthocyanin acyltransferase and might originate from GF. Its expression is also down regulated in mutants for other regulatory genes of the anthocyanin pathway (AN2, and AN11) (Quattrocchio et al., 1993) and RFLP analysis showed that DIFc is linked to the HF2 locus on chromosome V, just like GF (Wiering, 1974). The GF locus (GLUCOSYLATION AT FIVE) got its name because gf mutants lack the glucosyl group at the fifth position of the A-ring and therefore was initially thought to encode a 5-glucosyltransferase. However, another possibility was that actually the GF locus encodes an acyltransferase and that acylation is required for subsequent 5-glucosylation.

Phylogenetic analysis of known anthocyanin acyltransferases has shown that these proteins all belong to one specific subclade in the BAHD protein family (clade 1 Figure 1). The DIFc protein, however, clusters with Ss5MaT2 (the imperfect anthocyanin acyl
transferase of salvia), FaSAT (a strawberry enzyme that modifies benzyl esters), and CaPun1 (which in pepper works in the capsaicin pathway) in a distinct clade of the BAHD family.

Previously identified anthocyanin acyltransferase enzymes share a conserved domain (Supplemental Figure 1) that consists of three motifs (Nakayama et al., 2003). The second motif is hardly recognisable in PhDIFc, or in SsMaT2. Hence, the sequence of DIFc did not provide any obvious evidence for a role in anthocyanin acylation.

**Acyltransferase activity of the recombinant DIFc protein**

To determine the biochemical function of the DIFc gene, the entire coding sequence has been used to produce recombinant PhDIFc as His-tagged fusion protein in *E. coli*. The activity of the recombinant DIFc protein was assessed on the substrates delphinidin 3-glucoside and delphinidin 3-rutinoside using previously described assay conditions (Fujiwara et al., 1997) and the reaction products obtained were analysed by HPLC. We found that DIFc is able to acylate delphinidin 3-rutinoside using either caffeoyl-CoA or coumaroyl-CoA as an acyl donor. However, no activity has been observed when delphinidin 3-glucoside was used as substrate (Table 1).

**Table 1:** Acyltransferase activity of the recombinant DIFc protein in *E. coli*.

<table>
<thead>
<tr>
<th>Crude Homogenate</th>
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<th>Substrates added</th>
<th>Acylated products detected</th>
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<td>DIFc</td>
<td>coumaroyl CoA</td>
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1 EV empty vector  
2 D3R, Delphinidin 3 rutinoside; D3G, Delphinidin 3 glucoside  
n.d., not determined

**Expression pattern of the DIFc gene in Petunia hybrida**

To assess whether the expression of DIFc correlates with anthocyanin accumulation, we analyzed transcript levels in different plant tissues and at several stages of flower development in the petunia wild type hybrid M1xV30. RT–PCR of DIFc transcripts showed high expression in the limb and in the tube of the flower and a lower amount in the very early stages of anther development. No expression is detectable in roots and leaves, while very low transcript levels are present in stems and sepals, which are often slightly pigmented in this hybrid genotype.
The expression pattern of *DIFc* clearly mirrors the expression of several anthocyanin biosynthetic genes in petunia (Spelt et al., 2000) and supports a role for the *DIFc* gene in anthocyanin biosynthesis (see Figure 2A).
Identification of mutations in the DIFc gene in Petunia hybrida gf mutants

To assess whether the DIFc protein is encoded by the GF locus, we analyzed the expression of the DIFc gene in the Petunia lines V30, V23 and the hybrid M1xV30, which all contain the dominant GF allele, and line R78, which is a recessive gf mutant. Because DIFc RNA was equally expressed in GF and gf lines, this excludes that GF regulates DIFc transcription in trans. (Figure 2B).

