Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor

De-Yu Xie¹, Shashi B. Sharma¹, Elaine Wright², Zeng-Yu Wang² and Richard A. Dixon¹,*
¹Plant Biology Division, and
²Forage Improvement Division, Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

Received 17 September 2005; revised 12 November 2005; accepted 16 November 2005.
¹For correspondence (fax +1 580 224 6601; e-mail radixon@noble.org).

Summary

Proanthocyanidins (PAs) and their monomeric building blocks, the (epi)-flavan-3-ols, are plant antioxidants that confer multiple human health benefits. The presence of PAs in forage crops is an important agronomic trait, preventing pasture bloat in ruminant animals. However, many consumed plant materials lack PAs, and there has been little success to date in introducing monomeric or polymeric flavan-3-ols de novo into plant tissues for disease prevention by dietary means or development of ‘bloat-safe’ forages. We report the introduction of PAs into plants by combined expression of a MYB family transcription factor and anthocyanidin reductase for conversion of anthocyanidin into (epi)-flavan-3-ol. Tobacco leaves expressing both transgenes accumulated epicatechin and gallocatechin monomers, and a series of dimers and oligomers consisting primarily of epicatechin units. The levels of PAs reached values that would confer bloat reduction in forage species. Expression of anthocyanidin reductase in anthocyanin-containing leaves of the forage legume *Medicago truncatula* resulted in production of a specific subset of PA oligomers.

Keywords: condensed tannins, metabolic engineering, transcription factor, anthocyanidin reductase.

Introduction

Proanthocyanidins (PAs, also known as condensed tannins) are plant-derived flavonoid polymers with a wide range of benefits for human health. They are present in many foods and drinks, such as barley, sorghum, cranberry, tea and wine (Dixon *et al.*, 2005; Foo *et al.*, 2000) and are strong antioxidants, giving greater and more stable protection against free-radical-induced oxidative tissue damage than vitamins C or E (Bagchi *et al.*, 1998, 1999; Cossins *et al.*, 1998; Shi *et al.*, 2003). Proanthocyanidins from various plant species block the growth of human cancer cells in vitro (Tamagawa and Fukushima, 1998; Ye and Krohn, 1999), protect cells against UV light-induced damage (Carini *et al.*, 2000), protect against urinary tract infections (Foo *et al.*, 2000) or improve hair growth in mice (Takahashi *et al.*, 2000). Many commercial PA products are widely used as dietary supplements.

Proanthocyanidins are built by polymerization of monomeric flavan-3-ols. The monomers, namely the catechins and epicatechins and their derivatives, have themselves been ascribed a number of potential activities beneficial to health, including protection against Alzheimer’s disease (Okello *et al.*, 2004), oral and stomach cancers (Gonzalez de Mejía *et al.*, 2005; Okabe *et al.*, 1999) and cardiovascular disease (Serafini *et al.*, 2003).

The presence of PAs is an important agronomic trait in forage crops, protecting ruminant animals from potentially lethal pasture bloat associated with consumption of forages, such as alfalfa (*Medicago sativa*), with a high leaf protein content. By binding to proteins in the rumen, PAs reduce the rate of fermentation with resulting reduction in methane production, and allow more intact protein to exit the rumen, resulting in improved nitrogen nutrition. Alfalfa forage, the fourth most important crop in the United States, lacks PAs, and their introduction has been a major, but yet unrealized, goal of alfalfa breeding.

Engineering PAs in crop plants presents a particular challenge. Tissues that do not naturally synthesize these compounds may lack the necessary precursors. Proantho-
cyanidins share the same upstream biosynthetic pathway, from phenylalanine to leucanthocyanidin (flavan-3,4-diol), as utilized for biosynthesis of anthocyanin flower pigment (Figure 1; Springob et al., 2003). The downstream pathway to PAs involves leucanthocyanidin reductase (LAR) for production of (+)-flavan-3-ols such as (+)-catechin (Tanner et al., 2003) and anthocyanidin reductase (ANR), encoded by the BANYLUS gene in Arabidopsis (Xie et al., 2003), for production of (−)-(epi)-flavan-3-ols, such as (−)-epicatechin, from anthocyanidin (Figure 1). For a plant tissue to accumulate epicatechin-based PAs, it is therefore necessary for it to contain a functional anthocyanin biosynthetic pathway. In addition to this potential limitation, many questions remain as to the exact mechanisms of transport (to the vacuole) and polymerization of the epicatechin monomers (Dixon et al., 2005; Marles et al., 2003; Xie and Dixon, 2005). Thus, it remains unclear which additional biosynthetic and non-biosynthetic genes will be needed for engineering of PAs in any specific plant tissue that does naturally accumulate the compounds.

