Chapter 3

Genetic control of anthocyanin methylation

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Abstract

Anthocyanins are the most widespread pigments in the plant kingdom that colour flowers and fruits. After the synthesis, simple anthocyanin 3-glucosides can be “decorated” with additional sugar, acyl and methoxy groups to yield the wide variety of anthocyanins found in nature. How this structural diversity arose is unclear, as the enzymes involved in various decorations steps are poorly characterized. Here, we report the identification and characterization of three anthocyanin methyltransferase (AMT) genes from Petunia hybrid. We show that the P. hybrida genome contains at least three AMT genes that are located at the genetically defined loci METHYLATION AT THREE (MT), METHYLATION AT FIVE1 (MF1) and MF2 respectively. Transformation of mt mf1 mf2 mutants with expression constructs for these genes, or with putative AMT gene from grape, restores anthocyanin methylation. These genetic data provide irrefutable evidence that the identified proteins are AMTs, and revealed that these AMTs have, despite the high sequence similarity, substantial differences in substrate specificity which can account for the different substitution patterns of anthocyanins in grape and petunia. Our results suggest that the wide divergence of anthocyanin structures found in distinct plants evolved in part by relatively small alterations in the biosynthetic enzymes with great impact on substrate specificities.

Introduction

Flavonoids are a large and diverse family of secondary metabolites that are found in virtually all vascular plants. Distinct flavonoids have roles in protection against UV light, defense against pathogens, signaling to nodulating bacteria, transport of hormones, pollen germination and the coloration of flowers and fruits. This functional diversity is paralleled by an enormous chemical diversity as today over 9000 flavonoids with distinct structures have been identified (Buer et al., 2010).
In the first steps of the pathway, the core flavonoid skeleton, consisting of three carbon rings, is formed and subsequently the central C ring is reduced to yield precursors of the nine major flavonoid subclasses, which include, for example, flavones, flavonols and anthocyanins, that are synthesized by various side branches of the central pathway. Although the flavonoid pathway is arguably one of the most studied pathways in plants, that holds primarily for the earliest steps in the central pathway, and in the side branch leading to anthocyanins. However, much less is known about the subsequent modifications, such as glucosylation, acylation and methylation, which are responsible for most of the structural variation of flavonoids.

Anthocyanins are orange, red and purple flavonoid pigments that colour the majority of flowers and fruits. Mutants in the anthocyanin pathway have been isolated in many species as they are easy to recognize and do not harm plant viability. Mutants of maize (Zea mays), snapdragon (Antirrhinum majus), and petunia (Petunia hybrida) have been instrumental to identify many of the structural genes of the pathway, as well as transcription regulators that control their expression in distinct tissues.

In petunia several flower colour mutants have been found with defects in the “decoration” of anthocyanin with glucose, rhamnose, methyl and acyl groups. These mutants have been studied by genetic, biochemical and enzymological analysis in the 1980s (Wiering, 1974; Jonsson et al., 1983; Jonsson et al., 1984a), but the genes and proteins encoded by these loci were never identified. In the limb of the flower petals the simple anthocyanin 3-glycosides are subsequently rhamnosylated by a rhamnosyltransferase encoded by RHAMNOSYLATION AT THREE (RT) (Brugliera et al., 1994; Kroon et al., 1994), acylated by an acyltransferase encoded by GLUCOSYLATION AT FIVE (GF) (Chapter 4), glycosylated in the 5 position, and methylated in the 5’ and 3’ position (Figure 1A). The methylation of the 3’ hydroxyl group in the anthocyanin B-ring, to convert cyanidin into peonidin or delphinidin into malvidin, or of both the 3’ and the 5’ hydroxyl groups, converting delphinidin into malvidin, is thought to increase the stability of anthocyanins and to modify their water solubility, thus significantly contributing to the accumulation of coloured molecules in petals or fruits (Forkmann and Heller, 1999; Suzuki et al., 2002).

In Petunia hybrida multiple partially redundant genes control the methylation of anthocyanins in the flower. One locus, designated METHYLATION AT FIVE, directs the methylation of one or both the 3’ and 5’ hydroxyl group, to convert cyanidin into peonidin and delphinidin into malvidin, whereas the METHYLATION AT THREE locus directs methylation of the 3’ hydroxyl group only and converts cyanidin in peonidin or delphinidin into petunidin (Figure 1A). Early genetic studies indicated that the MT and MF loci are polymeric and that P. hybrida genome has two MT and two MF loci, named MT1 and MT2 or MF1 and MF2 respectively. The finding that active MF alleles of different inbred lines
mapped to two distinct chromosomes was taken as evidence for two distinct MF1 and MF2 loci (Wiering and De Vlaming, 1977) but could be explained equally well by a translocation of a single MF gene in some lines. The existence of duplicated MT1 and MT2 loci is highly questionable, since data in support of this duplication were never published and attempts of others to prove its existence in crosses were all negative (Jonsson et al., 1984b). Using molecular methods we also found no evidence for a duplication of the MT2 gene, suggesting that MT1 may indeed not exist. Therefore we refer here to the (single) locus controlling 3’ methylation as MT.

Jonsson and coworkers characterized the anthocyanin-methyltransferase enzymes biochemically and found evidence for at least four isoenzymes in flower extracts that exhibit distinct kinetic properties and appear to be controlled by distinct MT and MF genes (Jonsson et al., 1984a). However, to date the genes encoding these anthocyanin O-methyltransferases from Petunia have not yet been isolated.

In this work, we describe the isolation of MT and MF loci from Petunia hybrida. We show that these genes encode proteins that can methylate anthocyanins in vitro, and share similarity with caffeoyl-CoA methyltransferases of Populus trichocarpa, Vitis vinifera and other plants. We performed a molecular characterization of known mt and mf Petunia mutants, and we complemented these mutants with different transgenes to confirm function of the isolated genes. We also used the same petunia mutants for complementation studies with a methyltransferase from Vitis vinifera (AOMT) that we described in a previous study (Hugueney et al., 2009) (see Chapter 2), confirming the activity of this grape gene in vivo. This last experiment shows that petunia can be used as a model plant to investigate the role of genes from different species, in which the production of transgenics is a long and labor intensive process.

**Results**

**Identification of a putative anthocyanin methyltransferase gene from Petunia hybrida**

We previously obtained, by differential screens of a petal cDNA library, several cDNA clones from petunia petals whose expression was abolished by mutations in the regulatory gene AN1 (Kroon et al., 1994; Spelt et al., 2000). These cDNAs represent seven distinct genes, designated DIFa, DIFc, DIFE1, DIFf, DIFg, DIFh and DIFi, that are transcriptionally controlled by AN1, AN2, and AN11 (Kroon et al., 1994).

