Flavonoid biosynthesis is one of the most extensively studied secondary metabolic pathways in plants (Winkel-Shirley, 2001a, 2001b; Grotewold, 2006). It is regulated by a complex network of signals triggered by internal metabolic cues and external signals, including visible light, UV radiation, pathogen attack, nitrogen, phosphorus, and iron deficiencies, low temperature, and wounding (Smith, 1982; Beggs et al., 1987; Wojtaszek et al., 1993; Christle et al., 1994; Lois, 1994; Vogt et al., 1994; Dixon and Paiva, 1995; Manthey et al., 2000; Bogs et al., 2007; Khavari-Nejad et al., 2009). Regulation of the flavonoid pathway branch producing flavan 3-ols, the building blocks of proanthocyanidins, has been studied in species including Arabidopsis (Arabidopsis thaliana), legumes (Medicago sativa, Medicago truncatula, Desmodium uncinatum, Lotus corniculatus), grapevine (Vitis vinifera), apple (Malus × domestica), and tobacco (Nicotiana tabacum; for review, see Marles et al., 2003; Lepiniec et al., 2006).

Studies in Arabidopsis and other plant species have identified key genes and enzymes of the proanthocyanidin-specific branch of the flavonoid pathway controlling the biosynthesis of 2,3-trans-flavan 3-ols (afzelechin, catechin, and gallocatechin [GC]) and 2,3-cis-flavan 3-ols (epiafzelechin, epicatechin, and epigallocatechin [EGC]) from flavan 3,4-diols (leucoanthocyanidins; Tanner and Kristiansen, 1993; Saito et al., 1999; Tanner et al., 2003; Xie et al., 2003). The flavan 3,4-diols can be directly reduced to 2,3-trans-flavan 3-ols by leucoanthocyanidin reductase (LAR; EC 1.17.1.3). LAR genes have been characterized in species including D. uncinatum (Tanner et al., 2003), tea (Camellia sinensis; Punyasiri et al., 2004), grapevine (Bogs et al., 2005), and strawberry (Fragaria × ananassa; Almeida et al., 2007) by in vitro assays of the encoded enzymes and in some cases by correlating gene expression with proanthocyanidin production and enzymatic activity in vivo (Pfeiffer et al., 2006; Pang et al., 2007). The conversion of flavan 3,4-diols to 3-OH-anthocyanidin molecules by anthocyanidin synthase (ANS; EC 1.14.11.19) represents a branch point between the proanthocyanidin and anthocyanin pathways. The anthocyanin pathway modifies 3-OH-anthocyanidins by a chain of glycosylation and esterification reactions, and the proanthocyanidin pathway catalyzes the reduction of 3-OH-anthocyanidins to 2,3-cis-flavan 3-ols.
by anthocyanidin reductase (ANR; EC 1.3.1.77). Genes encoding ANR have been isolated and characterized in a number of species, including Arabidopsis (Xie et al., 2003), M. truncatula (Xie et al., 2004), apple (Pfeiffer et al., 2006), L. corniculatus (Paolocci et al., 2007), and grapevine (Bogs et al., 2005).

Transcriptional regulation of flavonoid biosynthetic genes underlies the cell-specific and developmental organization of flavonoid biosynthesis (Roth et al., 1991; Tuerck and Fromm, 1994; Johnson et al., 2002; Nesi et al., 2002; Baudry et al., 2004). Recent results from Arabidopsis suggest that members of the MYB and R/B-like basic helix-loop-helix (bHLH) families form part of a regulatory network that controls flavonoid biosynthesis as well as developmentally controlled epidermal cell differentiation and cell patterning (for review, see Schiefelbein, 2003; Zhang et al., 2003; Lepiniec et al., 2006; Zhao et al., 2008). A combinatorial model for TRANSPARENT TESTA GLABRA1-dependent regulation of these pathways proposes an activator/repressor system that is based on competition for binding sites. The regulation of transcriptional regulators can be controlled by developmental mechanisms (Nesi et al., 2001; Broun, 2005) or by environmental or stress stimuli (Endt et al., 2002; Zhang et al., 2005).

Legumes offer many opportunities for studying proanthocyanidin biosynthesis and include species that accumulate proanthocyanidins across a broad range of concentrations and in different tissues (for review, see Marles et al., 2003; Lepiniec et al., 2006). M. sativa and M. truncatula plants accumulate a high level of proanthocyanidins in seed coats but low levels in flowers, stems, roots, and leaves (Pang et al., 2007). White clover (Trifolium repens) is a major component of temperate improved pastures worldwide and is a key forage plant in countries with intensive livestock production systems (Forster and Spangenberg, 1999). A low level of proanthocyanidins (3% of dry weight) in forages is beneficial for preventing pasture bloat and increasing nutrient uptake in ruminant livestock (Jones and Lyttleton, 1971; Waghorn and Jones, 1989; Wang et al., 1996). Although white clover plants accumulate a high level of proanthocyanidins in flowers and seed coats, there are very low levels in vegetative tissues, where proanthocyanidins, and/or their flavan-3-ol monomers, are restricted to trichome cells (Young and Paterson, 1980; Foo et al., 2000). Floral proanthocyanidins in white clover consist of nearly equal proportions of EGCs and GCs (Foo et al., 2000; Sivakumaran et al., 2004; Meagher et al., 2006). This property and a relatively high genetic transformation efficiency make white clover a good system for the functional analysis of genes involved in the biosynthesis of both 2,3-trans-flavan-3-ols and 2,3-cis-flavan-3-ols (Ding et al., 2003). Colocalization of the anthocyanin and proanthocyanidin pathways in epidermal cells of floral tissues is an additional advantage of this system, allowing the possibility of metabolic cross talk (Devic et al., 1999; Abrahams et al., 2003; Xie et al., 2003; Jørgensen et al., 2005) to be investigated.