To explore the possibility that the GF locus contains the DIFc gene, we amplified and sequenced the DIFc gene from various petunia lines with GF or gf alleles. The gf mutant lines R78 and R63 both contain a defective difc allele. The difcR78 allele contains a 7-bp insertion in the coding sequence of exon 1, which shifts the reading frame and results in a premature stop codon. The difcR63 allele contains an 8-bp insertion at exactly the same position. Because the 7- and 8-bp insertions resemble typical transposon-footprints, we assume that the difcR78 and difcR63 derive from a progenitor allele that was inactivated by the insertion of a transposon in germinal tissues (Figure 2C). All together these data strongly support that the DIFc clone derives from the petunia GF locus.

Figure 1. Phylogenetic relation among plant acyltransferases.
The tree is based on the alignment of the full protein sequences of acyltransferases and the separation in clades I, II, III, IV, V as defined by (D’Auria, 2006). The proteins were aligned using the ClustalW 2 on line tool (Thompson et al., 1994) and the phylogenetic tree was built using the PHYLIP program (version 3.63). Protein sequences used: At5AT Arabidopsis thaliana BAB01191 (Luo et al., 2007), Sc3MaT Senecio cruentus AAO38058, Dv3MAT Dahlia Q8GSN8 (Suzuki et al., 2002), Ss5MAT1 Salvia splendens Q8W1W9 (Suzuki et al., 2001), Pf5MaT Petiolaris frutescens AF405204 (Yonekura-Sakakibara et al., 2000), CrDAT Catharanthus roseus AAC99311 (St-Pierre et al., 1998), PsSalAT Papaver somniferum AAK73661 (Grothe et al., 2001), CbBEAT Clarkia breweri AAC18062 (Dudareva et al., 1998), FaSAAT Fragaria x ananassa AF193789 (Aharoni et al., 2000), TcTAT Taxus cuspidata AF190130 (Walker et al., 2000), TcTBT Taxus cuspidata AAG3804, TcDBAT Taxus cuspidata AF193765 (Walker and Croteau, 2000), Gl5AT Gentiana triflora BAA74428 (Fujiiwara et al., 1998), CER2 AAM64817 Arabidopsis thaliana, Glossy2 CAA61258.1 Zea mays, RsVAT Rauwolfia serpentine CAD89104.2 (Bayer et al., 2004), Pun1 AAV66311.1 Capsicum annuum (Stewart et al., 2005), ACT AAO73071.1 Hordeum vulgare (Burhenne et al., 2003), CHAT AAN09797.1 Arabidopsis thaliana (D’Auria et al., 2002), MpaAT1 AAV18799.2 Malus x domestica, AMAT AAW22989.1 Vitis labrusca (Wang and De Luca, 2005), NtBEBT AAN09798.1 Nicotiana tabacum (D’Auria et al., 2002), DBNBTB AAM75818.1 Taxus Canadensis (Walker et al., 2002), At3g29635 NP_189605 Arabidopsis thaliana, At3g29670 AAP40017 Arabidopsis thaliana, At3g29680 NP_189610 Arabidopsis thaliana, At3g39050 NP_568561 Arabidopsis thaliana, At3g39090 AAP49516 Arabidopsis thaliana, At3g81160 NP_200924 Arabidopsis thaliana, At3g39080 AAP49522, At3AT1 NP_171890 Arabidopsis thaliana (Luo et al., 2007), At3AT2 NP_171849 (Luo et al., 2007), Arabidopsis thaliana, Ss5MaT2 AAR26385 Salvia splendens (Suzuki et al., 2004)
**Anthocyanin acyltransferases**

Figure 2. Expression pattern, genetic regulation and gene structure of PhDIFc.

Expression of DIFc in different buds at different developmental stages and in different tissues of *Petunia hybrida* (A). Effect of mutations in different petunia anthocyanin regulators on PhDIFc expression (B). Structure of the wild type and mutant alleles of PhDIFc (C) Exons and introns are indicated by grey rectangles and lines respectively. Lesions found in mutants alleles of specific lines are indicated below the gene maps. Sequence of the wild type in the first exon at the position where two gf alleles present an insertion (indicated in underlined italic letters).

