Introduction of apple ANR genes into tobacco inhibits expression of both CHI and DFR genes in flowers, leading to loss of anthocyanin

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Abstract

Three genes encoding anthocyanidin reductase (ANR) in apple (Malus domestica Borkh.), designated MdANR1, MdANR2a, and MdANR2b, have been cloned and characterized. MdANR1 shows 91% identity in coding DNA sequences with MdANR2a and MdANR2b, while MdANR2a and MdANR2b are allelic and share 99% nucleotide sequence identity in the coding region. MdANR1 and MdANR2 genes are located on linkage groups 10 and 5, respectively. Expression levels of both MdANR1 and MdANR2 genes are generally higher in yellow-skinned cv. Golden Delicious than in red-skinned cv. Red Delicious. Transcript accumulation of MdANR1 and MdANR2 genes in fruits gradually decreased throughout fruit development. Ectopic expression of apple MdANR genes in tobacco positively and negatively regulates the biosynthesis of proanthocyanidins (PAs) and anthocyanin, respectively, resulting in white, pale pink-coloured, and white/red variegated flowers. The accumulation of anthocyanin is significantly reduced in all tobacco transgenic flowers, while catechin and epicatechin contents in transgenic flowers are significantly higher than those in flowers of wild-type plants. The inhibition of anthocyanin synthesis in tobacco transgenic flowers overexpressing MdANR genes is probably attributed to down-regulation of CHALCONE ISOMERASE (CHI) and DIHYDROFLAVONOL-4-REDUCTASE (DFR) genes involved in the anthocyanin pathway. Interestingly, several transgenic lines show no detectable transcripts of the gene encoding leucoanthocyanidin reductase (LAR) in flowers, but accumulate higher levels of catechin in flowers of transgenic plants than those of wild-type plants. This finding suggests that the ANR gene may be capable of generating catechin via an alternative route, although this mechanism is yet to be further elucidated.

Key words: Anthocyanin, anthocyanidin reductase, flavonoid, Malus, proanthocyanidin.

Introduction

Proanthocyanidins (PAs), also known as condensed tannins, are phenolic polymers of condensed flavan-3-ols and are among the major flavonoid compounds found in higher plants (Winkel-Shirley, 2001). PAs are powerful antioxidants, and thus provide multiple health benefits to humans, including anti-inflammatory effects, immunity enhancement, as well as lowering risks of cardiovascular diseases and certain cancers (Santos-Buelga and Scalbert, 2000). PAs can also protect ruminants against pasture bloat disease and enhance ruminant nutrition (McMahon et al., 2000). Moreover, PAs can interact with proteins, particularly saliva proteins such as α-amylase, resulting in the astringent and bitter sensations in many fruits and fruit juices (Vidal et al., 2003; Obreque-Slier et al., 2010; Renard et al., 2011).
Therefore, it is important to further our understanding of PA biosynthesis, and to engineer PAs in fruit and forage crops to improve their nutritional and health protective values.

The biosynthesis pathway of PAs is a branch of the anthocyanin biosynthesis pathway, and begins with the synthesis of flavan-3-ol units, such as catechin and epicatechin (Fig. 1). Catechin is derived from leucocyanidin by leucoanthocyanidin reductase (LAR), while epicatechins are synthesized from cyanidin by anthocyanidin reductase (ANR). The functionality of LAR has been characterized in *Desmodium uncinatum* (Tanner *et al.*, 2003) and grapevine (Bogs *et al.*, 2005). ANR has been initially identified in *Arabidopsis*, and it is encoded by the *BANYULS (BAN)* gene (Xie *et al.*, 2003). ANR utilizes cyanidin as a substrate, rather than leucocyanidin, which is consistent with the fact that leucoanthocyanidin dioxygenase (LDOX) is essential for PA synthesis in *Arabidopsis* (Abrahams *et al.*, 2003). Ectopic expression of *BAN* in tobacco flower petals and *Arabidopsis* leaves results in loss of anthocyanins and accumulation of condensed tannins, suggesting that there is an interaction between anthocyanidin and PA pathways (Xie *et al.*, 2003). However, there are no reports on how overexpression of either *BAN* or other PA pathway genes influence expression of genes involved in the anthocyanin pathway.

