

RESEARCH PAPER

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 transcription factors, *TT2*, *TT8*, and *TTG1* (Nesi *et al.*, 2001; Baudry *et al.*, 2004). In persimmon fruits, expression levels of *DkMYB4* are positively correlated with accumulation of *DkANR* transcripts, but have little influence on accumulation of *DkLAR* transcripts (Akagi *et al.*, 2009). In contrast, grapevine *VvMYBPA1* can activate expression of both *VvANR* and *VvLAR* genes in grape berries and leaves (Bogs *et al.*, 2007).

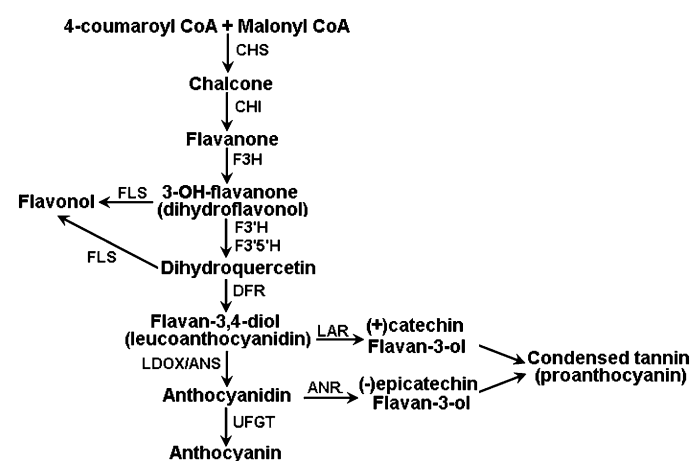


Fig. 1. A general schematic diagram of the flavonoid biosynthetic pathway. CHI, chalcone isomerase; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; F3'H, flavanone 3'-hydroxylase; FLS, flavonol synthase; DFR or BAN, dihydroflavonol reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, glucose transferase.

Apple (*Malus×domestica* Borkh.) is one of the most widely cultivated fruit crops grown around the world. Since red coloration is an important contributor to apple fruit quality, the molecular mechanism underlying apple red coloration has recently been extensively studied. For example, red coloration of the flesh of apple fruit is due to increased *MdMYB10* transcripts and subsequent accumulation of anthocyanins throughout the plant (Espley *et al.*, 2007). Two alleles of *MdMYB10*, *MdMYBA* and *MdMYB1*, play important roles in red coloration of the skin of apple fruit (Talos *et al.*, 2006; Ban *et al.*, 2007). In addition, apple fruits are also well known to be rich in PAs (Souquet, 1996). Thus, apple is an important edible source of PAs with known nutrient and health benefits for humans (Hammerstone *et al.*, 2000). However, few studies have been reported on genes involved in the PA pathway in apple. In fact, the PA pathway is in competition with anthocyanin synthesis, especially via the *ANR* gene as it utilizes the same substrate, anthocyanidin. Moreover, functional characterization of *ANR* and *LAR* genes has so far only been reported in *Medicago truncatula* (Tanner *et al.*, 2003), *Arabidopsis* (Xie *et al.*, 2003), and *Vitis vinifera* (Bogs *et al.*, 2005). Many questions related to the mechanism of how plants synthesize PAs still remain unanswered. Therefore, investigation of *MdANR* genes in apple will not only provide valuable insights into the molecular mechanism of PA accumulation in plants, but will also aid in our understanding of coloration of fruit.

In this study, isolation, genetic mapping, and functional characterization of the gene family encoding ANR in apple is reported. Expression profiles of apple *ANR* genes in fruit were also investigated at different stages of development. Moreover, the functionality of different members of the apple *ANR* gene family is characterized via ectopic expression in tobacco. The results demonstrate that ectopic expression of apple *ANR* genes in tobacco flowers inhibits expression of both *CHALCONE ISOMERASE* (*CHI*) and *DIHYDROFLAVONOL-4-REDUCTASE* (*DFR*) genes, resulting in loss of anthocyanin. These findings will aid in future attempts to manipulate flavonoid biosynthesis in apple as well as in other plants.