Further analysis showed that DIFf encodes a cytochrome b5 required for the activity of the cytochrome P450 enzyme flavonoid 3’,5’ hydroxylase (F3’5’H) (de Vetten et al., 1999), that DIFa encodes anthocyanin synthase (AS), that DIFg originates from the
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**RHAMNOSYLATION AT THREE (RT) locus and encodes a rhamnosyl-transferase** (Kroon et al., 1994), and that *Dif* originates from the AN9 locus and encodes a glutathione S-transferase (Alfenito et al., 1998).

Several initial observations made us suspect that *Dife1* encodes an anthocyanin methyltransferase. First, expression of mRNAs hybridizing to *Dife1* is strongly down-regulated by mutations in genes *ANTHOCYANIN 1 (AN1), AN2 and AN11* (Quattrocchio et al., 1993), as well as in lines with mutations in all *MT* and *MF* genes (Fig 1B). Second, RFLP analysis showed that *Dife1* gene is linked to the HF2 locus on chromosome V (unpublished data), as are the genes *MT2* and *MF2* (Wiering, 1974).

**Figure 1.** Role of genetic loci and enzymes required for methylation of anthocyanins. 
(A) The diagram depicts the last steps involved in the modification of simple anthocyanin 3 glucosides by subsequent rhamnosylation, 5-glucosylation, acylation and methylation. Enzymes involved in each reaction are indicated on the right of the arrows and genetic loci controlling the reaction on the left. 
(B) RNA gel blot analysis of *Dife1* expression in petals of four inbred petunia lines (R4, R43, R46 and R63), that are homozygous for functional (+) or mutant alleles (-) of *MT, MF1* and *MF2*. 

Abbreviations: 5GT, Anthocyanin 5-glucosyltransferase.; AAT, Anthocyanin acyl transferase.; A3'MT, Anthocyanins 3' methyltransferase.; A3', 5'MT, Anthocyanins 3', 5' methyltransferase; RT, RHAMNOSYLATION AT THREE; GF, GLUCOSYLATION AT FIVE; MT, METHYLATION AT THREE; MF, METHYLATION AT FIVE.
Plant O-methyltransferases constitute a diverse family that consists of two major clades, A and B, which diverged into 2 and 3 subgroups respectively (Lam et al., 2007). Figure 2 shows that DIFe1 belongs to the A1 subfamily, which contains mostly proteins that were annotated as caffeoyl-CoA O-methyltransferases (CCoA-OMTs) (Lam et al., 2007), and a recently identified protein from grape (VvAOMT) that can methylate anthocyanins in vitro (Hugueney et al., 2009). Other transferases implicated in the methylation of flavonoids, including 3’ and/or 5’ methyltransferases, like the flavonol O-methyltransferase of Catharantus, and a 3’ methyltransferase from Mentha for example, belong to the B1 and B2 groups (Lam et al., 2007) and thus are only distantly related to DIFe1.

**Recombinant DIFe1 protein can methylate anthocyanins in vitro**

To assess whether the petunia DIFe1 cDNA encodes a functional anthocyanin methyltransferase, we expressed DIFe1 as a His-tagged fusion protein in E. coli. Crude homogenates from DIFe1 expressing cells or cells containing the empty vector were assayed for methyltransferase activity using ^14C-S-adenosyl methionine (SAM) and either delphinidin 3-glucoside (D3G) or delphinidin 3-rutinoside (D3R) as substrates under assay conditions previously described (Jonsson et al., 1983). Crude homogenates from DIFe1 expressing cells catalyzed the conversion of both D3R and D3G into the corresponding methylated products, petunidin and malvidin, as judged by Thin Layer Chromatography (TLC), while no detectable production of petunidin and malvidin was seen in assays with homogenates from untransformed cells, if no crude homogenates were added at all or when ^14C-SAM was omitted (Table 1). This indicates that DIFe1 can indeed methylate anthocyanins.

**Table 1: Methyltransferase activity in E. coli cells expressing DIFe1**

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1^EV, empty vector

2^D3R, delphinidin 3-rutinoside; D3G, delphinidin 3-glucoside
To examine the reaction(s) catalyzed by DIFe1 in more detail, we used a modified assay in which we quantitatively measured the products produced by DIFe1 using HPLC (Fig 3A). In these assays DIFe1 converted the D3R, D3G and D3, 5G (delphinidin 3, 5-diglucoside) with roughly equal efficiency. In all cases the major products were petunidin derivatives, while malvidin derivatives constituted a much smaller but nevertheless substantial fraction. The D3R and in particular D3G substrates were not completely pure and
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... contained low amounts of cyanidin derivatives, which were converted by DIFe1 into peonidin derivatives.

Together these data show that recombinant DIFe1 can methylate the glucosides, rutinosides and the 3', 5' diglucosides of both delphinidin and cyanidin in vitro and that it has a predominant A3'MT activity and a lower, but still significant, A3'5'MT activity.

Silencing of DIFe1 gene expression inhibits methylation

To establish whether DIFe1 is involved in the methylation of anthocyanins in vivo, we generated transgenic plants in which DIFe1 was inactivated by RNA interference (RNAi). Therefore, the DIFe1 cDNA was cloned in antisense orientation behind a Mac promoter, a derivative of the 35S promoter of Cauliflower Mosaic Virus (Comai et al., 1990). The resulting construct MAC:asDIFe1 was introduced into the V23xR51 hybrid (“VR”), which has purple flowers and is dominant for all known anthocyanin biosynthetic genes.

In one out of the five MAC:asDIFe1 transformants the flower colour changed from bright purple to a more dull reddish colour (Figure 3B). HPLC analysis showed that this colour change was associated with a substantial alteration of the anthocyanin composition (Figure 3C). In VR flowers 80% of the anthocyanins are malvidin derivatives, whereas in the Mac:asDIFe1 transformant with an altered flower colour (transformant #A) the main anthocyanins were delphinidin derivatives, showing that DIFe1 is required for anthocyanin methylation in vivo.

Figure 3. Methylation of anthocyanins by DIFe1 and DIFe2 in vitro and in vivo.

(A) Graph showing conversion of delphinidin (Del) into petunidin (Pet) and malvidin (Mal) and cyanidin (Cya) into peonidin (Peo) by cell free E. coli extracts expressing DIFe1 protein and, as a controls, extracts from cells containing the empty vector of no extract at all (none).

(B) Phenotypes of a flower from a control plant and MAC:asDIFe1 transformant #A.

(C) Graph showing the composition of anthocyanin aglycones in flowers of distinct MAC:asDIFe1 and MAC:asDIFe2 lines and a control plant without transgene.
**DiFe1 is part of a small gene family**

When we screened a cDNA library made from petals of *P. hybrida* cv. Old Glory Blue (OGB) with a *DiFe1* probe, we obtained two clones (E20 and E33) that originated from distinct genes.