In this study, a transcriptomic approach in combination with a biochemical analysis of selected flavonoids was used for molecular dissection of the proanthocyanidin and anthocyanin pathways in flowers of white clover. Spatiotemporal profiles of flavonoid gene expression and the accumulation of corresponding metabolites suggest that components of the anthocyanin and proanthocyanidin pathways could be encoded by distinct members of multigene families. Our gene-to-metabolite approach, integrating transcriptomic and biochemical data, suggests that down-regulation of the ANR gene correlates with changes in the expression of flavonoid-related genes, leading to metabolic reprogramming of the flavonoid pathway in white clover flowers.

RESULTS

Epidermal Cells of Petals Are the Main Site for the Production of Both Proanthocyanidins and Anthocyanins

Proanthocyanidins and flavan-3-ol monomers were histochemically stained in white clover organs and tissues using 4-dimethylaminocinnamaldehyde (DMACA; Fig. 1). As expected, floral organs stained strongly, indicating that a high level of proanthocyanidins and flavan-3-ol monomers were present. The accumulation pattern of proanthocyanidins in inflorescences at immature, partially (50%) open, and mature stages of development is shown in Figure 1, A to G. The accumulation of proanthocyanidins appeared to be developmentally regulated within immature and 50% open inflorescences, as indicated by intense staining of the oldest flowers located at the base of each inflorescence (Fig. 1, B, D, and E). White clover flowers have a calyx that consists of five fused sepals in which proanthocyanidins or flavan-3-ol monomers were detected only in trichomes (Fig. 1H). The white or pale pink asymmetrical corolla contains five petals: a single large standard petal and two wing petals, which enclose two keel petals (Fig. 1I). Proanthocyanidins were shown to accumulate in all three petal types, with accumulation within petals progressing from epidermal cells located on the abaxial side to epidermal cells on the adaxial side during development (Fig. 1, J and K). The bases of all five petals are fused to a tube of 10 stamens. A mosaic pattern of proanthocyanidin accumulation was detected on the abaxial side of stamen filaments (Fig. 1L). Figure 1M shows the accumulation of proanthocyanidins in a carpel. Proanthocyanidins or flavan-3-ol monomers were detected only in trichomes of aerial vegetative organs of white clover (Fig. 1N), including peduncles, stolons, stipules, data not shown), leaves, and petioles (Fig. 1, O and P). Trichomes staining heavily with DMACA were seen in leaves at stage 0.2 of development (Thomas, 1987; Fig. 1O). The accumulation of proanthocyanidins in peduncles was similarly restricted to trichomes (Fig. 1P).
Anthocyanins accumulated in both epidermal and subepidermal cells of aerial vegetative organs, with no detectable accumulation in trichomes. The accumulation of anthocyanins in floral organs was also restricted to epidermal cells and was most clearly seen in a small group of cells on the sepals (Fig. 1, Q–T) and in petals (Fig. 1U). Anthocyanins accumulated in epidermal cells located on the adaxial side of leaves (Fig. 1, V and W).

Flavonoid Levels and Composition during Floral Development in White Clover

We divided the inflorescences transversely at three selected developmental stages, namely immature, 50% open, and mature stages, for quantitative analyses of flavonols, proanthocyanidins, and flavan 3-ols during flower development. This allowed the less developed flowers (upper part of inflorescence) and more developed flowers (lower part of inflorescence) within each inflorescence to be analyzed separately (Fig. 2A). As a result, flower development was represented by six stages, the youngest being the upper part of immature inflorescences (stage 1) and the most developed being the lower part of mature inflorescences (stage 6; Fig. 2A).

A quantitative polyvinylpolypyrrolidone (PVPP)-butanol-HCl assay showed that a very low level of proanthocyanidins accumulated in leaves (Fig. 2B), reflecting their presence only in trichomes. The accumulation of proanthocyanins was developmentally regulated in flowers, the highest level being detected at stage 4 (Fig. 2B). Analysis of free flavan 3-ol levels and composition in inflorescences using liquid chromatography-mass spectrometry (LC-MS)
revealed the presence of only GC and EGC monomeric units (Fig. 2C). The accumulation of EGC and GC was also found to be developmentally regulated in flowers, with free monomers detected at stage 2 and the highest levels recorded at stage 3. A higher level of GC than EGC was seen at all six stages of flower development. A very low level of anthocyanins was detected at all stages of flower development (data not shown), reflecting low levels of visible anthocyanin accumulation in petals (Fig. 1, C, F, and I). Analysis of the levels and composition of flavonols revealed four main flavonol glycoside species, with myricetin (F1, mass-to-charge ratio \([m/z] 479\)), quercetin (F2, \(m/z 463\); F4, \(m/z 505\)), and kaempferol (F3, \(m/z 447\)) backbones (Fig. 2D).

The stereochemistry of the sugar unit and the position of the acetate moiety in these molecules were not established. Levels of the four flavonol glycosides increased during flower development, each showing the highest levels in mature flowers. The myricetin glycosides (F1, \(m/z 479\); R3 =OH, R5 =OH) were predominant in immature inflorescences (stages 1–2), almost equal levels of myricetin and quercetin glycosides (F2, \(m/z 463\); F4, \(m/z 505\); R3 =OH, R5 =H) were found at flower stage 3, and quercetin glycosides were the most abundant species at later stages of development.