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 T₂ 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. DQ099803) was obtained from an apple expressed sequence tag (EST) database (<http://titan.biotech.uiuc.edu/apple/>), and a pair of primers (5'-CACGACCAAACCTGTTCCCTT-3'/5'-GTTGCAACCCCTGCAACTT-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 *Bam*HI, 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'-TTCTCTTATGGCCGGTCCTT-3'/5'-CGTCCTCCACATGTGCAATG-3') 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'-CTGCCGCATGATGATCTTCAC-3'/5'-GAATCTCTTCGTGGACACTCC-3' and 5'-ATCTGCTTCTGGTCGGTACA-3'/5'-TTGGCCTTGAAGTACTCCAC-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'. PCR products were separated on 2% (w/v) agarose gels. The linkage analysis was conducted using JoinMap version 4.0 according to a previously reported strategy (Han *et al.*, 2011).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from leaf and flower tissues were extracted using an RNAqueous Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Total RNA from apple fruits was isolated according to the protocol described by Gasic *et al.* (2004). A total of 3 µg of RNA was treated with DNase I (Invitrogen) to remove contaminating DNA, and then subjected to cDNA synthesis using the SuperScript III RT kit (Invitrogen) according to the manufacturer's instructions.

The SYBR Green real-time PCR assay was carried out in a total volume of 15 µl, containing 12.5 µl of 2× SYBR Green I Master Mix (Applied Biosystems), 0.2 µM (each) of specific primers, and 100 ng of template cDNA. The amplification program consisted of one cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The fluorescent product was detected at the last step of each cycle. Following amplification, melting temperatures of PCR products were analysed to determine the specificity of the PCR product. Melting curves were obtained by slow heating at 0.5 °C s⁻¹, from 60 °C to 90 °C while continuously monitoring the fluorescence signal. A negative control without a cDNA template was run with each analysis to normalize the data, and evaluate the overall specificity. The qRT-PCRs were carried out in 96-well plates in a 7300 Real Time PCR System (Applied Biosystems). All samples were run in triplicate. An apple actin gene was used as an endogenous control gene. Differences in cycle threshold (*C_t*) between target and actin genes were used to determine the relative transcript level of the target gene, and calculated as

$2^{-\text{exp}-(C_{t\text{target}}-C_{t\text{actin}})}$. Real-time PCR primer sequences are listed on Supplementary Table S1 available at *JXB* online.

Expression vector construction and tobacco transformation

Two pairs of primers, 5'-TGACTCTAGAATGGCCACCCAA-CAACCCATCT-3'/5'-ATACGAGCTCCTAGTTCTGCAGCAGC-CCCTTT-3' and 5'-TGACTCTAGAATGGCCACCCAAACAAC-CCA TCT-3'/5'-ATACGAGCTCTTTTATTTTCTTTTCTCAT C-3', were designed to amplify the whole coding sequences of *MdANR1* and *MdANR2*, respectively, using cDNA from leaves of cv. GoldRush as templates. The forward and reverse primers contained *Xho*I/*Sac*I sites at the 5' end, respectively. The PCR amplification was conducted using proofreading DNA polymerase Platinum® *Pfx* (Invitrogen), and PCR products, digested with *Xho*I and *Sac*I, were ligated into *Xho*I/*Sac*I-digested pBI121 binary vector. The binary vector containing each of the *MdANR* genes was sequenced. As a result, three constructs carrying coding regions of *MdANR1*, *MdANR2a*, and *MdANR2b*, respectively, were generated.

Each construct was immobilized into *Agrobacterium tumefaciens* strain GV3101, and co-cultivated with tobacco leaf sections following a previously reported protocol (Horsch *et al.*, 1985), with some modification. Briefly, leaf sections (~0.5 cm²) of 10-day-old seedlings were incubated with *Agrobacterium* suspension (*A*₆₀₀=0.5) for 30 min at 28 °C with shaking at 100 rpm. Leaf explants were transferred to a solid medium supplemented with B5 vitamins, 3% (w/v) sucrose, 1 mg l⁻¹ 6-benzyladenine (BA), 0.1 mg l⁻¹ α-naphthaleneacetic acid (NAA), 0.8% tissue culture agar, pH 5.6, and 100 mg l⁻¹ kanamycin. Explants were incubated in the dark for 2 weeks at 25 °C, and then transferred to a growth chamber at 25 °C under a 16 h photoperiod. Finally, kanamycin-resistant plantlets were transferred to soil mix, acclimatized, and grown in the greenhouse.

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 11A, 4.6×50 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, 70% A; 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 quantify 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. JN035299), *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).