The sequence of E20 was identical to that of the original *DiFe1* cDNA from V26, except that its 5’ untranslated region (5’UTR) was 25 nucleotides (nt.) longer, while the 3’UTR region was 96 nt. shorter. The latter might be due to either polyadenylation of the *DiFe1* mRNA at distinct sites, or promiscuous priming of oligo-dT on an A-rich region in the 3’UTR during synthesis of the OGB clone.

The second OGB cDNA (clone E33) is 1076 in length and shares 82% nucleotide identity over 797 bp with *DiFe1*, indicating that it originated from a paralogous gene, which we named *DiFe2*. The E33 clone contained a 2-bp deletion at position 469 that disrupted the reading frame, suggesting that the OGB cultivar contains a mutant *dife2*OGB allele. For further analysis of the *DiFe2* sequence we corrected the sequence of this *dife2*OGB allele in silico by inserting a CT dinucleotide. This “corrected *DiFe2*” proved similar to functional *DiFe2* alleles that we later isolated from other accessions (see below). The deduced protein encoded by the *DiFe2*-corrected clone shares 82% identity with *DiFe1* over a 243 amino acid overlap (Figure 4A), and clusters with *DiFe1* in a clade that is distinct from other methyltransferases of the A1 subfamily, indicating that *DiFe1* and *DiFe2* arose by a relative recent gene duplication (Figure 4B).

To assess the role of *DiFe2* in vivo, we generated transgenic VR plants containing a *MAC:asDiFe2* transgene that expresses the antisense RNA. One of five *MAC:asDiFe2* transformants displayed a flower colour phenotype that was associated with a strong reduction in anthocyanin methylation, similar to the phenotype of *MAC:asDiFe1* (Figure 3B). Given the high similarity of the *DiFe1* and *DiFe2* sequences, and of their RNAi phenotypes, it is likely that the RNAi constructs silenced the expression of both genes (and even a third gene, see below) simultaneously.

Because genetic and biochemical data indicated that petunia contains 3 or 4 *Mt/MF* genes and can express at least 4 anthocyanin methyltransferase isoenzymes, we wondered whether the petunia genome contains additional paralogs of *DiFe1* and *DiFe2*. Therefore, we subjected genomic DNA of the lines V30 (*MT mf1 mf2*), R78 (*mt mf1 mf2*) and M1 (*MT MF1 MF2*), to DNA gel-blot analysis. The *DiFe1* cDNA probe detected after high stringency washing single EcoRI and BamHI fragments in each line (Figure 5 right panel). However, when we probed the blot with the *DiFe2* cDNA we detected after low-stringency washing (2x SSC 25°C) three EcoRI fragments and at least two BamHI fragments in each of these lines (Figure 5, left panel). One of these EcoRI fragments contains *DiFe1*, because (i) it was eliminated by high stringency washing, and because (ii) it matches exactly in size with the
fragments containing DIFe1. The two remaining fragments EcoRI fragment contain two distinct genes with high similarity to DIFe2, because the DIFe2 alleles from V30 and M1 are not cut by EcoRI, and because both EcoRI fragments hybridize to DIFe2 with equal efficiency under high stringency conditions.

When we amplified genomic DNA of the P. hybrida lines V30 and M1 or from the parental species P. inflata S6, we found that most primers complementary to the DIFe2OGB cDNA amplified two distinct genes with exon sequences that are nearly identical, except for a few single nucleotide polymorphisms (SNPs). Because this low number of SNPs is similar to that observed between alleles from different P. hybrida accessions, we could not distinguish which of these two genes is allelic to the DIFe2OGB cDNA and therefore designated them DIFe2a and DIFe2b. DIFe2a and DIFe2b are not alleles of one locus, but are paralogous genes, because (i) their introns differ extensively in size and sequence, except for some short conserved motifs near the splice sites and (ii) because we can amplify both genes from a variety of lines that were inbred for > 30 generations.

**Expression pattern of DIFe genes**

To resolve the pattern and genetic regulation of DIFe1, DIFe2a and DIFe2b expression, we analyzed the abundance of their mRNAs by quantitative RT-PCR and Real Time-PCR in pigmented tissues that accumulate anthocyanins (limb and tube of petal, anthers) and various unpigmented tissues (Figure 6A). All three DIFe genes are active in a roughly similar pattern, as they are all primarily expressed in the limb and tube of the petal and not, or much less, in anthers or unpigmented tissues. However, there are some quantitative differences in the expression of DIFe1, as compared to DIFe2a and DIFe2b that are noteworthy. First, the expression of DIFe1 is almost constant during flower development and persists in the open flowers (stages 5 and 6), whereas DIFe2a and DIFe2b mRNAs peak in developing flower buds (stage 1-4) and rapidly decline after opening of the flower, similar to the expression of other anthocyanin genes such as DFR and CHS (Quattrocchio et al., 1993). Second, expression of DIFe2a and DIFe2b in the petal limb is several-fold higher than that in the tube, while DIFe1 is expressed at similar levels in both tissues. Third, DIFe1 displays a low background expression in a wide range of tissues, whereas DIFe2a and DIFe2b are expressed in ovaries at comparable levels as in the petal tube but display little or no activity in other tissues. Although ovaries do not accumulate anthocyanins, they do express structural and regulatory anthocyanins genes, for reasons that are unknown (Huists et al., 1994; Spelt et al., 2000).
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Figure 5. DNA gel blot analysis of DIFe1 and DIFe2 genes and paralogs in three P. hybrida lines M1, V30 and R78. Genomic DNA of lines M1, V30 and R78 was digested with EcoRI (E) or BamHI (B), size separated and hybridized to the DIFe2 cDNA from OGB and first washed at low stringency (2x SSX, 25°C; left panel), and subsequently at high stringency (0.1x SSC 68°C: middle panel), or hybridized with the DIFe1 cDNA and washed at low (not shown) and high stringency (right panel). Fragments hybridizing to DIFe1 and DIFe2 at high stringency are marked with white and black arrowheads respectively. The open arrowhead indicates a fragment that is seen only with the DIFe2 probe under low stringency conditions.