Flavonoid Gene Expression Is Developmentally Regulated in White Clover Flowers

Transcript accumulation patterns of 12,000 white clover genes at six stages of flower development were monitored using a custom-made 12K CombiMatrix oligonucleotide array. The normalized expression value of each gene was plotted on a log scale against the six developmental stages, and hierarchical clustering was used to identify groups of genes showing similar expression patterns across the developmental stages (Supplemental Fig. S1). All of these profiles passed the significance filter at \(P \leq 0.05\). A total of 2,398 genes showed expression differences when at least two of the six developmental stages were compared. This map had 738 output nodes and organized genes into 56 clusters of similar gene expression (Supplemental Fig. S2). Most of these differentially expressed genes showed expression peaks between either stages 1 and 3 (expression profile A) or 4 and 6 (expression profile B). Most of the flavonoid pathway-related genes conformed to one of these two expression profiles (Supplemental Tables S2 and S3). The differentially expressed genes were grouped into seven functional classes based on deduced amino acid sequence similarity. These classes were flavonoid enzymes, transcription factors, mediators of protein-protein interactions and protein stability, transporters, mediators of auxin biosynthesis and signal transduction, proteins involved in cell signaling, and metabolic enzymes not involved in flavonoid biosynthesis.

Two genes similar to molecular markers of proanthocyanidin biosynthesis, namely ANR and LAR, were identified within the group of genes showing expression profile A (Supplemental Table S3). The 338-amino acid predicted protein encoded by the ANR-like gene shared 92.4% sequence similarity (88.2% identity) with

Figure 2. Analysis of proanthocyanidin, flavan 3-ol, and flavonol glycoside levels in white clover inflorescences containing flowers at six stages of flower development. A, Appearance of white clover inflorescences and flowers at six stages of flower development. B, Level of proanthocyanidins (\(A_{510} \text{mg}^{-1}\text{dry weight [DW]} \times 10^{-3}\)). C, Level and composition of flavan 3-ols (\(\mu\text{g} \text{mg}^{-1}\text{dry weight}). D, Level and composition of flavonol glycosides (total ion current \(\times 10^6\)). L, Leaves; F1, myricetin glycoside, \(m/z 479\); F2, quercetin glycoside, \(m/z 463\); F3, kaempferol glycoside, \(m/z 447\); F4, quercetin acetyl-glycoside, \(m/z 505\). Chemical formulae of the flavonoids are shown in Supplemental Table S1. Data shown are means of three biological replicates. Error bars denote SE.
a functionally characterized ANR from *M. truncatula* and 84% similarity (75.4% identity) with the BA-NYULS protein from *Arabidopsis* (Xie et al., 2003; Supplemental Fig. S3). The deduced amino acid sequence of the LAR-like protein was similar to equivalent deduced sequences of LAR proteins from *M. truncatula* (86.5% amino acid identity) and *Phaseolus coccineus* (71.7%) as well as *L. corniculatus* LAR2-1 (71.6%; Bogs et al., 2005; Pang et al., 2007; Paolocci et al., 2007; Supplemental Fig. S3). Southern hybridization analysis of genomic DNA suggested that the TrANR gene is represented by a small multigene family in white clover (Supplemental Fig. S4). The TrANR and TrLAR genes showed developmentally regulated expression profiles with the highest levels of gene expression at stage 3 (Supplemental Fig. S5), correlating well with accumulation of the corresponding flavan 3-ols (Fig. 2C). The expression level of TrANR was higher than that of TrLAR at all stages of flower development.

Many candidate genes encoding flavonoid pathway enzymes, transcription factors possibly involved in the regulation of flavonoid pathway enzymes, and transporters potentially involved in the compartmentalization of flavonoids into the vacuole also shared this expression profile (see Fig. 6 below; Supplemental Table S2). It was interesting that different members of some gene families with representatives up-regulated between stages 1 and 3 were found to be induced at later stages of flower development (see Fig. 6 below; Supplemental Table S3). Real-time reverse transcription (RT)-PCR was used to validate the microarray data, with an emphasis on the expression of molecular markers of proanthocyanidin biosynthesis (ANR and LAR), anthocyanin biosynthesis (anthocyanin 5-aromatic acetylase [ANAT] and UDP-Glc glucosyl-transferase [UGT]), as well as CHALCONE SYNTHASE (CHS) and ANS genes. The expression profile A genes included ANR, LAR, CHS2, CHS6, CHS7, ANS1, and MYB1. The expression profile B genes included ANAT3, UGT4, CHS10, ANS3, MYB8, and MYB5. In all cases, there was a good correlation between the results of quantitative real-time RT-PCR and microarray analyses (Supplemental Fig. S5).

**Down-Regulation of the ANR Gene Correlates with Changed Levels of Flavonoids in Flowers**

The in planta function of TrANR was analyzed in 18 transgenic white clover plants in which TrANR expression was down-regulated using a double-stranded RNA interference-mediated posttranscriptional gene-silencing strategy involving a TrANR hairpin construct (TrANRhp). The presence of transgenes in the T0 generation of transgenic plants was verified by real-time PCR (data not shown). No significant phenotypic differences were found between the transgenic and wild-type plants in vegetative organs. However, the petals from flowers of the transgenic TrANRhp lines displayed three main color phenotypes: white/light pink, resembling wild-type flowers (lines W1, W9, and W10); pink (lines P9, P8, P1, and P10); and dark red (lines R10, R14, R11, R4, R2, R8, and R9; Fig. 3, A–C). The highest level of anthocyanin pigmentation in the red-flowered lines was observed at flower stages 3 and 4. The pigmentation of petals in red-flowered lines was less intense at later stages of inflorescence development (Fig. 3, D and E). No significant differences were seen between sepal of wild-type and transgenic lines at any developmental stages (Fig. 3F). Light microscopy revealed a high level of anthocyanin accumulation in proanthocyanidin-producing cells of red-flowered TrANRhp lines at early stages (2–3) of flower development, namely, epidermal cells of petals, carpels, and stamens (Fig. 3, G–K). Anthocyanins were not seen in these organs in wild-type plants at corresponding stages of development (data not shown). A mosaic pattern of anthocyanin accumulation in epidermal cells of stamen filaments and carpels in TrANRhp lines correlated with the distribution of proanthocyanidin-producing cells in wild-type plants (Fig. 3, N–T). Transcript levels of TrANR were measured in 50% open inflorescences (stages 3 and 4) of transgenic and wild-type plants using quantitative real-time RT-PCR. A red-flowered phenotype correlated with a reduction in the level of TrANR expression in TrANRhp lines (Fig. 4). Pink-flowered TrANRhp lines showed an intermediate level of TrANR expression that was higher than that of red-flowered transgenic lines but lower than that of most transgenic lines with white or light pink flowers and wild-type plants (Fig. 4).