Complementation of a petunia gf mutants by a DIFc transgene

We tested whether DIFc can functionally complement petunia gf mutants by transforming the gf line R78 and the gf F1 hybrid R78xR63, with a 35S:DIFc construct. The R78 line is mutant for all methylation loci (*mt, mf1 and mf2*) as well as for *gf, hf1 and hf2* (encoding 3', 5' hydroxylase) and accumulates cyanidin-rutinoside as the major anthocyanin. The R78xR63 hybrid has the same genotype, except that it contains a functional MT gene.

By *Agrobacterium*-mediated transformation, we obtained three stable transformed plants in the hybrid line R78xR63 and eight in the R78 line. The flowers of the transgenic plants are more strongly pigmented than the untransformed controls in both genetic backgrounds (Figure 3A and 3B). In the R78xV63 background the change in colour is more spectacular as it also includes stronger blueing of the petals, most probably as contribution...
of the methylation of the anthocyanins (Figure 3B). The expression level of the transcript, the endogenous GF gene and the transgene were measured by RT-PCR (not shown). This confirmed that the lines with the highest expression were those giving the strongest phenotype.

HPLC analysis of petal anthocyanins showed the presence of extra peaks in the chromatogram of the 35S: DIFc transgenics compared to the R78 untransformed control (Figure 3C). The position of the major new peak in the chromatogram of the transgenic R78 and the R78xR63 flowers is consistent their identity as an acylated and methylated products. Final proof of their identity by mass-spectrometry is currently underway.

The result of the functional complementation of gf mutants, together with the analysis of the sequence of DIFc alleles in gf mutants, proves that the GF locus encodes the DIFc gene.

Figure 3. Complementation of gf mutants by 35S: PhDIFc.
(A) Flowers of an untransformed control (line R78: mt mf1 mf2 gf hf1 hf2) and transgenic harbouring the 35S:PhDIFc construct.
(B) Flowers of an untransformed control (hybrid R78xR63: MT mf1 mf2 gf hf1 hf2) and transgenic harbouring the 35S:PhDIFc construct.
(C) Composition of anthocyanin aglycones in petals from transgenic and control plants, as determined by HPLC.
Identification of two candidate anthocyanin acyltransferases from *Vitis vinifera*

A blast search of the *Vitis vinifera* genome and EST collection with the sequence of the petunia GF protein as a query, identified two grape proteins that share about 50 % of amino acid identity with GF (Figure 4). We cloned the corresponding genes and cDNAs from the red grape cultivar, Barbera, and called them VvAAT1 (*Vitis vinifera Anthocyanin Acyltransferase1*) and VvAAT2 (*Vitis vinifera Anthocyanin Acyltransferase2*) and assessed by different approaches whether these clones (or one of them) might encode a grape anthocyanin acyltransferase. The gene VvAAT1 cDNA contains an open reading frame of 1290 bp, which encodes a polypeptide of 429 amino acids, while the VvAAT2 coding region is 1281 bp long and encodes a polypeptide of 426 amino acids. The two predicted proteins lack the second conserved motif typical for proteins from clade I, which contains all (with the exception of the "imperfect" Ss5MAT2 from salvia) the identified anthocyanin acyltransferases motifs (see Supplemental Figure 1 and Figure 1). This apparently odd feature is actually in common with the petunia GF and Ss5MAT2 enzymes.

Figure 4. Alignment and comparison of the PhDIFc/GF protein sequence with the two *Vitis vinifera* anthocyanin acyltransferases candidates VvAAT1 and VvAAT2. The alignment was performed by Clustal W2 online tool and afterword the aminoacid similarity was evidenced by Boxshade. Black coloured amino acid are identical, grey coloured are similar.
Expression pattern of VvAAT1 and VvAAT2 in grape

To study if the expression of VvAAT1 and VvAAT2 is correlated with anthocyanin synthesis and deposition, we analyzed their expression in different grape cultivars. The cultivar Chardonnay generates white berries that lack anthocyanins, whereas Pinot and Barbera generate red berries that accumulate anthocyanins in the skin. HPLC analysis revealed that Barbera berries contain multiple anthocyanins with different methylation and acylation patterns, while acylated anthocyanins are absent from the Pinot berries (Figure 5A and 5B).