Figure 4. Comparison of methyltransferases encoded by DIFe1 (MT), DIFe2a (MF1) and DIFe2b (MF2) from P. hybrida.
(A) Comparison of methyltransferases encoded by DIFe1 (MT), DIFe2a (MF1) and DIFe2b (MF2) genes of petunia with caffeoyl-CoA methyltransferases from Vitis vinifera (VvOMT1), Nicotiana tabacum (NtCCoAOMT), Populus trichocarpa (PtCCoAOMT), Mesembryanthemum crystallinum (McPFOMT), Vitis vinifera (VvCCoAOMT), Zinnia elegans (ZeCCoAOMT), Stellaria longipes (SlCCoAMT), Medicago sativa (MsCCoAMT) and Petroselinum crispum (PcCCoAMT). Amino acids that are identical or similar to those in DIFe1 (MT) are marked by black and grey shading respectively. Residues of MsCCoAOMT that are involved in SAM and/or SAH binding or recognition of the Caffeoyl or CoA moiety of the substrate (Ferrer et al., 2005) are indicated above the sequences.
(B) Phylogenetic tree of the caffeoyl CoA subfamily A1.
To determine whether \textit{DIFe1}, \textit{DIFe2a} and \textit{DIFe2b} are encoded or regulated by the \textit{MT} or \textit{MF} loci, we analyzed their expression in stage 3-4 petal limbs from \textit{P. hybrida} lines and F1 hybrids with different genotypes (Figure 6B and 6C). In general expression of \textit{DIFe} genes seemed reduced in \textit{mt}, \textit{mf1} or \textit{mf2} mutants, but it was hard to link the observed variation in \textit{DIFe} gene expression to \textit{mt}, \textit{mf1} and/or \textit{mf2} mutations with certainty, in part because of the divergent genetic backgrounds of the lines used. \textit{DIFe1} mRNA expression is strongly reduced in all accessions containing a mutant \textit{mt} allele, while \textit{DIFe1} expression seems hardly or not at all affected by \textit{mf1} and/or \textit{mf2} mutations seem to have no clear consistent effect. In V30 (\textit{mf1} \textit{mf2}) expression of \textit{DIFe2a} was strongly reduced, suggesting that \textit{DIFe2a} may be encoded/regulated by \textit{MF1} or \textit{MF2}. In V23 petals all three \textit{DIFe} genes seem normally expressed, and expression of \textit{DIFe2b} seems even somewhat enhanced, indicating that the \textit{mf2^{V32}} mutation does not eliminate/reduce the mRNA levels of any of the \textit{DIFe} genes.

Next we analyzed whether the expression of \textit{DIFe} genes requires the regulatory anthocyanin genes \textit{AN1}, \textit{AN2} and \textit{AN11}. As expected all three \textit{DIFe1} genes are expressed in petals of the line R27 (\textit{AN1}, \textit{AN2}, \textit{AN11}), while in petals of isogenic \textit{an1} and \textit{an11} mutants their expression is strongly down-regulated, to a similar extent as \textit{DFR} (Figure 6D). Since no \textit{an2} mutants are available in the R27 background, we used a mutant line with an unstable \textit{an2} allele harbouring a transposon insertion and a derived germinal revertant (\textit{AN2^{REV}}) and found that in this isogenic W242 background the \textit{an2} mutation also reduced expression of the three \textit{DIFe} genes, to a similar extent as \textit{DFR}.

In summary these data show that both \textit{DIFe} genes are co-expressed in time and place and co-regulated by the \textit{AN1-AN2-AN11} complex with other anthocyanin genes like \textit{DFR}, and may be encoded by one \textit{MT1}, \textit{MF1} or \textit{MF2}. 

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Figure 6. Pattern and regulation of expression of DIFe genes.
(A) RT-PCR analysis of MT, MF1 and MF2 mRNAs in different tissues of P. hybrida M1xV30. Floral tissues were from buds of three different stages: Stage 1/2, buds up to 20 mm; stage 3-4, bud 30-40 cm; stage 5-6, open flowers.
(B) RT-PCR analysis of MT, MF1 and MF2 mRNAs in genotypes with mt, mf1 and/or mf2 alleles.
(C) Q-PCR analysis of MT, MF1 and MF2 mRNAs in genotypes with mt, mf1 and/or mf2 alleles. Primers used amplified both MF1 and MF2.
(D) Q-PCR analysis of mRNAs expressed in stage 3-4 petals of regulatory mutants. Lines R27 (AN1 AN2 AN11), W225 (an1) and W134 (an11) are isogenic. Line W242 is in a distinct genetic background and harbours a mutable an2 allele (an2mut). As an isogenic wild type we used a germinal AN2REV revertant in which the transposon had excised from AN2.
mt and mf petunia lines contain mutations in the DIFE1 and DIFE2 genes respectively

To find out whether the DIFE genes are located at MT or MF loci we analyzed whether mt and mf mutants contain defective DIFE1, DIFE2a and DIFE2b alleles. PCR and sequence analysis revealed that the lines M29, and R78 contain a mutant dife1 allele that is disrupted by a ~ 2.4 kb insertion in the fifth exon (Figure 7), while that insertion was absent from the DIFE1 alleles of the lines M1 (MT, MF1, MF2) and V30 (MT, mf1, mf2) and P. inflata (line S6), which is one of the parental species from which P. hybrida originates. This insertion seems to consist of a tandem repeat of a novel 1210 bp transposon, designated dTPH11 that contains 125-bp imperfect terminal inverted repeats (TIRs) with 9 mismatches and is flanked by a target site duplication of 9 bp. Given that we found this double dTPH11 insertion in several P. hybrida lines and in one of the parental species from which P. hybrida derived (see below), we assume that these dTPH11 copies are immobile, either because of mutations in the elements themselves (e.g. one of the 9 mismatches in the TIRs) or because a corresponding transposase source is missing in most lines. We completely sequenced the DIFE1 allele from V30 (MT, mf1, mf2) and found only some SNPs, when compared to the alleles in the MT MF1 MF2 lines M1 and S6, but no clear defects, such as an insertions, deletions, or premature stop codons, that would indicate that DIFE1<sup>30</sup> is not functional. Hence we conclude that DIFE1 is located at the MT locus, not at MF1 or MF2.

Furthermore we found that the mt1 mt2 mf1 mf2 lines M29, R63, R78 and V32 all contain a 13-bp deletion in the fifth exon of DIFE2b that shifts in the reading frame for the last 36 nucleotides. Since the same mutation is found in V30 (MT, mf1, mf2) this rules out that DIFE2b originates from MT and instead suggests that DIFE2b corresponds to one of the two MF loci. Line V23 (MT, MF1, mf2) contains a distinct 2-bp deletion in exon 4, the same as was found in the cDNA clone E30 from the cultivar OGB, while the DIFE2b allele of the line R100 (MT, mf1, MF2) did not contain clear defects that would inactivate the gene. Hence, we concluded that DIFE2b derives from the MF2 locus. Finally, we found that the DIFE2a allele of lines V30 (MT, mf1, mf2), R78 (mt, mf1, mf2) and R100 (MT mf1, MF2) contained two distinct deletions that removed either the major part of exon 5 or part of exon 3 and intron 3, indicating that DIFE2a is at the MF1 locus.