Entire 50% open inflorescences (stages 3 and 4) of red-flowered TrANRhp lines (R10, R14, R11, R4, R2, R8, and R9) and the pink-flowered transgenic line P9 were analyzed biochemically to characterize proanthocyanidin, flavan 3-ol, anthocyanin, and flavonol glycoside accumulation. Analysis of 50% open inflorescences with a PVPP-butanol-HCl assay showed up to a 4-fold reduction in the level of total proanthocyanidins in red-flowered transgenic plants relative to wild-type plants, while the pink-flowered TrANRhp-P9 transgenic line and wild-type control plants showed similar levels of total proanthocyanidins (Fig. 5A). Biochemical analysis of flavan 3-ols showed that TrANRhp lines with red-flowered phenotypes had lower levels of EGC in 50% open inflorescences, in comparison with wild-type plants (Fig. 5B). Interestingly, flowers of these plants also showed a reduced level of GC. The TrANRhp-R10 line had a 4-fold lower level of EGC than wild-type plants and a 30% reduction in the level of GC. The TrANRhp-R8 line showed a similar reduction in the level of EGC as TrANRhp-R10 but up to a 70% reduction in the level of GC, in comparison with wild-type plants. The pink-flowered TrANRhp-P9 line did not show any significant reduction in EGC and GC levels, relative to wild-type plants.

Analysis of anthocyanins in 50% open flowers of wild-type plants revealed two major molecules, delphinidin 3-sambubioside (A1) and cyanidin 3-sambubioside (A2),...
the level of A1 being approximately 2- to 3-fold that of A2 (Fig. 5C). As expected, there was a positive correlation between anthocyanin levels in 50% open inflorescences of transgenic plants and the intensity of petal coloration (Fig. 5C). All lines with red-flowered phenotypes had higher levels of A1 than wild-type plants. No significant differences were observed in A1 and A2 levels between the pink-flowered TrANRhp-P9 line and wild-type plants.

The levels of two major flavonol glycosides were modified in 50% open flowers from some TrANRhp lines, in comparison with those of wild-type plants (Fig. 5D). The level of myricetin glycoside (F1, m/z 479) was up to 3-fold higher in red-flowered transgenic TrANRhp lines than in wild-type plants. An intermediate level of myricetin glycoside accumulation was detected in transgenic lines with a pink-flowered TrANRhp-P9 line and wild-type plants.

Down-Regulation of the ANR Gene Correlates with Changes in the Expression of Flavonoid-Related Genes

A comparison was made between transcript accumulation patterns of 12,000 white clover genes in 50% open inflorescences of three red-flowered TrANRhp lines (R2, R4, and R10) and three wild-type lines with the custom-made CombiMatrix oligonucleotide array, as used previously in this study. Only expression profiles that passed the significance filter at \( P \leq 0.05 \) were analyzed (Supplemental Tables S4 and S5). Approximately 900 genes were up-regulated and 500 genes were down-regulated in the TrANRhp lines, relative to wild type plants (Supplemental Fig. S6). A large proportion of these genes (approximately 400) showed no BLAST hits or matched only hypothetical proteins. Of the annotated genes potentially involved in metabolic pathways, 150 were up-regulated and 123 were down-regulated in the TrANRhp lines, relative to wild-type plants. Representatives of each of the same seven functional classes used for transcript abundance profiling in developing flowers were also up- or down-regulated in TrANRhp lines (Supplemental Tables S4 and S5).
Thirty putative flavonoid pathway genes were up-regulated in the TrANRhp lines, relative to wild-type plants, including 10 genes potentially involved in the modification of anthocyanins (Fig. 6; Supplemental Table S4). It was interesting that candidate genes encoding 11 enzymes catalyzing different steps of the flavonoid pathway upstream of ANR, and genes similar to those encoding six enzymes of the isoflavonoid pathway, were also up-regulated in inflorescences of TrANRhp lines, relative to wild-type plants (Supplemental Table S4). Ten genes that were down-regulated in TrANRhp plants encoded known flavonoid-related enzymes (Fig. 6; Supplemental Table S5). As expected, the expression of ANR was lower (3.8-fold) in these lines than in wild-type plants. Quantitative real-time RT-PCR was used to validate data from the second microarray experiment. A sample of genes up-regulated or down-regulated in TrANRhp lines relative to wild-type plants was selected, namely ANS1, CHS10, CHS2, CHS6, and ANR (Supplemental Fig. S7). The expression profiles of these genes, determined by quantitative RT-PCR, correlated well with the results of the microarray experiment.