Transcription factors often control the expression of multiple enzymatic steps in natural product pathways in plants, and their ectopic expression may provide a simple means of coordinately up-regulating a whole biosynthetic pathway (Broun, 2004). Over 25 different transcription factors, belonging to seven different protein families (MYB, bHLH, WD40, WKRY, WIP, homeodomain and bMADS), have been implicated as controlling anthocyanin and/or PA biosynthesis in plants (Broun, 2005; Dixon et al., 2005; Springob et al., 2003). Over-expression of the PAP1 MYB gene of Arabidopsis activates most of the genes in the anthocyanin pathway leading to production of anthocyanins throughout the plant (Borevitz et al., 2001; Tohge et al., 2005b), and is therefore a potential tool for the introduction of the necessary precursor pools for PA engineering in plants.

Several studies have reported increasing or decreasing the levels of PAs in tissues in which these compounds occur naturally (Bavage et al., 1997; Damiani et al., 1999; Robbins et al., 1998; Tohge et al., 2005a). However, the results of these studies have not always been predictable (reviewed in Dixon et al., 2005). In a recent example, levels of PAs were paradoxically decreased in seeds of Arabidopsis constitutively expressing PAP1 (Tohge et al., 2005a). Metabolic engineering to introduce PAs into tissues that do not naturally make them has been unsuccessful to date because of the lack of understanding of the factors necessary for PA synthesis and accumulation in such tissues. Over-expression of the maize Lc gene resulted in the accumulation of PA-like compounds in alfalfa, but only if the plants were under abiotic stress (Ray et al., 2003). Co-expression of three transcription factors, TT2, PAP1 and Lc, was required to overcome cell-type-specific expression of PAs in Arabidopsis leaves and stems, but this constitutive accumulation of PAs was accompanied by death of the plants (Sharma and Dixon, 2005).

Low levels of PAs accumulate in tobacco flowers expressing an ANR transgene (Dixon et al., 2005; Xie et al., 2003), although not in vegetative tissue, presumably because of the lack of an anthocyanidin substrate. We report here a strategy for engineering PAs involving co-expression of ANR and PAP1. The anthocyanin pathway induced by PAP1 in tobacco leaves is diverted into epicatechin, gallocatechin and PAs on co-expression of PAP1 with ANR. These studies provide evidence for ectopic accumulation of PAs at agronomically useful levels as a result of metabolic engineering. Expression of ANR alone results in the accumulation of PA in the leaves of *Medicago truncatula*, a close relative of alfalfa.

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**Figure 1.** Biosynthetic pathways from naringenin to anthocyanins and PAs. The enzymes are: F3'H, flavanone-3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3,5'-hydroxylase; DFR, dihydroflavonol reductase; LAR, leucanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGL, uridine diphosphate glucose-flavonoid 3-O-glucosyl transferase. Enzymes whose transcript levels are up-regulated by PAP1 are marked with an asterisk.
Results

Accumulation of anthocyanins in tobacco transformed with Arabidopsis PAP1

To provide an anthocyanidin substrate for subsequent production of PAs, tobacco was transformed with Arabidopsis thaliana PAP1 cDNA under the control of the constitutive cauliflower mosaic virus 35S promoter, and T2 progeny expressing the gene were selected by RT-PCR analysis of leaf tissues (Figure S1). Wild-type tobacco plants produce anthocyanin pigmentation in the upper section of the flower corolla, but not normally in vegetative organs (Figure 2a,b). Expression of PAP1 resulted in massive accumulation of anthocyanins in leaves, flowers, stems and roots, which appeared deep red or purple (Figure 2a–d).