In summary these findings show that the genes DIFE1, DIFE2a and DIFE2b genes are identical to MT, MF2 and MF1 respectively, and from here on we will refer to these gene as such.
Figure 7. Structure of wild type and mutant alleles of MT (DiFe1), MF1 (DiFe2a) and MF2 (DiFe2b).
Exons and introns are drawn at reduced height. Lesions found in mutants alleles of specific lines are indicated below the gene maps. The triangle below the MT map indicates the insertion of two dTPH11 copies, each terminated by a left and right terminal inverted repeat (LIR and RIR); the flanking MT sequence is shown below, with the target site duplication in bold italic type. Deletions in mf1 and mf2 alleles are indicated by bars below the MF2 map and by the actual sequences below that MF1 map.
Mutant mt and mf alleles originate from P. axillaris, the white flowering parent of P. hybrida

P. hybrida originates from crosses in the early 1800s between various accession and/or subspecies of P. axillaris and P. inflata. P inflata subspecies have bee-pollinated flowers that contain methylated anthocyanins (malvidin), while P. axillaris has white flowers that are pollinated by moths. Interestingly, all accessions of P. axillaris and subspecies that were tested lack functional MT and MF alleles, as they all fail to complement P. hybrida mt or mf mutants in crosses. To examine whether P axillaris harbors the same dTHP11 insertion allele as the P. hybrida lines described above, we amplified genomic DNA from two P. axillaris accessions (lines S1 and S26) and, as a control, from the P. inflata line S6 (MT MF1 MF2), with one primer complementary to dTHP11 and one primer complementary to DiFe1 (Figure 8A). The P. axillaris lines S1 and S26 yielded PCR products with a size that was expected for a dTHP11 insertion in intron 4, whereas P inflata S6 yielded no products at all, as expected. This indicates that the dTHP11 insertion allele that gave rise to the mutant alleles of P. hybrida, arose in nature in the P. axillaris ancestor and was subsequently introgressed in P. hybrida.

To analyze the mf1 and mf2 alleles of P. axillaris, we amplified genomic DNA with some 15 different combinations of primers, including some that are specific for either the MF1 or MF2 gene of various P. hybrida lines. Depending on the primers used, this yielded either a single product, which in all cases produced a single sequence confirming that it was homogeneous, or no product at all (not shown). As all PCR products derived from the same gene, it appears that P. axillaris has only a single MF gene. Even more surprising, we found that this single gene contained two distinct deletions: exon 3 contained the same 2-bp deletion that was found in the P. hybrida alleles mf1V23 and mf1OGB, and exon 5 contained the same 13-bp deletion found in the alleles mf2V30 and mf2R78. The complete similarity of these mutations strongly suggests that the mf1 and mf2 alleles of P. hybrida arose in a natural habitat in P. axillaris. However, it remains at this stage mysterious how the same two deletions found in a single P. axillaris gene ended up in two separate genes in P. hybrida.
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Figure 8. PCR analysis of the mt1 allele in two accessions of Petunia axillaris.

(A) Map of the MT gene. Exons and introns are indicated by rectangles and lines respectively; the tandem insertion of dTPH11 found in Petunia hybrida mt alleles is indicated by the triangle. The arrowheads, connected by a dotted line, indicate positions of three pairs of primers used for PCR analysis.

(B) PCR products amplified from the Petunia axillaris lines S1 and S26, the Petunia inflata line S6, and Petunia hybrida V30 after amplification with primer pairs a, b and c.

Complementation of Petunia hybrida mt and mf mutants by 35S:MT and 35S:MF

Since the wild type progenitors of mt and mf mutants are not available, the biochemical characterization of these mutants relied on comparison with MT or MF lines in unrelated genetic background (Wiering and De Vlaming, 1977; Jonsson et al., 1983; Jonsson et al., 1984a), which in petunia can cause significant differences in, for example, gene expression levels, plant size, anthocyanin composition etc. To ascertain that the observed mutations in DIFe1, DIFe2a and DIFe2b, indeed caused the methylation defect in mt and mf lines, and to establish isogenic mutants and “wild-types” we produced two different constructs, 35S:PhMT:NOS and 35S:PhMF:NOS, in which the 3SS promoter drives the expression of the open reading frames from DIFe1 and DIFe2b respectively, and introduced them into mt mf1 mf2 mutants.

Because only few inbred lines are transformable we used the F1 hybrid V32xR78 as a host. The major anthocyanins in R78xV32 petals are delphinidin–derivates, because this hybrid is homozygous recessive for all methylation loci (mt, mf1, mf2), but contains a functional GF gene, which is required for anthocyanin acylation and 5-glucosylation (Figure 1A; see also Chapter 4) and HF1 and HF2 loci, which are required for 3’ and 5’ hydroxylation. Line R78 is exceptional in that it proved to be transformable and was used as
an alternative host. R78 is homozygous recessive for all methylation genes, as well as for gf, hf1 and hf2 and, therefore, accumulates primarily cyanidins in the petal.

RT-PCR analysis of the mRNAs in petals revealed that thirteen of the twenty \(35S:MT\) and five out of six \(35S:MF2\) transformants in the V32xR78 background expressed the transgene to similar levels as in wild type (Figure 9A), which resulted in a slightly more reddish colour of the petals (Figure 9B). In the R78 background five out of eight \(35S:MT\) and ten out of twelve \(35S:MF2\) transformants expressed the transgene to similar levels as in wild type (Figure 9A). These transformants exhibited at best a very mild change in flower colour that was only evident in side-side comparisons of flowers and difficult to fully reproduce by photography (Fig 9B and C).

We used HPLC and LC-MS to analyze the anthocyanin composition in petals from transgenic and control plants. Therefore, we extracted anthocyanins using either methanolic extracts, which recovers the intact anthocyanins, or by hydrolysis in 2 M HCl, which removes sugar-acyl groups and simplifies the analysis of the anthocyanidin aglycones. Figure 9D shows that the main anthocyanins in control V32xR78 plants are delphinidin derivatives, while in plants expressing \(35S:MT\) most delphinidins are converted into petunidin derivatives, while a small fraction is converted to malvidins. Hence the MT protein has strong A3’MT activity and a much lower, but still well detectable, A3’5’MT activity. Expression of \(35S:MF2\) also converted the delphinidins into petunidins and malvidins, but the malvidin/petunidin ratio was much higher than in the \(35S:MT\) flowers. This indicates that \(MF2\) has both A3’MT and A3’5’MT activity.