DISCUSSION

Distinct Representatives of Flavonoid-Related Multigene Families Are Likely to Contribute to Spatiotemporal Profiles of Anthocyanin and Proanthocyanidin Accumulation in Flowers

The developmentally regulated anthocyanin and proanthocyanidin pathways were found to be spatially colocalized in epidermal cells of white clover flower petals. Accumulation of flavan 3-ol monomers and proanthocyanidins began in immature inflorescences and peaked in partially open inflorescences. This correlated well with the temporal expression profile of the TrANR gene during floral development in white clover. A low level of anthocyanin accumulation was detected visually in epidermal cells of

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Analysis of TrANR transcript levels in TrANRhp white clover lines. Normalized relative TrANR transcript levels were determined in 50% open inflorescences of the indicated lines by quantitative real-time RT-PCR. R lines, Transgenic lines with red petals; P lines, transgenic lines with pink petals; W lines, transgenic lines with white petals; WT, wild-type cv Mink plants. Real-time RT-PCR data were expressed as means of three technical replicates derived from a single pooled sample of three flowers from the same stage of development. Expression values were normalized relative to the endogenous EF1a control gene. Error bars, denoting s.e., are too small to be seen in bars representing transgenic lines.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Analysis of flavonoid levels in TrANRhp white clover lines. Level and composition of flavonoid pathway products in 50% open inflorescences are shown. A, Level of proanthocyanidins (A_{280} mg^{-1} dry weight × 10^{-3}). B, Level of flavan 3-ols (μg mg^{-1} dry weight). C, Level and composition of anthocyanins (A_{500-550} mg^{-1} dry weight × 10^{5}). D, Level and composition of flavonol glycosides (total ion current × 10^{6}). A1, Delphinidin 3-sambubioside; A2, cyanidin 3-sambubioside; F1, myricetin glycoside, m/z 479; F2, quercetin glycoside, m/z 463; F3, kaempferol glycoside, m/z 447; F4, quercetin acetyl-glycoside, m/z 505; R lines, transgenic lines with red petals; P lines, transgenic lines with pink petals; WT, wild-type cv Mink. Chemical formulae of flavonoids are shown in Supplemental Table S1. Data are means of three technical replicates derived from a single pooled sample of three flowers from the same stage of development. Error bars denote s.e.
petals when they emerged from the sepals and were exposed to light. The onset of light-induced pigmentation in maturing flowers coincided with declining levels of flavan-3-ols and \( \text{TRANR} \) transcripts (Fig. 2C; Supplemental Fig. S5). We conclude that the activities of the proanthocyanidin and anthocyanin pathways are temporally separated, the proanthocyanidin pathway being active at an earlier stage of flower development than the anthocyanin pathway. The activities of the two pathways may overlap in epidermal cells of partially open flowers (stages 3 and 4). This raises questions about the molecular organization of these pathways and potential cross talk between them.

Proanthocyanidins and anthocyanins are produced by two related but distinct branches of the flavonoid pathway (Ray et al., 2003). Both branches involve the conversion of 4-coumaroyl-CoA and malonyl-CoA to flavan-3,4-diol and 3-OH-anthocyanidin molecules.

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**Figure 6.** A model for flavonoid biosynthesis in white clover flowers based on biochemical and transcriptomic analyses of \( \text{TrANR} \) transgenic white clover lines in which the endogenous \( \text{ANR} \) gene was targeted for silencing. Specific compounds are listed in lowercase letters. Classes of compounds are listed in uppercase letters. Enzymes are shown as open boxes. Compounds and genes encoding enzymes marked in red were up-regulated in red-flowered transgenic lines, relative to wild type cv Mink white clover plants. Compounds and genes encoding enzymes marked in blue were down-regulated in the transgenic lines. Full names of enzymes and transcriptomic data, obtained from a microarray experiment in which inflorescences at the 50% open stage of development were harvested from three independent transgenic lines and three wild-type white clover plants of different genotypes and analyzed using a custom-made 12K CombiMatrix oligonucleotide array, are shown in Supplemental Tables S4 and S5.
(Winkel-Shirley, 2001a; Grotewold, 2006). Flavonoid enzymes are commonly encoded by members of multigene families (Nesi et al., 2000; Yonekura-Sakakibara et al., 2007), and it would be interesting to know whether the anthocyanin and proanthocyanidin pathways recruit exactly the same enzymes, or distinct isoforms encoded by paralogous genes, for shared steps in flavonoid production. In support of the possibility of anthocyanin- or proanthocyanidin-specific isoforms of enzymes catalyzing these shared steps, distinct members of gene families encoding potential CHS, DFR, F3H, and ANS enzymes and transporters showed different expression profiles during flower development in white clover (Fig. 6; Supplemental Tables S2 and S3). Although candidate genes encoding CHI, F3′H, and F3 5′H, conformed to only one expression profile, expression of these genes was detectable throughout flower development (Supplemental Tables S2 and S3). Since members of the MYB, bHLH, and WDR families of transcription factors also showed different expression profiles during flower development in white clover, it would be interesting to know whether proanthocyanidin- or anthocyanin-specific R2R3-MYB/bHLH/WDR transcriptional complexes (de Vetten et al., 1999; Spelt et al., 2000; Baudry et al., 2004; Gonzalez et al., 2008) cocomodulate regulatory genes encoding biosynthetic enzymes in these pathways and are themselves transcriptionally controlled by developmental cues.

**ANR Activity Is Necessary and Sufficient for 2,3-cis-Flavan 3-Ol Production in White Clover Flowers**

Loss of ANR function in Arabidopsis mutants with null alleles of the BANYULS gene results in a transparent testa phenotype without flavan 3-ols in seed coats (Devic et al., 1999; Abrahams et al., 2002, 2003). Recombinant ANR protein is capable of catalyzing the conversion of cyanidin, delphinidin, and pelargonidin molecules into epicatechin, EGC, and epiafzelechin, respectively, in vitro (Xie et al., 2004). Ectopic expression of ANR in tobacco flowers down-regulates anthocyanin accumulation and increases the production of proanthocyanidins (Xie et al., 2003). These findings suggest that ANR activity is necessary and sufficient for the production of 2,3-cis-flavan 3-ols from flavan 3,4-diols. An intriguing finding of our study was that down-regulation of the ANR gene was correlated with reduced levels of GC in some 