To monitor the expression of VvAAT1 and VvAAT2 in these cultivars we used Real time RT-PCR. We found that VvAAT2 transcripts are hardly detectable in the berry before the onset of ripening (véraison). After véraison, VvAAT2 expression level increases dramatically during the first weeks in Barbera, and decreases during later stages of ripening (Figure 5C). Also in Pinot berries, VvAAT2 expression follows the accumulation of anthocyanins, but the maximum expression level is lower and does not correspond with véraison. In the white cultivar Chardonnay, however, VvAAT2 transcripts are barely detectable during berry development. Control experiments showed that the Chardonnay and Pinot genome contain VvAAT2 gene, which excluded the possibility that VvAAT2 transcripts were absent from Chardonnay and Pinot berries because of a deletion of the gene or because of mismatches between the Real time PCR primers and the VvAAT2 transcript (Figure 5D). Instead the data support the hypothesis that the lack of VvAAT2 mRNA in Chardonnay berries is due to the mutation in the VvMybA1 gene that is required for the induction of transcription of the structural anthocyanin genes in berries (Kobayashi et al., 2002).

The analysis of VvAAT2 transcripts in different plant parts shows that this gene is expressed exclusively in pigmented tissues. The VvAAT2 transcript is present in Barbera only in the berry skin, where anthocyanin biosynthesis is active and not in the unpigmented pulp, or in vegetative tissues like leaves and petioles (Figure 5 E). In Teinturier, VvAAT2 expression is also detectable in the pulp, which is strongly pigmented in this cultivar (He et al., 2010).

The expression pattern of VvAAT1 is very different from that of VvAAT2 and does not correlate with anthocyanin synthesis. VvAAT1 is strongly expressed in leaves and petioles and, hardly, also in the berries of the three cultivars (Figure 5 F and 5G).

These data again strongly support a role for VvAAT2 in anthocyanin biosynthesis and indicate that VvAAT1 is probably not involved in this pathway.
Anthocyanin acyltransferases

Figure 5. Pattern of anthocyanin accumulation during berry development and expression analysis of VvAA1 and VvAAT2 in grape. Accumulation profile of anthocyanins respectively in the cultivars Barbera (A) and in Pinot noir (B). The arrow indicates véraison, the onset of ripening. Expression pattern of VvAAT2 (C) during berry development in different Vitis vinifera cultivars and in different tissues of Vitis vinifera (in the white flesh cultivar Barbera and the cultivar Teinturier which accumulates anthocyanin in the berry flesh) (E). Expression patterns of VvAAT1 in different cultivars (F) and in different tissues of Vitis vinifera (G). The arrow in A, B, C and F indicates the onset of berry ripening (véraison). In (D) PCR on genomic DNA of Barbera and Chardonnay.
Functional analysis of VvAAT1 and VvAAT2

To study the function of the two candidate anthocyanin acyltransferases from grape, we made constructs to express both proteins in plants (35S VvAAT1 and 35S:VvAAT2). These constructs were transformed in gf petunia mutants (the line R78 and the hybrid R78xV63) by leaf disk infection.

We have generated 6 transgenics for 35SVvAAT1 and over 20 for 35S:VvAAT2. At the moment of writing, the plants are not yet flowering. When all transgenics will flower, we will proceed to assess the effect of the expression of the grape enzymes on the anthocyanin composition of the petals.

Transient expression in petals of the same gf mutant lines was done by agroinfection and the analysis of the anthocyanin composition in the infected flowers will be performed soon.