Besides the two structural genes *BAN* and *ANR*, MYB-regulatory genes have also been identified as being involved in PA biosynthesis. For example, expression of *BAN* in *Arabidopsis* seed coat is co-regulated by at least three MYB regulatory genes in tobacco flowers inhibits expression of both *VvANR* and *VvLAR* genes in grape berries and leaves (Bogs *et al.*, 2007).

![Fig. 1. A general schematic diagram of the flavonoid biosynthetic pathway.](image)

Materials and methods

Plant material

Apple leaves, flowers, and fruits at different developmental stages were collected, and whole fruits were used for gene expression studies. Wild-type and *T* <sub>2</sub> transgenic plants of tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) were grown in a greenhouse, and flowers at the full-bloom stage were harvested for the analysis of gene expression and flavonoid compounds. All the samples were frozen in liquid nitrogen upon collection, and stored at –80 °C until needed.

Isolation of genes encoding *ANR* in apple

A full-length cDNA of *MdANR* (GenBank accession no. DQ999803) was obtained from an apple expressed sequence tag (EST) database (http://titan.biote.c.uic.edu/apple/), and a pair of primers (5’-CACGACCAACCTGTCCCTT-3’/5’-GTTGCAACCCCTGTC AACCT-3’) was designed to screen an apple bacterial artificial
chromosome (BAC) library (cv. GoldRush) according to a previously described PCR-based screening protocol (Xu et al., 2001). The PCR program consisted of 34 cycles of 30 s at 94 °C, 30 s at 58 °C, 60 s at 72 °C, and a final extension for 5 min at 72 °C. Positive BAC clones were then subjected to Southern blot analysis, and three clones representing different gene copies were selected and subjected to subcloning. BAC DNA was extracted from a 300 ml culture using the Plasmid Midi kit (QIAGEN, Valencia, CA, USA), and the subcloning of BAC DNA was carried out according to a previously reported protocol (Han et al., 2007). The primer walking strategy was then used to sequence positive subclones to recover full genomic DNA sequences encoding ANR in apple.

Southern blot hybridization of BAC and genomic DNA
Genomic DNA (30 μg) from leaves of cv. GoldRush and 25 ng of BAC DNA from each positive clone were digested with BamHI, separated on a 1.0% agarose gel, and then transferred onto nylon membranes (Amersham Biosciences, Pittsburgh, PA, USA) using the capillary transfer method. A pair of primers (5'-TTTCCTTT ATGGCCGGTCTCTT-3'/5'-CGTCTCCACAGTGGAATG) was designed to DNA probes using cDNA from leaves of cv. GoldRush as a template. Hybridization was carried out using the DIG Easy Hyb kit (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Blots were exposed to a Lumi-Film X-ray film (Hyperfilm™, Amersham) at room temperature for 25 min.

Mapping of MdANR genes onto the integrated apple genetic and physical map

Two markers were developed based on insertion/deletion (InDel) polymorphism to tag MdANR1 and MdANR2 genes, and their primer sequences were 5'-CTGCGCATGATGCTTCACA-3'/5'-GAATCCTTCTGGGACACTCC-3' and 5'-ATCTGCTTCTTGCGGTACA-3'/5'-TTGGGCTTGAAATGTCACAC-3', respectively. The two InDel markers were used for screening an F1 population derived from a cross between 'Co-op 16' and 'Co-op 17'. The two InDel markers were used for screening an F1 population derived from a cross between 'Co-op 16' and 'Co-op 17'. The two InDel markers were used for screening an F1 population derived from a cross between 'Co-op 16' and 'Co-op 17'. The two InDel markers were used for screening an F1 population derived from a cross between 'Co-op 16' and 'Co-op 17'.