**Cross Talk within the Flavonoid Pathway**

The anthocyanin and proanthocyanidin pathways share dihydroflavonols as precursor molecules. There are three classes of these molecules, differing only in the extent of B-ring hydroxylation (Abrahams et al., 2002; Pang et al., 2007; Tanaka et al., 2008). Modification of dihydrokaempferols (R3′=H, R5′=H), dihydroquercetins (R3′=OH, R5′=H), and dihydromyricetins (R3′=OH, R5′=OH) by DFR, ANS, and a range of anthocyanin-modifying enzymes leads to the biosynthesis of anthocyanins with pelargonidin (R3′=H, R5′=H), cyanidin (R3′=OH, R5′=H), and delphinidin (R3′=OH, R5′=OH) backbones, respectively (Tanaka et al., 2008). Alternatively, dihydroflavonols can be converted to cis- and trans-epimeric forms of afzelechins (R3′=H, R5′=H), catechins (R3′=OH, R5′=H), and GCS (R3′=OH, R5′=OH) by the DFR, LAR, ANS, and ANR enzymes of the proanthocyanidin pathway.

Glycosylated forms of three flavonols, representing all three B-ring hydroxylated variants of dihydroflavonols, were found in white clover flowers, with an abundance of myricetin glycosides (F1, m/z 479; R3′=OH, R5′=OH) at the most immature stages and an increased level of quercetin glycosides (F2, m/z 463; F4, m/z 505; R3′=OH, R5′=H) at later developmental stages. Anthocyanin composition at all of these stages showed a predominance of delphinidin-based anthocyanins and a much lower level of cyanidin-based anthocyanins. A low level of kaempferol-based anthocyanins (F3, m/z 447; R3′=H, R5′=H) and virtually no pelargonidin-based anthocyanins were found at all developmental stages. Analysis of flavan 3-ols in white clover flowers resulted in the detection of only GCS and EGCs throughout development, with a higher level of GCs.

Cross talk between proanthocyanidin- and anthocyanin-specific branches of the flavonoid pathways was shown in seed coats of the Arabidopsis banyuls mutant, where down-regulation of ANR leads to the precocious accumulation of anthocyanins in proanthocyanidin-producing tissues (Devic et al., 1999; Abrahams et al., 2003). As mentioned earlier, overexpression of ANR in tobacco down-regulates anthocyanin production and results in the accumulation of proanthocyanidins in flower petals (Xie et al., 2003). Ectopic expression of ANR in M. truncatula plants resulted in a decrease of approximately 50% in anthocyanin production and up to a 3-fold increase in proanthocyanidin production in a subset of leaf cells.
(Xie et al., 2006). This change was attributed to the diversion of metabolic flux away from anthocyanin production and toward proanthocyanidin biosynthesis. In white clover plants with reduced expression of the ANR gene, a lower level of EGCs was associated with higher levels of products of the flavonol and anthocyanin pathways (Fig. 6). Enhanced accumulation of delphinidin-based anthocyanins could be explained by the diversion of intermediates, such as delphinidins, from flavan-3-ol to anthocyanin production. The finding that anthocyanins accumulated in epidermal cells of the inner whorls of immature flowers of transgenic lines, which produce only proanthocyanidins in wild-type plants (Fig. 3, L–T), supports this explanation as being the basis for the phenotype. It is unlikely that light-induced up-regulation of anthocyanin biosynthesis in carpels and stamens at stages 2 and 3 may have occurred, as these organs are covered by petals and sepals and anthocyanins were never visualized in carpels and stamens of flowers from wild-type white clover plants at these stages of development (Supplemental Fig. S8). The greatly increased levels of anthocyanins in red-flowered TrANRhp lines were developmentally regulated, with petals showing the most intense coloration approximately halfway through flower maturation and fading at later stages (Fig. 3, C–E). This observation is consistent with the idea that a temporary excess of intermediate molecules, due to down-regulation of the ANR gene, may have been the trigger for this metabolic diversion.

Differences in the levels of flavonoids between wild-type and TrANRhp white clover plants correlated with dramatic differences in the expression levels of (1) genes functioning downstream of ANS and ANR and encoding members of the glucosyltransferase, UDP-glucuronosyl/UDP-glucosyltransferase, glutathione transferase, methyltransferase, and anthocyanidin rhamnosyltransferase families and (2) genes functioning upstream of ANR in the general flavonoid pathway or in isoflavone biosynthesis, namely CHS-, CHI-, F3H-, F3’/5’H-, DFR-, ANS-, CHR-, IF3’H-, IFOMT-, and VR-like genes. Some transcription factors were also up-regulated in TrANRhp lines, providing further support for transcriptional regulation of this pathway (Johnson et al., 2002; Nesi et al., 2002; Baudry et al., 2004; Dubos et al., 2008). Another interesting consequence of down-regulating the TrANR gene was differential changes in the expression of members of the same gene family. The cumulative data are summarized in Figure 6.

In this study, we present experimental data correlating spatiotemporal patterns of anthocyanin and proanthocyanidin biosynthesis with differential expression patterns of flavonoid-related genes in developing white clover flowers. Our findings support a model where the anthocyanin and proanthocyanidin pathways are spatially colocalized within epidermal cells of petals but temporally separated, overlapping only in partially open flowers. These pathways may recruit distinct representatives of flavonoid-related enzymes encoded by multigene families. Altered levels of flavonoid pathway products and changes in the expression of many flavonoid-related genes provide evidence for metabolic reprogramming in TrANRhp lines due to the down-regulation of ANR gene expression and the possibility of cross talk between metabolic channels producing proanthocyanidins, anthocyanins, and flavonol glycosides. Our findings support a role for ANR in the biosynthesis of 2,3-trans-flavan 3-ols, in addition to its known function in the reduction of anthocyanidins to 2,3-cis-flavan 3-ols (Xie and Dixon, 2005).