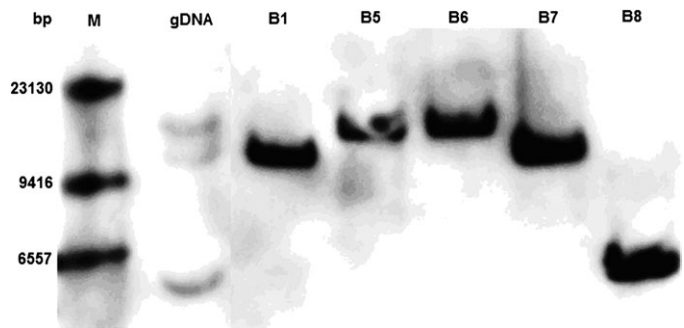


Fig. 2. Southern blot of BAC clones containing *MdANR* genes. Genomic DNA (gDNA) from apple ‘GoldRush’ was used to determine the number of gene copies of *MdANR* genes in the apple genome. The five positive BAC clones were designated as B1, B5, B6, B7, and B8. The digoxigenin (DIG)-labelled DNA probes correspond to cDNA fragments of the last two exons.

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.

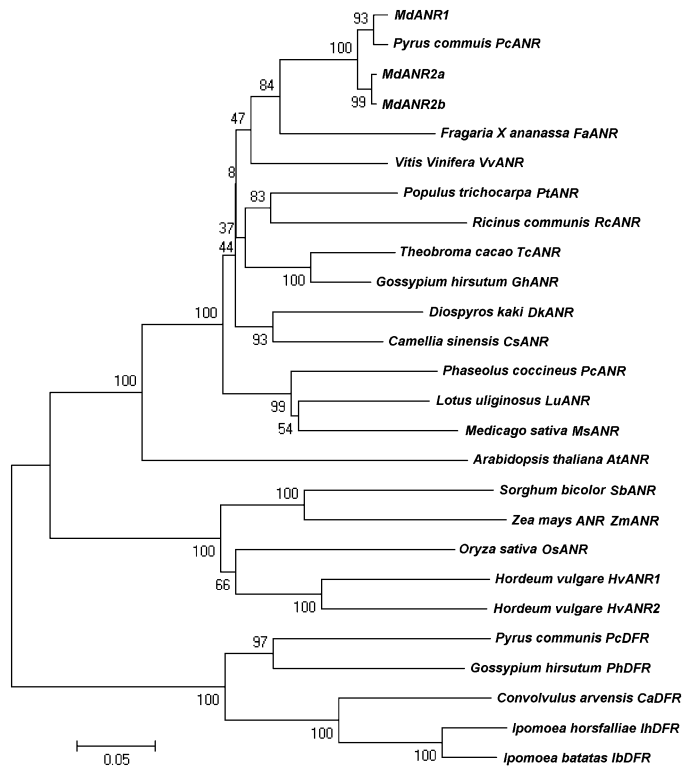


Fig. 3. Phylogenetic tree derived from nucleotide sequences of genes encoding ANR and DFR in plants. The scale bar represents 0.05 substitutions per site, and the numbers next to the nodes are bootstrap values from 1000 replicates. The GenBank accession numbers are as follows: *PcANR* (DQ251189), *FaANR* (DQ664192), *VvANR* (XM_002271336), *PtANR* (XM_002317234), *TcANR* (GU324348), *GhANR* (EF187443), *DkANR* (AB195284), *CsANR* (AY641729), *LuANR* (EF197823), *RcANR* (XM_002518532), *PcANR* (BN000164), *MsANR* (HM754630), *AtANR* (NM_104854), *HvANR1* (AK373696), *HvANR2* (AK365124), *OsANR* (NM_001060512), *SbANR* (XM_002447113), *ZmANR* (BT064433), *PcDFR* (AY227730), *lhDFR* (GQ180935), *lbDFR* (EU402466), *CaDFR* (EU189076), and *GhDFR* (FJ713480).