In spite of the intense, uniform appearance of pigment at the whole plant level in PAP1 transgenic plants, microscopic examination indicated that, in leaves, anthocyanin accumulated mainly in trichomes and epidermal cells located next to stomata (Figure 3c,f). Anthocyanin was found in epidermal cells on both abaxial and adaxial sides of the leaf, and in hypodermal and vascular parenchyma cells (Figure 3h). No anthocyanin was observed in any of these cell types in wild-type plants (Figure 3a,d,g). Palisade parenchyma cells of the leaf laminae on the adaxial side were weakly pigmented. In the flower corolla of PAP1 plants, anthocyanins accumulated in trichomes and in epidermal, hypodermal and parenchyma cells (data not shown).

Core anthocyanidins in PAP1 plants were analyzed after acid hydrolysis of the corresponding anthocyanins. The HPLC profiles of leaf extracts showed the presence of cyanidin as the major anthocyanidin, with smaller amounts of pelargonidin and a trace of delphinidin, none of which was present in extracts from leaves of wild-type plants (Figure 4). Flowers of PAP1 plants had a similar profile, whereas only cyanidin was found in hydrolysates of flowers from wild-type plants.

The levels of individual anthocyanidins in two independent PAP1 transgenic lines are shown in Table 1. Cyanidin was the major anthocyanidin in both leaves and flowers. Expression of PAP1 increased the level of total anthocyanidins by over three orders of magnitude compared with that in the corolla tissues of wild-type flowers (Table 1).

Generation of PAP + MtANR transgenic tobacco

To explore the possibility of converting the anthocyanidin accumulating in PAP1-expressing plants to epi-flavan-3-ols and further to PAs, Medicago truncatula anthocyanidin reductase (MtANR) was introduced into PAP1 transgenic tobacco by crossing. A total of 250 kanamycin-resistant PAP1 + MtANR transgenic seedlings, which showed segra-

gating leaf/stem phenotypes of purple, green-red or green (Figure 2), were grown in the greenhouse in pots. Controls were wild-type plants, T3 PAP1 plants, T1 and T2 progeny of MtANR transgenic plants and PAP1 + pBI121 transgenic plants generated by crossing the PAP1 line with plants transformed with empty pBI121 vector, all grown in parallel under the same conditions. To determine the expression levels of PAP1 and ANR, total RNA was isolated from young leaf tissue from 80 PAP1 × MtANR progeny representing all leaf color phenotypes, and analyzed by RT-PCR (Figure S1).

Plants with high expression of both PAP1 and MtANR exhibited reduced visible purple pigmentation compared with that in PAP1 or PAP1 + pBI121 controls (Figure 2). Extraction of anthocyanin from flowers and determination of individual anthocyanidins by HPLC (Table 1) or measurement of the overall absorption of the extracts at 524 nm (Figure S2) confirmed this conclusion. Lines expressing both transgenes had reduced extractable anthocyanin levels compared with those in lines expressing PAP1 alone, or PAP1 + pBI121. This suggests that ANR expression either inhibits anthocyanin formation or diverts flux into other pathways. Some of the PAP1 × MtANR or PAP1 × pBI121 progeny (e.g. lines 23, 282) had lost the PAP1 transgene by segregation (Figure S1), and therefore had anthocyanin levels similar to those of wild-type plants (Figure S2).

PA formation in flowers of PAP1 + MtANR tobacco

Wild-type tobacco plants do not produce PAs in floral tissues (Dixon et al., 2005). Flower tissue from 210 out of the 230 PAP1 + MtANR transgenic lines stained blue with 0.1% dimethylaminocinnamaldehyde (DMACA) reagent in the upper section of the corolla (Figure 5a). Blue staining with DMACA reagent is often used as a diagnostic test for PAs, although it can also detect flavan 3-ol monomers (Li et al., 1996). The intensity of the blue coloration was higher in PAP1 + MtANR transgenic flowers than in flowers of plants expressing MtANR alone. No PA staining was observed in flowers of PAP1 transgenic plants (Figure 5a).