Flavonoid analysis
Anthocyanins and flavonols were extracted from 50 mg of finely-ground tissues in 1 ml of 1% HCl/methanol (v/v) at room temperature in the dark with continuous shaking for 1 h, and centrifuged at 13, 000 rpm for 15 min. A total of 100 μl of supernatant was transferred to a fresh tube, acid-hydrolysed by adding 30 μl of 3 N HCl, and incubated at 70 °C for 1 h in a Thermo Hybaid MBS 0.25s thermocycler (Thermo Scientific). PAs were extracted using 1 ml of 70% (v/v) acetone containing 0.1% (w/v) ascorbate, and incubated at room temperature for 24 h in darkness. The extract was centrifuged at 13, 000 rpm for 15 min at room temperature, and the supernatant was transferred to a new 1.5 ml microfuge tube. An aliquot of 200 μl of extract was dried at 35 °C and resuspended in 100 μl of 1% (v/v) HCl/methanol and 100 μl of 200 mM sodium acetate (pH 7.5).

Flavonoids were identified using liquid chromatography–tandem mass spectrometry (LC-MS/MS) and their contents were calculated by comparison with commercial standards, including kaempferol, quercetin, cyanidin, catechin, and epicatechin (Sigma). The LC-MS/MS analysis was performed on a 5500 QTRAP mass spectrometer (AB Sciex) which was equipped with a 1200 Agilent HPLC. An Analyst (version 1.5.1, Applied Biosystems) was used for data acquisition and processing. A Phenomenex column (3u C6-Phenyl 1A, 4.6x50 mm) was used for separation. The HPLC flow rate was set at 0.3 ml min⁻¹. HPLC mobile phases consisted of A (0.1% formic acid in H₂O) and B (0.1% formic acid in acetonitrile). The autosampler was kept at 5 °C. The gradient for catechin and epicatechin was as follows: 0 min, 90% A; 10 min, 50% A; 13–18 min, 0% A; and 18.1–25 min, 90% A. The gradients for cyanidin, kaempferol, and quercetin were as follows: 0 min, 10%; 7–12.5 min, 0% A; and 13–20 min, 70% A. The injection volumes were 20 μl or 10 μl for anthocyanin or PA analysis, respectively. Multiple reaction monitoring was used to quantitatively detect catechin and epicatechin (m/z 291.0→139.2), cyanidin (m/z 287.2→213.2), kaempferol (m/z 287.1→153.2), and quercetin (m/z 303.1→153.1). The electrospray voltage was set to 5500 V, the
heater was set at 600°C, the curtain gas was 35 psi, and both GS1 and GS2 were at 60 psi. All samples were run three times.

Results

Three members of a gene family encode anthocyanin reductase in apple

To identify all gene copies encoding ANR present in the apple genome, positive BAC clones and genomic DNA from ‘GoldRush’ leaves were subjected to Southern blot analysis. The genomic DNA consisted of three bands (Fig. 2), indicating that there are three copies of MdANR genes in the apple genome. The three BAC clones, designated B5, B6, and B7, yielded high, middle, and low bands, respectively (Fig. 1), and were thus selected to recover genomic DNA sequences of MdANR genes in apple.

Three ANR genes, designated MdANR1 (GenBank accession no. JN035302), MdANR2a (GenBank accession no. JN035300), and MdANR2b (GenBank accession no. JN035301), were identified in apple. All three MdANR genes are composed of six exons and five introns, and contain an open reading frame of 1332 bp encoding a putative protein of 339 amino acids. Phylogenetic analysis was performed using coding DNA sequences of genes encoding ANR as well as closely related genes encoding DFR in plants. The results indicated that all three apple MdANR genes were clustered together with plant ANR genes and separated from plant DFR genes, indicating that all three genes encoded ANR (Fig. 3).

MdANR1 shows ~79% and ~95% nucleotide sequence identities in genomic and coding regions, respectively, with either MdANR2a or MdANR2b. MdANR2a and MdANR2b share 97.0% and 99.9% nucleotide sequence identities in genomic and coding regions, respectively. MdANR2a and MdANR2b were deemed allelic, as described later. Differences in nucleotide sequences between the two genomic fragments of MdANR2a and MdANR2b are mainly attributed to several small InDels. The amino acid sequence of MdANR1 has 97% identity with sequences of both MdANR2a and MdANR2b. The deduced amino acid sequences of MdANR2a and MdANR2b are almost identical, except for two sequences that are different.