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

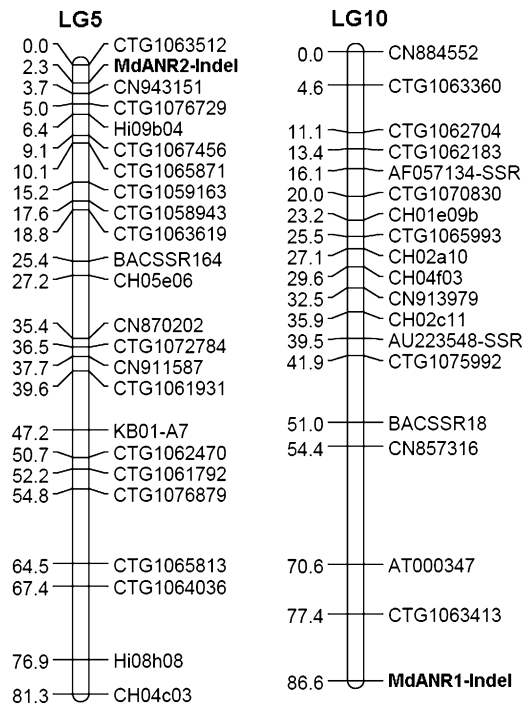


Fig. 4. Mapping of *MdANR1* and *MdANR2* genes onto apple genetic linkage groups 10 and 5, respectively.

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 *MdANR2a-6*, accumulated higher levels of quercetin when compared with flowers of the wild-type control. Moreover, 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.

Following qRT-PCR analysis, it was revealed that transgenic line *MdANR2b-4*, producing pure white flowers, exhibited the highest levels of transgene expression. *MdANR1-3* and *MdANR1-13* lines, producing white and red variegated flowers, respectively, exhibited relatively lower levels of transgene expression in their flowers when compared with those of other transgenic lines (Fig. 6b). Taken together, ectopic expression of *MdANR* genes in tobacco promoted the biosynthesis of PAs in flowers, but inhibited anthocyanin accumulation.

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

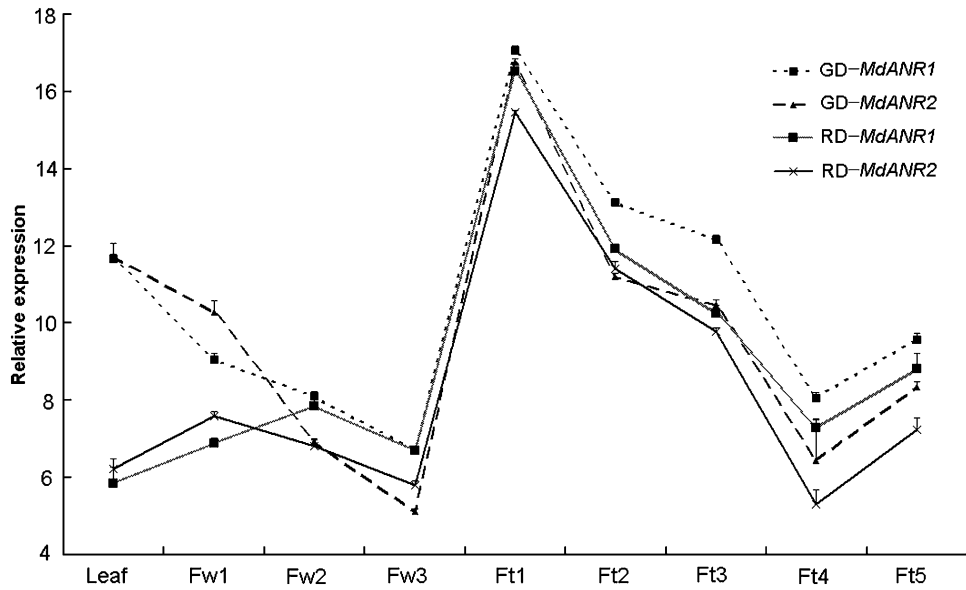


Fig. 5. Real-time PCR analysis of *MdANR1* and *MdANR2a/b* gene expression in various floral and fruit tissues of ‘Red Delicious’ and ‘Golden Delicious’. Differential expression of *MdANR1* and *MdANR2* between the two apple cultivars Red Delicious and Golden Delicious was compared. Abbreviations for stages of development are as follows: Fw1, flower buds at the pink stage; Fw2, flower buds at the balloon stage; Fw3, flowers at full bloom; Ft1, fruitlets 9 DAP; Ft2, fruitlets 16 DAP; Ft3, fruitlets 44 DAP; Ft4, fruits at 104 DAP; Ft5, fruits at 145 DAP; RD, ‘Red Delicious’; GD, ‘Golden Delicious’. All expression data were normalized to the apple actin gene, and values are means of three technical replicates.