Although \(35S:MT\) and \(35S:MF2\) were expressed in R78 to similar levels as in the V32xR78 background, this increased anthocyanin methylation only modestly, because only a small fraction of the cyanidins was converted into peonidins. LC-MS analysis showed that most of the cyanidins and peonidins in transgenic and control R78 plants accumulated as rutinosides and lacked the acyl and 5-glucosyl moiety, because of the gf mutation. This suggests that anthocyanins lacking either the acyl and/or 5-glucosyl groups are poor substrates for MT and MF2, which is in line with previous genetic data.
Figure 9. Complementation of mf and mt mutants by 35S:PMF and 35S:PMT.
(A) RT-PCR analysis of transgene expression in distinct transformants.
(B) Flowers of an untransformed control R78xV32 plant (mt mf1 mf2 GF HF1 HF2) and transgenic siblings that express 35S:MT, 35S:MF2 or 35S:VvAOMT.
(C) Flowers of an untransformed control R78 plant (mt mf1 mf2 gf hf1 hf2) and transgenic siblings that express 35S:MT, 35S:MF2 or 35S:VvAOMT.
(D) Composition of anthocyanin aglycones in petals from distinct transgenic and control plants, as determined by LC-MS.
Complementation of Petunia hybrida anthocyanin methyltransferase mutants by the Vitis vinifera VvAOMT

As above reported we have characterized a series of Petunia hybrida anthocyanin methyltransferase mutant lines and shown that these can be complemented by the expression of Petunia anthocyanin methyltransferase genes. To test whether these mutants provide a useful tool to characterize genes controlling a similar reaction in other species, we assessed whether a previously identified methyltransferase from Vitis vinifera (VvAOMT) (Hugueney et al., 2009) would complement these Petunia mt and mf mutants. The function of the VvAOMT as anthocyanin methyltransferase was previously demonstrated by in vitro experiments and in transiently transformed Nicotiana bentamiana leaves (Hugueney et al., 2009). To better assess the capacity of VvAOMT to methylate anthocyanins in vivo and compare its activity to that of the petunia methyltransferases, we expressed VvAOMT from the 35S promoter in the Petunia mt mf1 mf2 line R78 and the hybrid V32xR78. Among the six transgenic 35S:VvAOMT plants in the V32xR78 background, three exhibited a more bluish corolla when compared to untransformed plants (Figure 9A and 9B), and contained high amounts of malvidins and petunidins as judged by TLC analysis of the hydrolyzed anthocyanin aglycones (not shown). More detailed LC-MS analysis showed that in the V32xR78 background 35S:VvAOMT directed the conversion of delphinidins into roughly equal amounts of petunidins and malvidins, demonstrating that VvAOMT has both A3'MT and A3'S'MT activity in vivo (Figure 9D).

In R78 background 35S:VvAOMT induced the conversion of cyanidin-3-rutinoside, into peonidin-rutinosides, a pigment that is not usually found in petunia, and causes a mild change in the flower colour. RT-PCR confirmed that the transformants with the strongest phenotype expressed the transgene at the highest level (see Figure 9A and C).

Transient expression assay in petunia petals: a new tool to test the anthocyanin methyltransferase activity of transgenes.

To test whether petunia flower petals can be a convenient tool for rapid screening of the activity of genes possibly involved in anthocyanin modification, we used the same 35S:PhMT, 35S:PhMF and 35S:VvAOMT genes described above to transiently transform petals from the mt mf line R78 by agro-infiltration (Verweij et al., 2008a).

The petals were agroinfected on the plant and kept at 24°C. After 48 hours, we extracted anthocyanins for analysis by TLC. With this technique, we detected an extra product similar to that observed in flowers of the transgenic 35S:VvAOMT plants. Because by this method, generally, small portions of the petal are transformed, the relative amount of the methylated product is, however, lower than in permanently transformed petals (see Figure 10).
Further analysis of the products recovered from the transiently transformed petals is on its way, but we think that these results are a good indication that petunia petal agroinfiltration can be a useful approach for a fast screening of genes with a candidate function in the flavonol pathway.

Figure 10. TLC analysis of anthocyanins accumulating in mf, mt mutant flowers (V32xR78) after infiltration with the 35S:VvAOMT. The activity of VvAOMT gene involved in anthocyanins methylation is indicated as an orange spot (petunidin) in the lane #3. The extracts of mutant flowers used as controls are spotted in lane #1 and lane #5.

Discussion

The last steps of the biosynthesis of anthocyanins involve modification of the pigment molecules that include glycosylation, acylation and methylation. These ‘decorations’ are responsible for much of the chemical variation between anthocyanins of different species, but yet, represent the least understood part of anthocyanin synthesis. Here we report the identification and characterizations on anthocyanin methyltransferases from petunia and grape.

Using a reverse genetic approach, we identified and molecularly characterized the MT, MF1 and MF2 loci from Petunia hybrida, which had been previously identified in genetic studies. The finding that (i) the mt, mf1 and mf2 mutants contain deletions and insertions in the identified methyltransferase genes, (ii) that the mutants can be complemented by ectopic expression of the methyltransferase genes in transgenic plants and (3) that silencing of the
methyltransferase genes by RNAi phenocopies the mt mf1 mf2 mutants prove that the identified methyltransferases are encoded by MT, MF1 and MF2.

In various reviews Wiering and de Vlaming indicated that petunia may have two MT and two MF loci (Wiering, 1974; de Vlaming et al., 1984; Wiering and De Vlaming, 1984). The existence of two MF loci was inferred from the segregation ratios of dominant and recessive phenotypes in certain crosses, and linkage to markers on two different chromosomes (Wiering and De Vlaming, 1977). However, we were unable to trace the data (published or unpublished) that suggested the existence of two MT loci. Using the genetic stocks developed by Wiering and co-workers, Jonsson et al (1984) found no evidence for the existence of the MT1 locus, although they could not rule out that MT1 was genetically tightly linked to MF1. Our molecular data show that P. hybrida lines M1 and V30 contain (no more than) 2 MF genes, and only 1 MT gene. It is, however, possible that copy numbers of MT genes do vary between Petunia accessions, in particular when closely related paralogs are concerned. For example, within the CHALCONE SYNTHASE (CHS) gene family, several specific CHS genes got duplicated one or more times in only one of the parents of P. hybrida, either in P. axillaris or P. inflata, resulting subfamilies of closely related paralogs of a variable size in P. hybrida varieties (Koes et al., 1987).

Given the very high similarity of the MF1 and MF2 genes it is possible that the duplication that gave rise to these two P. hybrida paralogs took place in the P. inflata parent only, explaining why P. axillaris contains only 1 (inactive) MF gene. Alternatively the gene duplication may have occurred in the common ancestor of P. inflata and P. axillaris, followed by mutation of one paralog and complete deletion of the other in P. axillaris.