Mapping of MdANR1 and MdANR2 onto the apple genetic linkage map

The genomic DNA sequence of MdANR1 from apple cv. GoldRush was BLASTed against the genome sequence database of apple cv. Golden Delicious (http://www.rosaceae.org/gb/gbrowse/malus_x_domestica/), and an InDel, ~2.2 kb downstream of the stop codon, was identified. Based on this InDel, a marker was developed, designated as MdANR1-indel, and used for screening a segregating population derived from a ‘Coop 17’×’Coop 16’ cross (Supplementary Fig. S1 at JXB online). As a result, the apple MdANR1 gene was anchored onto linkage group 10 (Fig. 4).

Similarly, alignment of genomic DNA sequences of MdANR2a and MdANR2b revealed an InDel in the last intron, and a marker, designated MdANR2-indel, was developed based on this Indel. The MdANR2-indel was subsequently used to screen the ‘Coop 17’×’Coop 16’ mapping population, and all progeny segregated into three genotypes (Supplementary Fig. S2 at JXB online). The first genotype had a high band corresponding to the MdANR2a allele, and the second genotype had a low band corresponding to the MdANR2b allele. However, the third genotype carried two bands that corresponded to both alleles. This result clearly demonstrated that MdANR2a and MdANR2b were alleles of the MdANR2 gene. Analysis of the genetic map indicated that the MdANR2 gene was located on linkage group 5 (Fig. 4).

Expression profiles of MdANR1 and MdANR2 genes in apple

Two genotypes, a red-skinned cv. Red Delicious and a yellow-skinned cv. Golden Delicious, were selected to investigate expression profiles of genes encoding ANR in apple. qRT-PCR analysis indicated that MdANR1 and MdANR2 genes were expressed in all analysed tissues, including leaf, flower, and fruit (Fig. 5). Transcript accumulation of both MdANR1 and MdANR2 genes was highest in young fruitlets [9 days after pollination (DAP)] of both ‘Red Delicious’ and ‘Golden Delicious’, and then declined as fruitlets continued to develop. Transcript levels of both MdANR1 and MdANR2 genes in fruits reached their lowest levels at 104 DAP, but then slightly increased at fruit maturity.

Overall, transcript accumulation of both MdANR1 and MdANR2 was higher in ‘Golden Delicious’ than in ‘Red Delicious’. Transcript levels of both MdANR1 and MdANR2 were relatively lower in flowers than in both leaves and fruits at early and middle stages of development. Accumulation of transcripts of both MdANR1 and MdANR2 were lower in flowers of cv. Golden Delicious, and continued to decline throughout flower development; however, transcripts of both genes remained relatively constant in flowers of cv. Red Delicious throughout flower development.
Functional analysis of MdANR genes in tobacco

Coding region sequences encoding MdANR1, MdANR2a, and MdANR2b were separately transferred into tobacco under control of the Cauliflower mosaic virus (CaMV) 35S promoter, and several transgenic lines were generated for each construct. Flower colours of all T2 transgenic lines were different from those of wild-type plants, indicating that all three genes encoding ANR in apple were functional (Fig. 6a). Moreover, T2 transgenic lines produced flowers of different pigmentation patterns. For example, transgenic lines carrying MdANR1 produced either pale or white variegation with red pendant flowers. All three transgenic lines carrying MdANR2a produced pale-pink flowers, and two transgenic lines overexpressing MdANR2b produced either pale pink-coloured or pure white flowers, respectively.

Following LC-MS/MS analysis, it was revealed that flowers of all transgenic lines accumulated lower levels of anthocyanin than wild-type flowers (Table 1). Anthocyanin contents in either white- or pale pink-coloured transgenic flowers were too low to be detected. White and red variegated transgenic flowers accumulated certain amounts of anthocyanin, but these levels were significantly lower than those of wild-type flowers. Moreover, flowers of all transgenic lines accumulated higher levels of both catechin and epicatechin than did wild-type flowers. Transgenic line MdANR2b-4 producing pure white flowers accumulated the highest levels of epicatechin, while transgenic lines MdANR1-3 and MdANR1-13, producing white and red variegated flowers, respectively, had lower levels of epicatechin when compared with other transgenic lines. Flowers of all transgenic lines, except for MdANR2b-3 and MdANR2b-4, produced lower levels of kaempferol when compared with flowers of wild-type control.