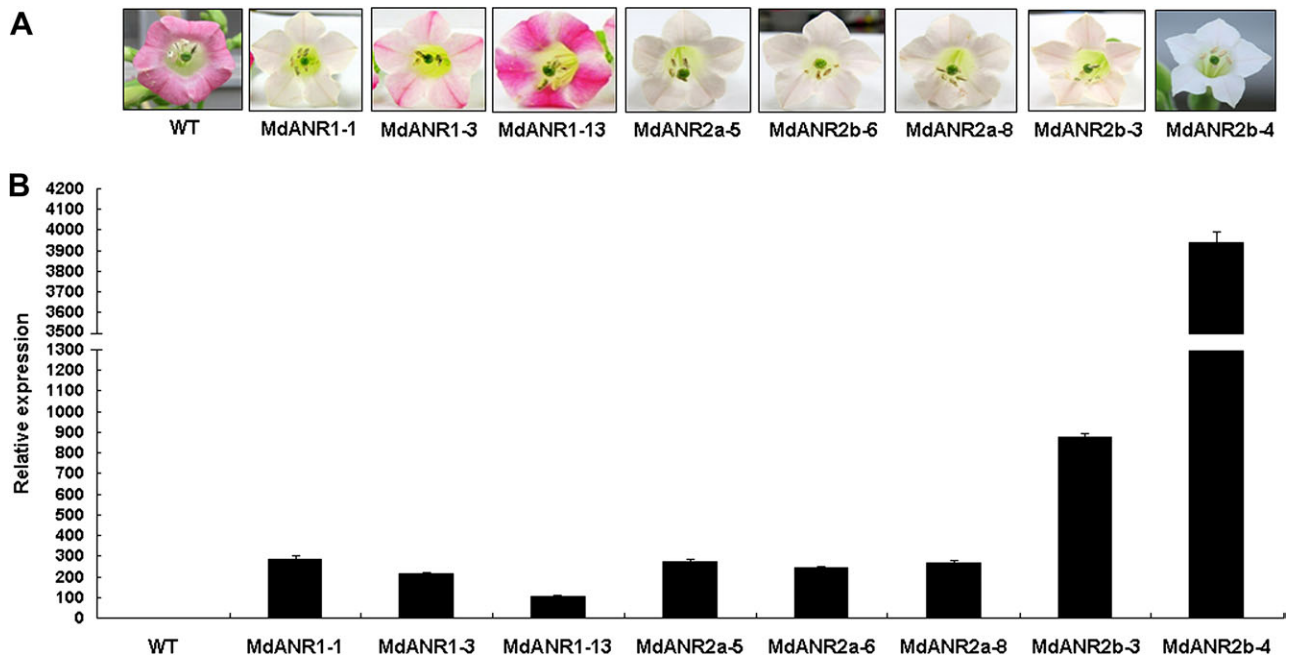


Fig. 6. Ectopic expression of *MdANR1* and *MdANR2* genes in tobacco. (A) Tobacco flowers of wild-type (WT) and T_2 transgenic lines. Transgenic lines containing *MdANR1*, *MdANR2a*, and *MdANR2b* genes were pre-fixed with *MdANR1*, *MdANR2a*, and *MdANR2b*, respectively. (B) Expression profiles of *MdANR* genes in flowers of transgenic tobacco lines.

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

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

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

^a All data correspond to mean values \pm SD of three biological replicates. N/A, not available.