Flower tubes of plants transgenic for PAP1 alone were highly pigmented. Anthocyanin levels appeared, on the basis of both visual examination and quantitative determination, to be significantly lower in T2 progeny of PAP1 + MtANR transgenic plants than in plants transgenic for PAP1 alone (Figures 2b and 3b, Table 1), but no PA staining was observed in the tubes (Figure 5a). To better analyze the PA content and composition in flowers, ethyl acetate extracts were analyzed by TLC. Extracts from leaves of the tannin-rich legume Desmodium uncinatum were included as positive controls. Expression of PAP1 alone resulted in detection of bright pink anthocyanin material on DMACA-sprayed TLC plates, but no blue material indicative of PAs (Figure 5b). In contrast, extracts from flowers expressing both PAP1 and

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Figure 2. Phenotypic comparisons of leaf (a), flower (b), stem (c) and root (d) of wild-type (W), MtANR, PAP1 and PAP1 - MtANR transgenic tobacco plants.
MtANR did not show pink anthocyanin but instead contained DMACA-positive material of various molecular weights, from monomeric (running at the solvent front) to highly polymeric (remaining on the origin of the TLC plate (Figure 5b)). Corolla tissues corresponding to the region above (upper) and below (lower) the arrow in Figure 5a were separated, and samples extracted with ethyl acetate. A TLC analysis demonstrated blue-staining oligomeric PAs in extracts from the lower sections (Figure 5b), although of a low degree of polymerization (dp) and at apparently lower levels than in corresponding extracts from upper sections or from whole flowers (Figure 5c). Note that the TLC assay is only semiquantitative. Staining with DMACA reagent may not detect PAs in flower tubes, either because of interference by anthocyanin or because of extraction of low-dp oligomers in the 50% ethanol staining solution.

The monomeric composition of PAs from pooled upper and lower corolla sections was determined by butanol:HCl hydrolysis (Li et al., 1996) of ethyl acetate extracts followed by TLC analysis. Because ethyl acetate cannot extract anthocyanin, the pigments produced from hydrolysis are derived from PAs. Cyanidin was the major product, with a trace of pelargonidin (Figure 5c). Thus, the extension units in the engineered PAs mainly consisted of flavan-3-ols with two hydroxyl groups on the B ring, most likely corresponding to units derived from (−)-epicatechin, since epi-flavan-3-ols are the main products of ANR (Xie et al., 2003, 2004). Using procyanidin B2 [epicatechin(4→beta→8)-epicatechin] as standard in butanol:HCl assays, PA levels in open flower corolla tissues were shown to be in the range of 25–263 μg g⁻¹ fresh weight (FW; Table 2, Table S1). Note that the low levels of PA reported in corolla tissues of wild-
Table 1 Levels of anthocyanidins\(^a\) in leaf and flower tissues of wild-type tobacco plants, two independent transgenic lines constitutively expressing Arabidopsis PAP1 and three lines co-expressing PAP1 and MtANR

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Delphinidin ((\mu g) g(^{-1}) FW(^b))</th>
<th>Cyanidin ((\mu g) g(^{-1}) FW(^b))</th>
<th>Pelargonidin ((\mu g) g(^{-1}) FW(^b))</th>
<th>Total ((\mu g) g(^{-1}) FW(^b))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># 292-leaf</td>
<td>6.3 ± 0.1</td>
<td>912.1 ± 21.4</td>
<td>31.0 ± 0.3</td>
<td>949.4</td>
</tr>
<tr>
<td># 292-flower</td>
<td>7.5 ± 0.7</td>
<td>958.6 ± 4.1</td>
<td>106.6 ± 1.6</td>
<td>1072.7</td>
</tr>
<tr>
<td># 293-leaf</td>
<td>6.7 ± 0.1</td>
<td>1344.9 ± 3.4</td>
<td>47.4 ± 0.8</td>
<td>1399.0</td>
</tr>
<tr>
<td># 293-flower</td>
<td>8.2 ± 0.1</td>
<td>531.4 ± 1.5</td>
<td>79.0 ± 2.9</td>
<td>618.6</td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Flower</td>
<td>0.0</td>
<td>0.5 ± 0.2</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>PAP1 – MtANR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># 13-leaf</td>
<td>3.3 ± 0.1</td>
<td>487.7 ± 2.3</td>
<td>13.6 ± 1.0</td>
<td>504.5</td>
</tr>
<tr>
<td># 13-flower</td>
<td>0.0</td>
<td>33.2 ± 1.6</td>
<td>10.4 ± 0.3</td>
<td>62.4</td>
</tr>
<tr>
<td># 47-leaf</td>
<td>0.0</td>
<td>546.6 ± 6.4</td>
<td>15.2 ± 0.8</td>
<td>561.8</td>
</tr>
<tr>
<td># 47-flower</td>
<td>2.8 ± 0.1</td>
<td>171.0 ± 7.0</td>
<td>15.0 ± 0.5</td>
<td>188.8</td>
</tr>
<tr>
<td># 136-leaf</td>
<td>0.0</td>
<td>321.2 ± 36.6</td>
<td>8.2 ± 0.3</td>
<td>329.5</td>
</tr>
<tr>
<td># 136-flower</td>
<td>0.0</td>
<td>135.5 ± 20.1</td>
<td>19.1 ± 3.6</td>
<td>154.8</td>
</tr>
</tbody>
</table>