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Influence of overexpressing MdANR genes on expression of other flavonoid pathway genes in transgenic tobacco flowers

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Influence of overexpressing MdANR genes on expression of other flavonoid pathway genes in transgenic tobacco flowers

qRT-PCR analysis was conducted to investigate the coordinate interaction of MdANR genes with other flavonoid pathway genes in transgenic tobacco flowers, including.
structural genes such as NtCHS, NtCHI, NtF3H, NtF3’H, NtFLS, NtDFR, NtUFGT, NtLAR, NtANR1, and NtANR2, and the regulator gene NtAn2. Expression of all flavonoid structural genes in flowers of all different transgenic lines carrying apple MdANR genes showed significant differences (Fig. 7). Overexpression of MdANR genes in tobacco greatly influenced expression of flavonoid structural genes in these flowers. For example, expression of NtANR, NtUFGT, and NtF3’H genes was up-regulated in flowers of transgenic plants carrying either MdANR1 or MdANR2a, but these
were down-regulated in transgenic flowers overexpressing *MdANR2b*. Flowers of transgenic lines such as *MdANR1-1*, *MdANR2a-5*, and *MdANR2a-6* showed no detectable expression of the *NtLAR* gene. Of all genes investigated, the two genes *NtCHI* and *NtDFR* showed lower levels of expression in flowers of all transgenic lines. Moreover,  

**Table 1.** Flavonoid contents in transgenic and wild-type tobacco flowers

| Flower       | Flavonol (μg g⁻¹) | Anthocyanin (ng g⁻¹) | Proanthocyanidin (ng g⁻¹) |
|--------------|-------------------|----------------------|---------------------------|
|              | Kaempferol        | Quercetin | Cyanidin | Catechin | Epicatechin |
| Wild type    | 60.57±0.83        | 71.80±1.51 | 1722±64.37 | 4.80±0.53 | 6.47±0.76 |
| *MdANR1-1*   | 53.73±0.64        | 87.60±4.42 | N/A      | 9.07±0.99 | 9.93±1.01 |
| *MdANR1-3*   | 32.53±0.96        | 78.17±5.58 | 759±5.77 | 5.33±0.23 | 8.40±0.87 |
| *MdANR1-13*  | 43.57±1.81        | 86.57±1.80 | 1063±18.38 | 5.47±0.64 | 7.20±0.20 |
| *MdANR2a-5*  | 42.10±1.67        | 99.67±4.89 | N/A      | 9.53±0.58 | 9.60±0.20 |
| *MdANR2a-6*  | 26.30±1.47        | 68.40±6.62 | N/A      | 6.07±0.42 | 8.80±0.20 |
| *MdANR2a-8*  | 42.20±2.73        | 83.13±6.65 | N/A      | 10.33±1.48 | 11.93±1.97 |
| *MdANR2b-3*  | 68.77±3.37        | 92.90±4.03 | N/A      | 6.80±0.20 | 8.73±0.61 |
| *MdANR2b-4*  | 145.33±10.21      | 493.67±21.92 | N/A      | 5.60±0.20 | 16.73±0.50 |

* All data correspond to mean values ± SD of three biological replicates. N/A, not available.

**Fig. 7.** Expression profiles of flavonoid-related structural biosynthetic genes in flowers of transgenic tobacco lines carrying *MdANR* genes. All mRNA transcripts expressed in transgenic flowers were quantified relative to those expressed in wild-type tobacco flowers.

Apple *ANR* genes inhibit expression of both *CHI* and *DFR* genes in tobacco flowers.
transcript accumulation of the R2R3 MYB gene *NtAn2* was significantly higher in flowers of all transgenic plants than those of the wild-type control.