MATERIALS AND METHODS

Plant Growth Conditions

Wild-type and transgenic white clover (Trifolium repens ‘Mink’) plants were grown at 22°C with a 16-h photoperiod and a light intensity of 240 ± 30 μmol m⁻² s⁻¹. The plants were vernalized in a controlled growth room for 6 weeks at 5°C with an 8-h photoperiod and a light intensity of 41 ± 5 μmol m⁻² s⁻¹ at canopy height. Flowering was then induced in a controlled growth cabinet (Enconair) by growing plants for 4 weeks at 22°C with a 16-h photoperiod and a light intensity of 240 ± 30 μmol m⁻² s⁻¹ at canopy height.

Southern Hybridization Analysis

Southern hybridization analysis was performed as described previously (Ludlow et al., 2009). Genomic DNA (20 μg) was digested with 10 units of each restriction enzyme (EcoRI, EcoRV, and HindIII) and hybridized with a digoxigenin-labeled probe (Supplemental Table S5) corresponding to the coding region of the TrANR gene.

Generation and Analysis of Transgenic Plants

Transgenic white clover plants (cv Mink) were generated by Agrobacterium tumefaciens-mediated transformation using cotyledonary explants and selection with 50 mg L⁻¹ kanamycin sulfate as described previously (Ding et al., 2003). DNA was extracted from leaf tissue of putative transgenic lines using the DNeasy Plant Mini Kit (Qiagen; catalog no. 69104) according to the manufacturer’s protocol and screened by real-time PCR for the presence of the npt2 selectable marker gene, using the primers qPCR-F/R listed in Supplemental Table S6. The real-time PCR master mix consisted of 5 μL of 2× SYBR Green Master Mix (Applied Biosystems; catalog no. 4309155), 0.2 μL of each primer (10 μM), and denonized water to a total volume of 10 μL. The real-time PCR Master Mix was added to the DNA sample or the control sample (10 ng–1.5 μg). An Mx3000P (Stratagene) real-time PCR thermo cycler was used. Thermo cycling conditions were as follows: 95°C for 10 min; followed by 40 cycles of 95°C denaturation for 30 s, 55°C annealing for 1 min, and 72°C extension for 30 s; and a final cycle of 95°C denaturation for 1 min, 55°C annealing for 30 s, and 95°C denaturation for 30 s.

Visualization of Proanthocyanidins and Anthocyanins

Plant material was stained for the presence of proanthocyanidins and monomeric flavan 3-ols using 0.01% (w/v) DMACA in absolute ethanol containing 1% (w/v) concentrated hydrochloric acid (McMurrough and McDowell, 1978). Anthocyanins were visualized in untreated white clover tissues. Images were captured using an MZFLIII light microscope (Leica Microsystems) fitted with a CCD camera.

Biochemical Analysis of Flavonoids

A quantitative PVPP-butanol-HCl assay (Ray et al., 2003) was used to measure total proanthocyanidin levels in three biological replicates of 5- to 10- mg samples of freeze-dried, finely ground white clover material. Flavonol glycosides, flavan 3-ols, and anthocyanins were identified and quantified by
LC-MS analysis. Three technical replicates of freeze-dried, finely ground plant material (approximately 5 mg) were extracted three times in 0.5-mL aliquots of methanol:water (4:1, v/v). The combined extracts were dried with gentle warming under a stream of nitrogen and reconstituted in 200 μL of methanol: water (4:1, v/v). An Agilent 1100 series HPLC system equipped with a quaternary gradient pump, column heater, autosampler with sample cooler (maintained at 4°C), and diode array detector (data acquired over 190–800 nm), coupled to a Thermo Electron LTQ ion-trap mass spectrometer, was used for LC-MS analysis. Aliquots (5 μL) of each sample were injected onto a 150× 2.1-mm i.d., 3μm Thermo BDS Hypersil C18 column maintained at 40°C. The mobile phase consisted of two components: A (water and 0.1% [v/v] formic acid) and B (acetoniitile and 0.1% [v/v] formic acid), and followed the gradient at a flow rate of 0.2 mL min⁻¹: 0 to 5 min, 98% A; 5 to 25 min, 62% A; 26 to 35 min (0.3 mL min⁻¹), 98% A. For the identification of metabolites, LC-MS was run in polarity switching mode with MS² data acquired in both negative and positive modes. Analysis of the electrospray ionization (ESI) negative mode MS and MS² data allowed the identification of four flavanol glycosides, and analysis of the ESI positive mode MS and MS² data along with the photodiode array data allowed the identification of two anthocyanins. For enhanced sensitivity needed to quantify metabolites, LC-MS data were acquired in ESI negative mode with a mass range limited to 200 to 1,000 atomic mass units. The system was tuned using a 20 μg mL⁻¹ standard of epicatechin prior to data acquisition. Standard curves for GC and EGC were prepared by serial dilution of stock solutions and analyzed in conjunction with the samples. The results were linear over the range examined (8–285 ng for EGC, 5–81 ng for GC). Standards for the flavonol and for the UV-visible absorption (500–550 nm) were prepared by serial dilution of stock solutions and a no-template control in duplicate. Stratagene software generated a standard curve and calculated the quantity in fmol μL⁻¹ of the unknowns.

Preparation of Constructs for Plant Transformation

A full-length cDNA clone encoding a candidate ANR gene was identified by analysis of ESTs generated from a previously described white clover cDNA library (Sawbridge et al., 2003). A PCR product containing a 331-bp fragment of the TrANR gene corresponding to nucleotides 693 to 1,025 after the start codon was amplified from a complete cDNA clone (Supplemental Table S5). The PCR product was cloned into pDONR221, sequenced, and then subcloned into a Gateway-enabled plant expression vector to create inverted repeats of the TrANR gene in a hairpin expression cassette under the control of an enhanced cauliflower mosaic virus 35S promoter and the 35S terminator (Smith et al., 2007). The final transformation vector was named pTrANRhp.