\(^a\) Determined by HPLC after hydrolysis of anthocyanins.
\(^b\) Values represent the average and standard deviation from three replicate determinations.

Type and PAP1 transgenic tobacco by the butanol:HCl assay represent non-specific background absorption resulting from subtraction of the absorption of an independent wild-type extract from the absorption of the test samples (see Experimental procedures). No PAs were detected in corresponding extracts from these tissues by TLC (Figure 5).

Formation of PAs in leaves and stems of PAP1 + MtANR tobacco

Staining of PAP1 + MtANR tobacco leaves with DMACA reagent resulted in rapid formation of a blue coloration. However, this color was lost following de-staining in 70% ethanol, suggesting that the leaves contained oligomers with a lower molecular weight than did flowers. This was confirmed by TLC analysis. The TLC profiles of ethyl acetate extracts from leaf tissue of PAP1 + MtANR transgenic plants revealed a range of PAs of different molecular weights in addition to monomeric epicatechin (Figure 5d). Low levels of oligomers comparable in size to the mid-range PA oligomers from the tannin-rich legume D. uncinatum or from 70% acetone extracts of PAP1 + MtANR flowers were observed (Figure 5d). No PAs or epicatechin were detected in leaves from PAP1 + pBI121, PAP1 or wild-type plants.

Proanthocyanidins extracted with ethyl acetate from PAP1 + MtANR transgenic leaf tissue were hydrolyzed with butanol:HCl. The TLC analysis revealed cyanidin and pelargonidin as the major products (Figure 5e). No anthocyanidins were detected from parallel analysis of leaves of PAP1, PAP1 + pBI121, MtANR or wild-type plants. A greater
percentage of units in the engineered PAs was derived from (epi)-afzelechin (producing pelargonidin on hydrolysis) in leaves than in flowers (compare Figure 5c,e).

Mature leaf tissues of lines that co-expressed PAP1 and MtANR contained extractable PAs in the range of 12–363 μg PA B2 g⁻¹ FW (Table 2, Table S1), corresponding to 0.12–3.6 mg g⁻¹ dry weight (DW). The threshold level of PAs for bloat safety of forages is estimated to be in the range of 1–5 mg g⁻¹ DW (Li et al., 1996). Seven out of the 21 lines analyzed fell within this range.
Table 2 Levels of PAs and flavan-3-ols in various tissues of transgenic and control tobacco plants

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Mature leaf</th>
<th>Young leaf</th>
<th>Corolla tissues of open flowers</th>
<th>Corolla tissues of flower buds</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP1 – MtANR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#13</td>
<td>293.5 ± 18.3</td>
<td>21.6 ± 1.2</td>
<td>44.3 ± 14.3</td>
<td>0</td>
<td>55.2 ± 8.3</td>
</tr>
<tr>
<td>#23</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>#42</td>
<td>0</td>
<td>0</td>
<td>25.8 ± 2.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>#47</td>
<td>126.4 ± 1.7</td>
<td>28.3 ± 1.2</td>
<td>262.7 ± 28.5</td>
<td>34.2 ± 4.7</td>
<td>46.8 ± 10.7</td>
</tr>
<tr>
<td>#67</td>
<td>87.6 ± 16.6</td>
<td>20.3 ± 0.6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>#136</td>
<td>363.0 ± 1.5</td>
<td>28.4 ± 14.1</td>
<td>197.2 ± 11.9</td>
<td>5.6 ± 11.9</td>
<td>55.7 ± 5.7</td>
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<tr>
<td>PAP1 – pBI121</td>
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<td></td>
</tr>
<tr>
<td>#257</td>
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<tr>
<td>#282</td>
<td>0</td>
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<td>Wild type</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MtANR.#-B-21-B</td>
<td></td>
<td></td>
<td>42.6 ± 21.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PAP1,#292</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Flavan-3-ol level in leaf tissues (µg g⁻¹ FW)