Discussion

ANR and LAR catalyze the synthesis of flavan-3-ol, an initiating monomer of condensed tannin or PA synthesis, from cyanidin and leucoanthocyanidin, respectively. ANR is not only important for PA synthesis, but it also influences the synthesis of anthocyanin by competing with UFGT (UDP-glucose:flavonoid 3-O-glucosyltransferase) activity by which it converts anthocyanidin to anthocyanin (Bog et al., 2005). Thus, ANR plays an important role in both anthocyanin and PA synthesis in plants. To date, genes encoding ANR have been isolated and characterized in Arabidopsis and grapevines (Xie et al., 2003; Bogs et al., 2005). However, it is not clear how ANR genes interact with other flavonoid structural genes to coordinate the biosynthesis of anthocyanin. Herein, a gene family encoding ANR in apple has been isolated and functional analysis has been conducted. The results will provide insights into the interaction between *MdANR* genes and other genes involved in flavonoid biosynthesis.

Duplication of *MdANR* genes is related to the polyploid origin of the apple genome

Two *MdANR* genes, *MdANR1* and *MdANR2*, have been identified in the apple genome. *MdANR1* and *MdANR2* genes share 91% nucleotide sequence identity in their coding regions, and are located on linkage groups 10 and 5, respectively. The apple is a diploid (2n=34), but it has an allopolyploid origin, and has a wide genetic diversity (Korban and Tartarini, 2009; Velasco et al., 2003; Zhang et al., 2011). Recently, integrated physical and genetic maps of the apple have demonstrated that linkage groups 5 and 10 are of homologous chromosome pairs (Han et al., 2011). Moreover, regulation of genes during early stages of fruit development has been investigated (Soria-Guerra et al., 2011). Interestingly, two FLAVONOID 3’ HYDROXY-LASE (F3’H) genes, *MdF3’H1* and *MdF3’H2*, have been identified in the apple genome (Han et al., 2010). *MdF3’H1* and *MdF3’H2* share 91% nucleotide sequence identity in their coding regions, and are located on linkage groups 6 and 14, respectively. The apple linkage groups 6 and 14 are also homologous chromosome pairs (Han et al., 2011). Thus, it is clear that duplication of both ANR and F3’H genes in apple must be simultaneously derived from whole-genome duplication during the process of speciation (Xu and Korban, 2004). Moreover, the two apple *MdANR* genes are functional, and are expressed in fruits throughout fruit development. Multiple genes expressed in fruits may be responsible for the presence of high contents of PAs in apple (Souquet, 1996). In addition, coloration patterns of flowers of transgenic tobacco overexpressing *MdANR1* are different from those of transgenic flowers overexpressing *MdANR2*. These differences may be attributed to transgene positional effects. However, it cannot be ruled out that *MdANR1* and *MdANR2* genes may have also functionally diverged during the course of evolutionary development of apple.

Incomplete inhibition of anthocyanin production results in novel floral pigmentation patterns

Floral colours and pigmentation patterns, such as white and red variegation, are of important ornamental value. Novel floral pigmentation patterns have been obtained by genetic modification of the anthocyanin biosynthesis pathway in several ornamental plants such as petunia, lisianthus, and torenia that naturally produce patterned flowers, with some white and coloured petal areas (Napoli et al., 1990; Davies, 2009). Variegated pigmentation patterns are pre-determined by morphological signals within petals that control levels of gene inhibition, and in turn those signals would interact with environmental signals that influence pigmentation (van der Krol et al., 1990). Tobacco plants do not produce flowers that are naturally patterned or variegated. However, variegated flower phenotypes have been observed in tobacco plants overexpressing an *Arabidopsis* transposable element, *Tagl-R* (Liu et al., 2001). Yet, no patterned tobacco flowers have been reported following inhibition of anthocyanin biosynthesis.

It has been reported that anthocyanin biosynthesis can be inhibited by overproduction of enzymes that compete for the substrate (Joung et al., 2003). For example, introduction of a *Medicago CHALCONE REDUCTASE (CHR)* gene into petunia altered flower coloration from deep purple to pale purple, but did not yield any white-coloured flowers (Davies et al., 1998). The ANR enzyme is known to compete with the UFGT enzyme to convert anthocyanidin to epicatechin. In a previous study, it has been demonstrated that ectopic expression of an *Arabidopsis BAN* gene in tobacco can significantly inhibit the biosynthesis of anthocyanin, resulting in white-coloured flowers (Xie et al., 2003). Here, it is further found that overexpression of apple ANR genes in tobacco not only produces white flowers or pale pink-coloured flowers, but has also resulted in recovery of flowers with novel pigmentation patterns, with some petal areas that are white and others that are red.