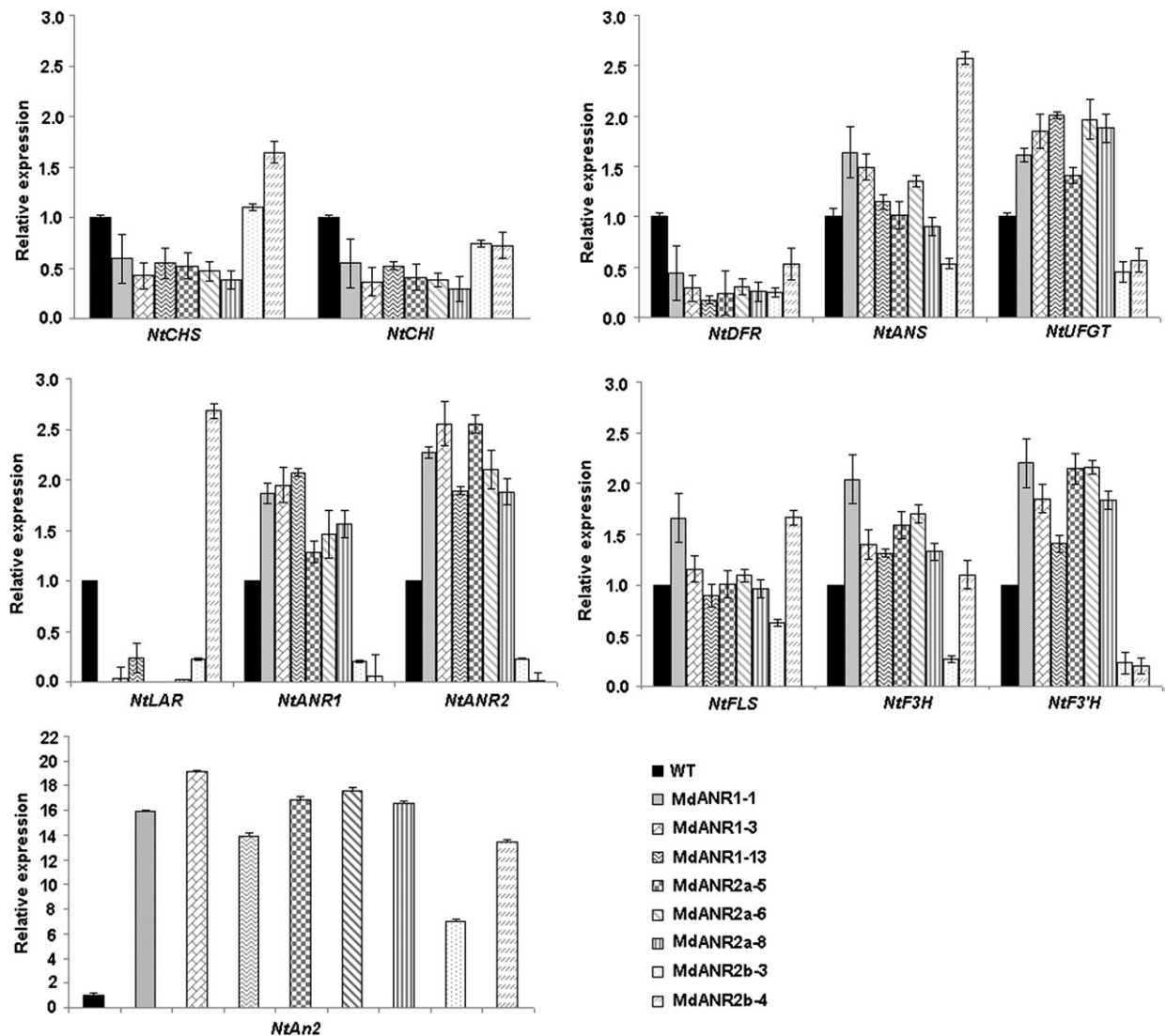


Fig. 7. Expression profiles of flavonoid-related structural biosynthetic genes in flowers of transgenic tobacco lines carrying *MdANR* genes. All mRNA transcripts expressed in transgenic flowers were quantified relative to those expressed in wild-type tobacco flowers.

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,

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 catalyse 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.*, 2010; 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' HYDROXYLASE (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, *Tag1-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 *NtLAR*, *NtDFR*, and *NtCHI* genes in transgenic tobacco lines. Of these three genes, *NtLAR* and *NtDFR* are related to *MdANR*. Collectively, *MdANR*, *NtLAR*, and *NtDFR* belong to the reductase-epimerase-dehydrogenase (RED) superfamily although they share low identity in their DNA coding sequences. However, co-suppression of *NtLAR* and *NtDFR* 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 *NtANR* genes in tobacco. Secondly, *ANR* is more closely related to *DFR* than to *LAR*; however, overexpression of an *MdANR* gene suppresses expression of *NtLAR* more severely than that of *NtDFR*. Therefore, the mechanism of co-suppression is rather complicated, and requires further investigation.

LAR converts leucoanthocyanidin into catechin and it competes with anthocyanidin 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 *NtLAR* to accept NADPH, resulting in low levels of expression of *NtLAR* 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 *NtLAR* and *NtDFR* may be due to interactions among enzymes involved in the flavonoid biosynthetic pathway.

Variiegated 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

(Grotewold, 2006). 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 coordinately 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 F₁ apple population.

Figure S2. Segregation for the *MdANR2* gene in an F₁ 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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