<table>
<thead>
<tr>
<th>PAP1 – MtANR</th>
<th>(–)-Epicatechin</th>
<th>(–)-Gallocatechin</th>
</tr>
</thead>
<tbody>
<tr>
<td>#13</td>
<td>50.6 ± 7.9</td>
<td>13.8 ± 2.3</td>
</tr>
<tr>
<td>#67</td>
<td>54.5 ± 3.0</td>
<td>15.8 ± 1.6</td>
</tr>
<tr>
<td>#136</td>
<td>39.0 ± 10.4</td>
<td>15.5 ± 2.7</td>
</tr>
<tr>
<td>Wild type</td>
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<td>0</td>
</tr>
<tr>
<td>MtANR.#-B-21-B</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PAP1,#292</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values for a larger selection of transgenic and control lines are given in Table S1.
*0 means not detected or negative value; ND, not determined.

The TLC profiles of intact and hydrolyzed PAs from stem tissue were similar to those from leaf tissue (data not shown). Levels of PAs in stem tissues of PAP1 + MtANR transgenic plants were in the range of 21–159 µg g⁻¹ FW (Table S1), with three out of eight lines analyzed producing levels within the region recommended for biofilm safety in alfalfa. However, no PAs were detected in root tissue.

Accumulation of flavan-3-ols

PAP1 + MtANR transgenic plants accumulated free epicatechin and galloylacetechin (Figure S3, Table 2). Analysis of several lines by HPLC revealed levels of (–)-epicatechin and (–)-gallocatechin in leaf tissues to be around 40–50 and 15 µg g⁻¹ FW, respectively (Table 2). Flower tissues accumulated (–)-gallocatechin and (–)-epigallocatechin at similar levels (data not shown). Free (–)-epiafzelechin, the product of the action of ANR on pelargonidin (Xie et al., 2003), was not detected in leaf extracts even though pelargonidin was produced following hydrolysis of PAs from leaves (Figure 5e).

Engineering PAs in Medicago truncatula

Leaves of the model legume M. truncatula accumulate anthocyanins in a central red spot (Figure 5f). Unlike...
tobacco, *M. truncatula* leaves contain low levels of PAs localized to small glandular trichomes tightly appressed to the epidermal surface of the leaf (Figure S4). These trichomes are easily missed if not stained with DMACA, and were not recognized in an earlier study (Aziz *et al.*, 2005). Proanthocyanidins were not detected by TLC analysis of ethyl acetate extracts from leaves of wild-type plants (Figure 5g).

In *M. truncatula* expression of MtANR driven by the 35S promoter (Figure S4) resulted in a decrease of approximately 50% in anthocyanin pigmentation in the red spot compared with that of wild-type plants at the same stage of development (Figure 5f, Table S2). Leaves of transgenic *M. truncatula* constitutively expressing MtANR contained up to three times more PAs than those of wild-type plants at the same stage of development (Table S2), and these were now clearly detected by TLC. Surprisingly, oligomers of two distinct size classes predominated (Figure 5g). Increased staining of trichomes, or changes in trichome density, were not observed in MtANR lines (data not shown). Synthesis of PAs appeared to be the result of diversion of flux away from anthocyanin formation within the red spot region.