To determine whether or not transgene copy number had an effect on coloration of transgenic flowers, Southern blot analysis was conducted for those transgenic lines carrying the *MdANR1* gene. All three transgenic lines producing different coloured flowers contained a single copy of the *MdANR1* transgene (Supplementary Fig. S3 at JXB online). Thus, differences in flower pigmentation could not be attributed to transgene copy number. Moreover, two transgenic lines of *MdANR1* produced white and red variegated flowers and showed lower levels of transgene expression when compared with other transgenic lines of *MdANR2* producing either white or pale pink-coloured flowers. In addition, previous studies demonstrated that introducing an enzyme to compete with anthocyanin biosynthetic enzymes
could successfully lower pigment levels, but this was not sufficient for depleting pigmentation in flowers and resulting in white-coloured flowers (Davies et al., 1998; Xie et al., 2003). Interestingly in this study, a single transgenic line, MdANR2b-4, produced pure white flowers, yet showed very high levels of MdANR2b expression. Taken together, these findings suggested that low levels of expression of MdANR genes might be partially responsible for incomplete inhibition of anthocyanin synthesis in transgenic tobacco flowers.

**The apple ANR gene may also have a redundant function in converting anthocyanidin into catechin**

It is well documented that LAR and ANR catalyse the conversion of leucocyanidin and cyanidin into catechin and epicatechin, respectively (Bogs et al., 2005; Szankowski et al., 2009). In this study, flowers of all transgenic tobacco lines overexpressing either MdANR1 or MdANR2 genes have accumulated higher levels of both catechin and epicatechin compared with those of wild-type tobacco plants. However, overexpression of an MdANR gene has contributed to significant suppression of the function of the LAR native gene, as expression levels of the LAR gene in most transgenic tobacco lines has been too low to be detected. Thus, other genes in anthocyanin biosynthesis, besides LAR, must be capable of promoting accumulation of catechin.

Incubation of BAN proteins of *M. truncatula* and *Arabidopsis* with anthocyanidins, cyanidin, and pelargonidin has resulted in the formation of epicatechin as the major product and catechin as a minor product (Xie et al., 2003). Similarly, findings in this study also suggest that ANR or BAN could synthesize both epicatechin and catechin. This in turn raises a question as to whether the synthesis of catechin results from epimerization of epicatechin or whether it is a product that is catalysed by ANR. It has been reported that incubating either epicatechin or catechin with either MtANR or AtANR in the presence of NADPH does not result in epimerization into catechin or epicatechin, respectively (Xie et al., 2004). LDOX is essential for PA synthesis in *Arabidopsis* (Abrahams et al., 2003), thus suggesting that ANR cannot efficiently use leucoanthocyanidin as a substrate to produce catechin. Thus, it is likely that BAN could directly convert anthocyanidin into catechin. In addition, it is worth noting that catechin might be formed through chemical epimerization of epicatechin (Xie et al., 2004). Further studies are needed to clarify whether the catechin detected in this study is attributed to the epimerization of epicatechin to catechin. It has been reported that high temperatures and alkalization are two main factors that induce the epimerization of epicatechin into catechin (Kofink et al., 2007). However, in this study, PA extraction has been conducted under pH 7.5 and 35 °C, which is not high. Moreover, the accumulated catechin, presumed to be formed through chemical epimerization, is observed as a minor byproduct (Xie et al., 2003). In this study, the amounts of epicatechin and catechin accumulating in flowers of some transgenic tobacco lines overexpressing an MdANR gene are almost equal; thus, formation of catechin in these lines cannot be fully explained by chemical epimerization alone.