Discussion

In recent years, several acyltrasferases from different plant species have been characterized but we know, only for a few species, the enzymes belonging to the AAT class, able to catalyze the acylation of the C-ring of flavonoid substrates (Nakayama et al., 2003).

Here we report the characterization of a new gene, DIFc/GF, that is activated by the regulators of anthocyanin biosynthesis (AN1, AN2, AN11) and show that it encoded a protein able to acylate in vitro delphinidin 3-rutinoside using either caffeoyl Co-A or coumaroyl Co-A as acyl donor. We demonstrated that the DIFc is encoded by the GF locus as: i) the DIFc maps, like GF, to chromosome V, ii) the DIFc coding sequence contains two different mutations in two gf mutant lines, iii) the expression of the DIFc cDNA from the 35S promoter rescues the defect of the gf mutant and restores the production of acylated anthocyanins.

The GLUCOSYLATION AT FIVE (GF) locus was identified via flower colour mutants and found to be required for the 5-glucosylation of the anthocyanins, hence the name of the locus (de Vlaming et al., 1982). However, it is unlikely to be directly involved in the 5-glucosylation, because gf mutants express normal amounts of 5-glucosyltransferase (5GT) activity (Jonsson et al., 1984a). Given that GF encodes an anthocyanin acyltransferase and that the anthocyanin 5-O-glucosyltransferase from petunia can, in vitro, only glucosylate (acylated) anthocyanin derivatives, like 3-(p-coumaroyl)-rutinosides, but not the 3-rutinosides (Jonsson et al., 1984a) is appears that the anthocyanin rutinosides need to be first acylated by GF, before they can be 5-glucosylated by 5GT. Furthermore, GF is also required for methylation of the 3 and 5’ hydroxyl on the B ring, which is catalysed by
methyltransferases encoded by the loci MT, MF1 and MF2. Genetic data indicate that the methyltransferases require acylated and/or 5 glucosylated anthocyanins as a substrate to carry out their function, because simple rutinosides lacking both acyl and glycosil groups are not efficiently methylated by any of these enzymes \textit{in vivo} (Chapter 3). This implies that the different “decorations” of the anthocyanin in petunia petals occur in the order acylation, 5-glucosylation, methylation, which is dictated by the substrate specificities of the acyltransferase, 5-glucosyltransferase and the methyltransferases.

Interesting, the decoration of the anthocyanins in grape does not seem to follow the same order of events. First the berries of the cultivar Pinot lack acylated anthocyanins (Figure 5), although the expression of the VvAAT2 gene is not much lower than in Barbera, which accumulates acylated anthocyanins. This suggests that the lack of acylation in Pinot is due to an earlier block in the pathway that is required for the function of the VvAAT2 enzyme. Alternatively it might be that one of the SNPs in the VvAAT2 coding sequence reduces the activity of the enzyme encoded by the Pinot allele. Expression of the VvAAT2 gene from Pinot in heterologous systems, like petunia, can shed light on this point. Despite the absence of acylation, the berries of Pinot accumulate methylated anthocyanins, like peonidin, petunidin and malvidin, at similar level as in Barbera. This is in agreement with the finding that the grape methyltransferase VvAOMT does not require acylated substrates and, when expressed in petunia, can methylate anthocyanins even in a \textit{gf} background, in contrast to the homologous methyltransferases from petunia (Chapter 3). Together these findings suggest that enzymes involved in the terminal decoration of the anthocyanins have undergone “small” changes in substrate specificity, which are not easily recognized from the protein sequences, with “large” impact on anthocyanins that distinct species can accumulate. That is, given the order of events, petunia cannot make malvidin 3-rutinosides, instead grape can synthesize acylated compounds using methylated or glycosylated anthocyanins as substrates.