**Discussion**

**Engineering the epicatechin building block of PAs**

Both (+)-catechin and (−)-epicatechin are common components of PAs (Xie and Dixon, 2005). Genetic engineering of catechin-based PAs requires operation of the anthocyanin pathway only as far as leuconanthocyanidin, whereas epicatechin-derived PAs require a source of anthocyanidin in addition to expression of ANR (Xie *et al.*, 2003), provided in the present work by ectopic expression of PAP1. Orthologs of ANR and LAR have yet to be described in tobacco. However, PAP1 expression does not induce ANR in Arabidopsis (Sharma and Dixon, 2005), and this is most likely also the case in tobacco, since no epicatechin or PAs were detected in floral tissues expressing PAP1 alone. LAR does not appear to exist in Arabidopsis; if the gene is present in tobacco, it is unlikely to be induced by PAP1, since no free (+)-catechin was detected in PAP1 or PAP1 + MtANR transgenic tobacco. Surprisingly, although ANR expression has been observed to reduce anthocyanin levels and lead to production of PAs in transgenic tobacco flowers (Xie *et al.*, 2003 and the present work), there are no reports of a phenotypic effect of expressing LAR in tobacco (Tanner *et al.*, 2003).

The levels of free flavan-3-ols in tobacco leaves expressing PAP1 and MtANR were very low compared with those in young tea shoots (almost 30% of the dry weight; Balentine *et al.*, 1988). However, a significant proportion of the (−)-epicatechin produced in transgenic tobacco is probably converted to PAs. Preventing polymerization will therefore be important for engineering plants to produce (−)-epicatechin as a health-promoting nutraceutical. Arabidopsis plants lacking expression of the AHA10 gene, encoding a proton-pumping ATPase, fail to accumulate PAs but instead accumulate large amounts of epicatechin in the seed coat (Baxter *et al.*, 2005). Whether targeted inhibition of putative vacuolar transport components such as AHA10 will block PA polymerization in leaves of plants engineered for epicatechin production remains to be seen.

**Conversion of flavan-3-ols to PAs in transgenic tobacco and Medicago truncatula**

Factors necessary for conversion of monomeric precursors to PAs in Arabidopsis include two putative flavonoid transporters, encoded by the genes TT12 and TT19 (Debeaujon *et al.*, 2001; Kitamura *et al.*, 2004), and the AHA10 ATPase (Baxter *et al.*, 2005). Functional orthologs of these genes are presumably present in tobacco and *M. truncatula* leaves and stems, allowing for accumulation of oligomeric and polymeric PAs following supply of epicatechin monomers as a result of co-expression of PAP1 and ANR. Additional factors may limit accumulation of PAs in other plant species. For example, constitutive co-expression of the anthocyanin pathway plus ANR, TT12, TT19, AHA10 and a range of transcription factors necessary for accumulation of PA in the Arabidopsis seed coat is insufficient to result in accumulation of PAs in Arabidopsis leaves; rather, PAs accumulate only in a small subset of cells in which the Arabidopsis ANR promoter is naturally expressed (Sharma and Dixon, 2005).

Other than a supply of suitable monomer units, nothing is known of the factors that further control the composition or degree of polymerization of PAs. Expression of PAP1 in tobacco leaf tissue led to production of three anthocyanins, with a relative abundance cyanidin > pelargonidin > delphinidin. *Medicago* ANR has a relative substrate preference in the same order (Xie *et al.*, 2004), consistent with the formation of PAs consisting primarily of (−)-epicatechin (derived from cyanidin) with some (−)-epiafzelechin (derived from pelargonidin) units. The reason for the clear difference in dp of the PAs produced in leaves, flowers or stems of the two species studied here is not obvious, particularly the very specific subset of oligomers found in *M. truncatula* tissues.

Although PAP1 expression in tobacco resulted in accumulation of delphinidin, very few units derived from this compound were recovered from the PAs, perhaps because of the low levels of delphinidin compared with cyanidin and pelargonidin. Conversion of delphinidin to (−)-gallo catechin by reduction followed by epimerization at C2, occurs with MtANR in *vitro* (Xie *et al.*, 2004), and this may explain the detection of free gallo catechin in the present work.
Substrate for the polymerization reaction of PA biosynthesis

To date, many models have been proposed for the polymerization reaction of PA biosynthesis, but there as is yet no firm biochemical or genetic evidence in favor of any one specific mechanism (Dixon et al., 2005; Xie and Dixon, 2005). Models for PA polymerization based on in vitro chemical studies propose that (+)-catechin or (-)-epicatechin only function as starter units, with extension units being derived from non-enzymatic condensation with leucoanthocyanins. This scheme is hard to reconcile with the fact that leucoanthocyanidin possesses the 2,3-trans stereochemistry of the (+)-flavan-3-ol (+)-catechin (Figure 1), whereas the commonly found epicatechin-type extension units possess 2,3-cis stereochemistry and are therefore likely to be derived from the (-)-flavan-3-ol (-)-epicatechin formed via the ANR reaction (Xie et al., 2003). The present results, in which co-expression of ANR with PAP1 leads to accumulation of PAs associated with reduced anthocyanin levels, are consistent with, but do not yet prove, a model in which (-)-epicatechin is both a starter and extension unit for PA biosynthesis (Xie and Dixon, 2005).