Analysis of Gene Expression in White Clover

Proprietary CombiMatrix (Mukilteo) custom Array software was used to design the oligonucleotide probes of 35 to 40 bases in length for each white clover unigene. The resulting probe set was then assigned to a custom-made 12K CombiMatrix oligonucleotide array. In the first microarray experiment, gene expression was studied at different stages of flower development. The upper and lower halves were collected from the immature inflorescences, from the inflorescences in which approximately 50% of flowers were opened, and from the mature inflorescences in which 100% of flowers were opened. Samples were collected from three independent batches of plants. Inflorescences were excised from plants and cut with a scalpel blade, snap frozen in liquid nitrogen, and stored at −80°C. In the second microarray experiment, three wild-type lines and three independent transgenic white clover lines containing a construct to silence the TrANR gene and having a red-flowered phenotype were vernalized as already described. Samples of 50% open inflorescences from wild-type plants and red-flowered TrANRhp white clover lines were excised from plants, snap frozen in liquid nitrogen, and stored at −80°C. Frozen inflorescences were ground to a fine powder with a mortar and pestle under liquid nitrogen, and total RNA was extracted using a cetyltrimethyl-ammonium bromide-based method (Chang et al., 1993). The total RNA samples were further purified using the RNeasy Plant Mini kit (Qiagen; catalog no. 74104) following the manufacturer’s protocol. Total RNA was quantified using spectrophotometry, and the quality was verified using an Agilent 2100 Bioanalyzer (RNA 6000 Nano Assay). The RNA samples were amplified, and labeling was performed using the MessageAmp II aRNA Amplification Kit (Ambion; catalog no. AM1751) and the Biotin-ULS aRNA Fluorescent Labeling Kit (Kreatech; catalog no. GEOA026) according to the manufacturers’ protocols. Each sample was hybridized to a separate array following the protocol recommended by the manufacturer (CombiMatrix). The hybridized arrays were scanned with an Axon GenePix 4000B instrument. Data were extracted using proprietary CombiMatrix Microarray Imager software. Background subtraction was performed by computing the mean signal intensity from the faintest 5% of all probes plus 2 standard units and deducting this value from all spots on the array. A minimum floor value was then set to 20 to eliminate any zero or negative spot values. The data on each array were then normalized using global median normalization (Draghici, 2003) prior to being log₂ transformed. Significant differences in gene expression levels between treatments were identified using ANOVA using the MAANOVA Bioconductor package (http://www.bioconductor.org/packages/2.3/bioc/html/maanova.html; Wu et al., 2003). Genes that showed a difference with a significance of P ≤ 0.05 were identified as showing markedly different gene expression between the treatments. Genes showing similar expression profiles across the six phenological ranges of flower development in the first experiment were identified using self-organizing maps in the SOM package from the R statistical programming environment (http://www.r-project.org/) and hierarchical clustering from the Bioconductor package (http://www.bioconductor.org). Gene annotations were assigned to the unigenes based on sequence similarity to entries within the UniProt and GenBank public databases, using the Bioinformatics Advanced Scientific Computing platform (Erwin et al., 2007).

Thirteen white clover flavonoid genes representing different expression profiles and four internal control genes were selected for the validation of microarray data using real-time RT-PCR. A list of the primers used is shown in Supplemental Table S6. All reactions were performed in duplicate 10-μL volumes using SYBR Green Master Mix (Applied Biosystems), and the optimized concentrations of forward and reverse primers (200–600 nM). The thermal profile (95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 30 s) was followed immediately by a dissociation curve protocol (95°C for 1 min, 60°C for 1 min, 20-min ramp time from 60°C to 95°C, followed by 95°C for 30 s). Controls included a no-reverse-transcriptase reaction for each sample and a no-template control in duplicate. Stratagene software generated a standard curve and calculated the quantity in fmol μL⁻¹ of the unknowns. The absence of more than one PCR product, genomic DNA contamination, and primer-dimers was ascertained from the dissociation curve profile. Expression values were normalized by geometric averaging of four internal control genes (Vandesompele et al., 2002) encoding glyceraldehyde 3-phosphate dehydrogenase, elongation factor 1α (Efiα), histone H4, and 5′-adenosyl-Met synthetase using geNorm software (PrimerDesign).

Analysis of TrANR transcript levels in TrANRhp and wild-type white clover lines was measured by real-time RT-PCR as described above. The data were expressed as the means of three technical replicates derived from a single pooled sample of three flowers from the same stage of development. Comparative expression of TrANR was calculated relative to that of the endogenous Efiα control gene using a relative quantification method (Dorak, 2006).

Sequence data from this article can be found in the GenBank/EMBL database under the following accession numbers: TrANR, GU300807; TrANRhp, GU300808. The microarray data have been submitted to ArrayExpress. The submission accession number is E-MEXP-1688.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Normalized microarray data from white clover genes showing significant differences in expression (P < 0.05) during the six stages of flower development shown in Figure 2.

Supplemental Figure S2. A self-organizing map with 7 x 8 output nodes organizing white clover genes into 56 clusters with similar gene expression profiles during flower development.

Supplemental Figure S3. Phylogenetic tree of several classes of reducetase-epimerase-dehydrogenase proteins.

Supplemental Figure S4. Southern hybridization analysis of the TrANR gene in wild-type white clover.

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gapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. Plant Physiol 143: 1347–1361
Manthey JA, Grohmann K, Berhow MA, Tisserat B (2000) Changes in...
citrus leaf flavonoid concentrations resulting from blight-induced zinc deficiency. Plant Physiol Biochem 38: 333–343
Winkel-Shirley B (2001b) It takes a garden: how work on diverse plant species has contributed to an understanding of flavonoid metabolism. Plant Physiol 127: 1399–1404