Plants possess many genes encoding BADH proteins, with over 70 genes in Arabidopsis and 113 in rice (D'Auria, 2006) that can be divided in at least 5 distinct clades (D'Auria, 2006). Several AATs have been identified to date. Although these enzymes display substantial functional differences, they can acylate the anthocyanin glucoside or rutinoside substrates in different positions and they all belong to clade I, indicating that they all evolved from a common ancestor. The GF protein, however, belongs to clade III and is only distantly related to AATs in clade I. Biochemical work in Salvia identified another member of clade III, Ss5MaT2, which can acylate anthocyanins \textit{in vitro} (Suzuki et al., 2004). \textit{In vitro} Ss5MaT2 conjugates a malonyl group to the manolynated 5-glucoside moiety of an anthocyanin 3 coumaroyl glucoside (“mono demanolyl salvianin”). However, Ss5MaT2 catalyzes this reaction with very low efficiency, suggesting that its major function may be a different
catalysis. The genetic data provide irrefutable evidence that in the clade III the protein PhGF is required for the acylation of the rhamnose moiety of an anthocyanin 3-rutinosides. This function of GF is dispensable under laboratory conditions, but apparently essential in a natural habitat, as the acylation and/or subsequent 5-glucosylation and methylation reactions that depend in this acylation, have a significant impact on the colour of the flower and thereby on the attraction of pollinators.

The PhGF protein is therefore not a case of ‘odd’ convergent evolution, resulting in a suboptimal enzyme, as was suggested for Ss5MaT2 (Suzuki et al., 2004), but rather the acquisition of a new substrate specificity by a group of proteins with common origin. This finding also makes clear that anthocyanin acyltransferases have originated from at least two different ancestors. Analysis of the gape homolog VvAAT2 suggests that also this protein has a role in anthocyanin modification and this implies that anthocyanin acyltransferases from clade III may be widely conserved and acquired role that the attraction of pollinators.

**Materials and methods**

**Plant Material, RNA and DNA extraction and grape anthocyanin analysis**

Roots, shoots, leaves and berries were collected and extracted as reported in the Chapter 3 from grapevine plants (*Vitis vinifera* L.) cv. Barbera, cv. Pinot, cv. Teinturies and cv. Chardonnay.

**DNA and RNA analysis**

DNA and total RNA from petunia and grape tissues were extracted as described in Chapter 3. RT-PCR of petunia mRNA was performed as described in the Chapter 3. For RT-PCR analysis of the DIFc/GF RNA we used the following primers: 4573 PhDIFc_FW CACCATGAATCAAAGTTTGAATGGTTCTT and 4574 PhDIFc_Rv CTATTTTGGCATAAGACTAAACTCCA. Primers used for RT-PCR analysis of ACTIN and GAPDH mRNA are given in Chapter 3.

Expression of grape genes was measured by Real Time PCR. Therefore, 10 µg of total RNA was reverse transcribed in duplicate using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Power SYBR Green (Applied Biosystems) for quantitation of amplification results. The gene-specific primers were for the VvAAT1 forward 5'-GCCCATGTGCTGTGGAAAC-3' and reverse 5'-CTGTCGTCCGCCATGAATCT-3' and for the VvAAT2 forward 5'-TCCCCACCTCAGGCATTTGA-3' and reverse 5'-TGCTTTTGATGGAGCTTGCA-3'. These primers give a fragment of 59 bp for VvAT1 and
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60 bp for VvATT2. Normalization was done based on the expression of VvUbiquitin1 (Bogs et al., 2005) and Vvactin.

Anthocyanin extraction and analysis

Analyses of the anthocyanin content in berries of Barbera, Chardonnay and Pinot was performed by HPLC on 250 mg frozen tissue by HPLC following a previously published protocol (Ferrandino and Guidoni, 2010).