Plant O-methyltransferases constitute a large family of proteins that can methylate a range of low molecular weight metabolites, including phenylpropanoids, flavonoids and alkaloids (Lam et al., 2007). Given the large number of potential substrates, it is not trivial to identify the substrates of a given methyltransferase protein. In the majority of cases the substrates are inferred by testing various (potential) substrates in a trial and error approach using in vitro assays with either purified or recombinant proteins. Alternatively the substrate specificity is inferred from sequence similarity to O-methyltransferases for which in vitro data are available. Our results show, however, that such data should be interpreted with caution. For example, the MT and MF enzymes exhibit high similarity to proteins that are annotated as caffeoyl-CoA O-MTs or hydroxy-cinnamic acid CoA OMTs, but apparently cannot methylate these substrates, as petunia flowers only express activities that can methylate anthocyanins, but not cinnamic acids, and because mutant flowers do not contain any ferulic or sinapic acids (Jonsson et al., 1982). Furthermore, although recombinant DIFe1 protein could methylate cyanidin 3-glucoside, delphinidin 3-glucoside and delphinidin 3, 5-diglucosides in vitro, these compounds prove poor substrates in vivo, as the anthocyanin 3-
glycosides or 3-rutinosides that accumulate in rt and gf mutants respectively cannot be efficiently methylated by MT, MF1 or MF2 in vivo. Even when $K_m$ values for potential substrates are determined, it remains difficult to infer the in vivo function from in vitro data, as the concentrations of the substrate in vivo (in the cytoplasm!) are usually unknown and difficult to determine.

The in vivo data show that the MT and MF enzymes of petunia and VvAOMT from grape display, despite the high sequence similarity, significant differences in substrate specificities. MF and VvAOMT have a strong A3'5'MT activity, indicating that they can accept both 3'-hydroxy and 3'-methoxy anthocyanin derivatives as a substrate, while MT has at best a weak A3'5'MT activity indicating that 3'-methoxy derivates are a poor substrate. Furthermore, VvAOMT differs from MT and MF in that it can efficiently methylate the anthocyanin-rutinosides that accumulate in a gf background, whereas MT and MF cannot. This explains why the grape cultivar Pinot can accumulate anthocyanin-glucoside that are methylated, but not acylated (Chapter 4). The finding that VvAOMT can also methylate anthocyanins in a GF background, however, cannot be taken as evidence that VvAOMT can also methylate acylated and/or 5-glucosylated anthocyanins, because it cannot be excluded that in these transgenic plants, which accumulate anthocyanins derivatives that are not normally seen in P. hybrida, methylation takes place before acylation and glucosylation.

This result, anyway, provides in vivo evidence for the function of Vitis gene, and suggests that the use of petunia as a model plant to investigate the function of heterologous anthocyanin genes is a powerful approach, especially for species like grape, for which no mutants are available and transformation is not a routine procedure. Since petunia flowers are easy to transiently transform by agro-infection (Verweij et al., 2008a), this can be used as a fast method to screen candidate genes and avoid the time consuming procedure to generate stable transformants.

As described by Ibrahim et al, O methyltransferases share about 32-71% of sequence identity and they can be grouped on the basis of their substrate specificity (Ibrahim et al., 1998). The sequences of anthocyanin methyltransferases, as well as caffeoyl CoA methyltransferases, display high similarity throughout the protein, and residues/motifs that are involved in dimerization and binding of the SAM substrate are fully conserved. However, the region between residues 200-215 stands out as being highly variable. In the CCoA-MT of Alfalfa (Medicago sativa) this region forms one side of the pocket that holds the phenolic moiety of the caffeoyl-CoA substrate (Ferrer et al., 2005), and is well conserved in various CCoA-OMTs, but diverged extensively in MT, MF and VvAOMT. Hence, the alterations in this region are likely to be elementary for functional divergence of anthocyanin- and caffeoyl-CoA methyltransferases. The residues 50-64 constitute a second region where CCoMs diverge from the anthocyanin MTs. In MsCCoA-MT this domain is close to, but not
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in direct contact with either the phenolic or CoA moiety of the substrate, and may therefore affect substrate specificity in a more indirect way, if at all. More direct evidence for a role of these two domains in the functional divergence of these methyl-transferases, might be obtained by a more detailed comparison of the structures of the anthocyanin methyltransferases and their substrates (though modeling or crystallography) combined with functional assays of mutant proteins in which specific domains are swapped between distinct methyltransferases.

Biochemical work on petunia flowers showed that anthocyanin methyltransferase activities in petal extracts could be separated by chromatofocusing into 4 distinct species with different isoelectric points (pIs) and analysis of mt and mf mutants (in heterologous genetic backgrounds) suggested that the expression of each species was controlled by a specific MT or MF gene (Jonsson et al., 1984a). These data suggested that the pIs of these proteins range from 4.8, for the enzyme controlled by MF1, to 5.4 for the enzyme controlled by MF2, while the enzymes controlled by MT1 and/or MT2 have intermediate pIs. Furthermore experiments with an antiserum raised against a partially purified enzyme that was controlled by MF2, could inhibit/pull down the activities controlled by MF2 and MT1, but not the activity controlled by MF1. These biochemical data are completely at odds with the properties of the MT and MF enzymes predicted from the gene sequences. For example, the predicted MF1 and MF2 proteins from distinct P. hybrida and P. inflata accessions have nearly identical sequences, and thus are expected to immunologically cross react, and also have nearly identical predicted pIs that are in the range 5.05 to 5.15 (differences between MF1 or MF2 from one line are of similar size as differences between homologous proteins from different lines), while MT (pI = 5.48) is more basic, but would still be expected to cross-react with anti-MF2 given the high similarity of MT and MF2. It might be that because of the unrelated genetic backgrounds of the mutants used by Jonsson et al, some of the methyltransferase species were incorrectly linked to specific MT/MF genes, and therefore their results should be interpreted with caution. However, the discrepancies between the predicted and in vivo observed properties of the methyltransferases seem too large and plentiful to be explained by such misinterpretations alone, suggesting that these methyltransferase are in vivo subject to post-translational modifications, which might affect their activity and/or substrate-specificity in unforeseen ways.
Materials and methods

Plant Material

Plant varieties used were Petunia hybrida hybrids. Old Glory Red (OGB) (Ball Seed, USA) and R78 X V32 (mt1, mt2, mf1, mf2, GF); and the inbred lines R78 (mt1, mt2, mf1, mf2, gf), V30 (MT1, MT2, mf1, mf2), M1 (MT1, MT2, MF1, MF2), and M29 (mt1, mt2, mf1, mf2).