**Overexpression of MdANR genes causes co-suppression of other structural genes involved in flavonoid biosynthesis**

Overexpression of the CHALCONE SYNTHASE (CHS) gene results in co-suppression of homologous genes in petunia, and this co-suppression is related to an RNA silencing mechanism (Baulcombe, 2004). In this study, overexpression of *MdANR* genes has resulted in co-suppression of *NiLAR, NiDFR*, and *NiCHI* genes in transgenic tobacco lines. Of these three genes, *NiLAR* and *NiDFR* are related to *MdANR*. Collectively, *MdANR, NiLAR, and NiDFR* belong to the reductase-epimerase-dehydrogenase (RED) superfamily although they share low identity in their DNA coding sequences. However, co-suppression of *NiLAR* and *NiDFR* may not be related to RNA silencing for the following reasons. First, *ANR* genes from apple and tobacco share ~52% identity in their DNA coding sequences, and overexpression of *MdANR* does not result in co-suppression of *NiANR* genes in tobacco. Secondly, *ANR* is more closely related to *DFR* than to *LAR*; however, overexpression of an *MdANR* gene suppresses expression of *NiLAR* more severely than that of *NiDFR*. Therefore, the mechanism of co-suppression is rather complicated, and requires further investigation.

LAR converts leucoanthocyanidin into catechin and it competes with anthocyanin synthase (ANS)/LDOX for the substrate to produce an alternative initiating unit for PA biosynthesis. It is known that ANR and LAR are NADPH-dependent reductases. Thus, overexpression of *MdANR* in this study will offer little opportunity for *NiLAR* to accept NADPH, resulting in low levels of expression of *NiDFR* in flowers of transgenic tobacco lines. Interactions among DFR, CHS, and CHI have been previously identified in *Arabidopsis* (Burbulis and Winkel-Shirley, 1999). Therefore, it is likely that co-suppression of *NiLAR* and *NiDFR* may be due to interactions among enzymes involved in the flavonoid biosynthetic pathway.

**Variegated patterns of flower coloration of transgenic tobacco overexpressing MdANR are related to low levels of expression of both *NtCHI* and *NtDFR***

In this study, expression of structural and regulatory genes involved in the flavonoid biosynthesis pathway has been investigated in flowers of transgenic tobacco lines overexpressing *MdANR* genes. Of the flavonoid structural genes, *NtCHI* and *NtDFR* have demonstrated significantly lower levels of expression in flowers of all transgenic tobacco lines compared with those of wild-type plants. It is well known that expression of *CHI* and *DFR* genes is controlled by regulatory genes such as *R2R3 MYB* transcription factors.
More recently, an R2R3 MYB regulator from tobacco 
(NtAn2) has been isolated and reported to be a key gene controlling anthocyanin production in reproductive tissues of tobacco (Pattanaik et al., 2010). Interestingly, in this study, flowers of all transgenic tobacco lines have demonstrated significantly higher levels of expression of NtAn2 than those of wild-type tobacco. Thus, it seems that overexpression of MdANR may affect the regulatory role of the NtAn2 gene. However, it is not yet clear as to whether or not the ANR protein can interact with R2R3 MYB proteins, thus leading to their enhanced transcriptional activities.

Transgenic tobacco lines carrying MdANR genes accumulate up to 10 ng g\(^{-1}\) more PAs than wild-type plants, and most show slight changes in flavonol accumulation compared with wild-type plants. However, most MdANR transgenic lines have not accumulated anthocyanins, whereas wild-type plants have accumulated >1700 ng g\(^{-1}\) anthocyanins. These results suggest that ANR genes may coordinate interact with other flavonoid genes to regulate flavonoid biosynthesis. Overall, overexpression of MdANR genes in tobacco results in a significant decrease in the total amount of flavonoids accumulated in flowers. The loss of anthocyanin in tobacco transgenic flowers overexpressing MdANR genes is probably attributed to suppression of the two genes NtCHI and NtDFR.

**Supplementary data**

Supplementary data are available at *JXB* online.  

Figure S1. Segregation for the MdANR1 gene in an F\(_1\) apple population.  

Figure S2. Segregation for the MdANR2 gene in an F\(_1\) apple population.  

Figure S3. Copy numbers of MdANR1 genes in transgenic tobacco lines determined by Southern blot hybridization.  

Table S1. Primer sequences for qRT-PCR.

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