Cloning of VvAAT1, VvAAT2 cDNA

The open reading frames of VvAAT1 and VvAAT2 (spanning from the ATG to the STOP codon) were amplified from cDNA of mature berries (cv. Barbera) with high fidelity Taq Polymerase (AccuPrime™ Taq DNA Polymerase System, Invitrogen) using the forward primer for the VvAAT2 5’-ATGGAGGTCAAAATACTGTCAAAG-3’ and the reverse primer 5’-TCAAGGAGCTCCATTGGA-3’, for the VvAAT1 the forward primer 5’-ATGAAGGTCCAGATGCTGT-3’ and the reverse primer 5’-CTAGACAGAGGAAGTGAAGGC-3’. The amplified cDNA fragments for both VvAATs were cloned into the pGEM-T easy vector (Promega) and the resulting plasmids were sequenced.

Characterization of Recombinant PhDIFc

The cDNA of PhDIFc was cloned into an E. coli expression vector, pQE60 (QIAGEN). Two primers petatF 5’- GAGATACACCATGGATCAAAGTTT-3’ and petatR 5’- CGGGATCTTTTTGGCATAGAAGTTCTT-3’ were used with the pCGP1904 plasmid (containing the PhDIFc cDNA) as template to amplify the PhDIFc full-size coding sequence with an NcoI recognition site at introduced at the initiating AUG and BamHI recognition site just prior to the putative stop codon. The resulting PCR product was a fragment of about 1.2kb. The petatF oligonucleotide used in the PCR contained three bases that differed from the petunia PhAAT sequence. As a consequence the translated amino acids around the putative initiating Methionine were changed from RYIMQ to RYTMDQ. The cloned PCR product was ligated with NcoI/BamHI ends of pQE60 vector (QIAGEN). The new construct was sequenced and transformed in BL21 E.coli strain to produce the recombinant protein as a His-tagged fusion protein.
Constructs for stable transformation

For both transient and stable expression in plants, VvAAT1, VvAAT2 and DIFc/GF were amplified from ATG to STOP codon from the cDNA of Barbera and V30 respectively. We used the pDONR221 Gateway®-adapted vector (Invitrogen) to generate entry clone containing the gene of interest. We used specific primers to generate the PCR products with attB sites and then we generated the entry clone by performing a BP recombination reaction between a pDONOR vector (pDONOR221) and the attB PCR product. For VvAAT1 we used VvAAT1-FW (5' - GGGGACAAAGTTTGTACAAGAAAGCAGGCTcaATGAAGGTCCAGATGCTGT-3') and VvAAT1-RV (5' - GGGGACCAAAGTTTGTACAAGAAAGCAGGCTcAATGAAGGTCCAGATGCTGT-3'), For VvAAT2 we used VvAAT2-FW (5' - GGGGACAAAGTTTGTACAAGAAAGCAGGCTcaATGGAGGTCAAATACTGTCAAAG-3') and VvAAT2-RV (5' - GGGGACAAAGTTTGTACAAGAAAGCAGGCTcAATGGAGGTCAAATACTGTCAAAG-3'), and for PhDIFc/GF we used PhDIFc/GF-FW (5' - GGGGACAAAGTTTGTACAAGAAAGCAGGCTcaATGAATCAGAATGGTTCTT-3') and PhDIFc/GF-RV (5' - GGGGACAAAGTTTGTACAAGAAAGCAGGCTcAATGAATCAGAATGGTTCTT-3'). The VvAAT1, VvAAT2 and PhDIFc/GF entry clones were then recombined into destination vector pK2GW7.0. All constructs were introduced into Agrobacterium tumefaciens strain AGL0 by electroporation. A single colony was used for an overnight culture and used to infect leaf discs (van der Meer, 1999) or to agroinfect petals (Verweij et al., 2008a).
Protein alignment of Petunia DIFc and grape acyltransferases

with others acyltransferases members. Black coloured amino acid are identical, grey coloured amino acid are similar.

**Figure S1:** Protein alignment of Petunia DIFc and grape acyltransferases VaA1 and VaAT2 with others acyltransferases members. Black coloured amino acid are identical, grey coloured amino acid are similar.
References


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