Petals from OGB flowers were harvested at developmental stages defined as follows:

- stage 3: dark red bud with emerging corolla (> 35 mm in length); stage 4: dark red opened flower pre-anther dehiscence (> 50 mm in length).

cDNA library construction

Total RNA was isolated from the petal tissue of P. hybrida cv OGB of stage 1-3 flowers using the method of Turpen and Griffith (1986). PolyA⁺ RNA was selected from the total RNA, using oligotex-dTTM (Qiagen). A ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene) was used to construct a directional cDNA library in λZAP using 5 µg of poly(A)⁺ RNA isolated as described above. The total number of recombinants obtained was 1 x 10⁶ pfu. One hundred thousand pfu of the amplified library were plated and duplicate lifts were taken onto Colony/Plaque ScreenTM filters (Dupont) and hybridized with the original DIFe1 cDNA, which originated from petals of line V26, using standard procedures.

DNA and RNA blot analysis

Total RNA was isolated using a Plant RNA Isolation Kit (Qiagen). Genomic DNA was isolated as described elsewhere (Brugliera et al., 1994).

Enzymatic activity of PhMT

In order to clone the Petunia DIFe1 (MT) into an E. coli expression vector, pQE30 (QIAGEN), the DIFe1 cDNA from OGB was amplified with the primers 1907BamHI_FW GCATGGATCCACAGGCAAAACC GCCCACCCTG and 1907PstI_RV GCATCTGCAGCTAGGAGAGACGCCTGCAAG, digested with BamHI and PstI and ligated into vector pQE30 (Khorshed Alam et al., 2008) to yield the expression construct pCGP3086.

Purified recombinant AOMT was assayed in a final volume of 50 µl, the assay reactions were incubated at 30°C for 30 minutes and were stopped with fifty microlitres of a chloroform mix (CHCl₃:methanol/1% HCl, 2:1). The enzyme activity of the Petunia E20 clone contained in pCGP3086 was initially assessed using the substrates delphinidin 3-glucoside and delphinidin 3-rutinoside under assay conditions as described in (Jonsson et al., 1983).
The residue of the enzymatic assay was resuspended in 2-3 µL of methanol 1% HCl and spotted onto a TLC plate alongside standard samples of petunidin, malvidin and delphinidin. The anthocyanidins were separated in a Forestal system (HOAc: water: HCl; 30:10:3) (Markham, *Techniques of flavonoid identification*, Academic Press, London, 1982) and the TLC was exposed to an autoradiographic film (Kodak) for 16 hours at -70°C.

**Southern-Blot Hybridization**

Genomic DNA was isolated from the leaves of young *Petunia hybrida* plants and the DNA (6 µg) was digested with EcoRI or BamHI restriction enzymes, size-separated on a 0.9% agarose gel and blotted to nylon membranes by downward capillary transfer. The blots were hybridized at 42°C overnight in DIG Easy Hyb buffer (Roche) using a digoxigenin-labelled *MF* or *MT* cDNA probe, washed under low stringency conditions (25°C, 2xSSC), and treated with high affinity anti-digoxigenin (anti-DIG) antibodies conjugated to alkaline phosphatase. After incubation with the chemiluminescent substrate CSPD the membrane was exposed for 4-16 hrs. to X-ray film. Subsequently the blot was rewashed at high stringency (0.1x SSC, 68°C) and again exposed. The same membrane was hybridized two times, checking that any residual signal was completely eliminated from the membrane before the following hybridization.

**Real time PCR analysis of gene expression**

The total RNA was extracted as described elsewhere (Spelt et al., 2000). Reverse transcription was performed for each sample from 2.5 µg of purified RNA. Gene specific primers were as follows: *VvAOMT*-F (5'-GATGAATGTCCTGTCGAGT-3') and *VvAOMT*-R (5'-GCAAGAGCTTTGCTGAGTGA-3'), *PhMT*-F (5'-GCAGCGAGCTTTATGGGTCTT-3') and *PhMT*-R (5'-TGAGCTTTTAGAACATCGTAGAAC-3'), *PhMF*-F (5'-CTTTTGGCTACTGCTCTTGCATT-3') and *PhMF*-R (5'-CTCTGTCCGGATCAATCGCTAT-3'). The efficiency of the primer pair was measured on a plasmid serial dilution. Real time PCR was performed using Power SYBER Green (Applied Biosystems). Normalization was done based on the expression of *Actin*.

The RT-PCR were performed using the following primers: For *PhMT* 5101 FW ATATTACTACACCAAGGATTTTACTGAAAGC and 629 RV TGCTCAACATGAGCGAAAC for *VvAOMT* 4567FW CACCATGTCCAGCTCAAGTCATAGGGGC and 629RV TGCTCAACACATGAGCGAAAC for *PhMF*. 
Constructs for stable transformation

For the stable expression of VvAOMT and PhMT, we cloned the cDNA sequence from ATG to STOP codon from Barbera and M1. 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. The primers used for VvAOMT were: VvAOMT-F (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCCAGCTCAAGTCATA-3') and VvAOMT-R (5'-GGGGACCACCTTTGTACAAAGAGAAGCTGGGTA CTAATAGAGGCGCTGCAGA-3'), and for PhMT for PhMT-F (5'-GGGGACCAAGCTTGACAAGAGACCGCTCA ATGACAGGCAAAACCGCC-3') and PhMT-R (5'-GGGGACCACCTTTGTACAAAGAGAAGCTGGGTA CTAATAGAGGCGCTGCAGA-3'). The VvAOMT and PhMT were then transferred into destination vector pK2GW7.0 (Dubin et al., 2008). The PhMF was amplified with the primers: PhMF-F and (5'-CACCATGGCAGGCAA AAGCG-3') PhMF-R (5'-CTAGCTGAGACGCTGCAGC-3'), cloned in the pGem T Easy. The plasmid was cut with Ncol and EcoRI, transferred in the entry vector pENTR4, and subsequently recombined in the 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).

Analysis of anthocyanin content in petunia flowers

TLC analysis were performed as described in (Koes et al., 1995). For HPLC analysis 0.5 g of petals was freeze dried and extracted with 4.5 ml 50% acetonitrile containing 0.1 % TFA. Anthocyanins were analyzed as described previously (Katsumoto et al., 2007). Anthocyanins were further analyzed with LC-IT-TOF-MS (Shimadzu Co) analysis using a column of Inertsil ODS-4 (250 x 4.6 mm) (GL-Sciences Inc). The elution profile consisted of a linear gradient from A:B=90:10 to 50:50 in 30 min (in which A is 0.1 % formic acid and B is 90 % acetonitrile containing 0.1 % formic acid) and then from A:B=50:50 to A:B= 0:100 in 15 min. The flow rate was 0.6ml/min. Data of MS+ and MS- between 250 and 1000 m/z were collected.
References


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