PA engineering in forage crops

In spite of many attempts (reviewed in Dixon et al., 2005), there has so far been little success in introducing PAs into forage crops for prevention of pasture bloat, as opposed to modulating levels of PAs in forages that naturally produce these compounds. One report has suggested that expression of the maize Lc transcription factor results in limited accumulation of PAs in alfalfa leaves, although the plants appeared to require abiotic stress for PA biosynthesis to be manifested (Ray et al., 2003). The levels of PA reported to prevent pasture bloat in forages are in the range of 1–5 mg g⁻¹ DW (Li et al., 1996), with at least tenfold higher concentrations being optimal for improvement of efficiency of use of protein nitrogen by ruminant animals. Co-expression of PAP1 and ANR leads to levels of PAs in tobacco that, if translated to alfalfa, could potentially provide bloat protection. Although the levels of PA produced in M. truncatula fell short of those necessary for improved agronomic performance by a factor of approximately five, increasing anthocyanin availability may result in the necessary improvements.

Experimental procedures

Plant material

The full-length open reading frame of the Arabidopsis PAP1 cDNA, under control of the cauliflower mosaic virus 35S promoter in the binary construct pCHF3:PAP1 (Borevitz et al., 2001) was transferred to Nicotiana tabacum cv. Xanthi using Agrobacterium-mediated transformation (Horsch et al., 1988). Tobacco plants constitutively expressing M. truncatula ANR have been described previously (Xie et al., 2003). Transgenic tobacco plants separately expressing PAP1 (T₂ progeny) or MAnR (T₄, four lines respectively representing one, two, three or multiple copies of the MAnR gene) were crossed in both ♂ × ♀ and ♀ × ♂ directions. PAP1 and pBI121 empty vector control transgenic plants were also crossed for controls. F₁ seeds of PAP1 × MAnR or PAP1 × pBI121 plants were harvested, sterilized using 10% bleach and gminated on solid MS medium (Murashige and Skoog, 1962) supplemented with kanamycin. A total of 302 antibiotic-resistant lines, including 250 lines of PAP1 × MAnR, 42 lines of PAP1 × pBI121, 10 PAP1 progeny and four MAnR lines were grown in the greenhouse for tissue sampling. Six non-transgenic wild-type tobacco plants were grown under the same conditions as additional controls. A copy of each line was made through vegetative propagation using auxiliary buds for collection of stem and root tissues.

Transformation of Medicago truncatula

The binary vector pBI121-MAnR containing the MAnR cDNA (Xie et al., 2003) driven by the 35S promoter was transferred into Agrobacterium tumefaciens strain C58 by the freeze-thaw method. Transgenic M. truncatula (cv. Jemalong A17) plants were generated by Agrobacterium-mediated transformation of cotyledons (Wright et al., 2005).

RT-PCR and RNA gel blot analysis

Total RNA was isolated using TRI-reagent following the manufacturer’s protocol (Molecular Research Center Inc., Cincinnati, OH, USA). For screening MAnR transgenic M. truncatula plants, 20 µg RNA was loaded for each sample and RNA gel blotting carried out according to standard protocols (Sambrook et al., 1989). Hybridizations were performed using a [³²P]dCTP-labeled MAnR probe following the High Efficiency Hybridization System protocol (Molecular Research Center). For RT-PCR screening of transgenic tobacco plants, first strand cDNA was synthesized from 2 µg total RNA using Ready-To-Go™ RT-PCR beads (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer’s two-step protocol. Two microliters of first strand cDNA was used for each PCR reaction in a volume of 30 µl. Primer pairs for MAnR amplification were 5’-CCTCATAGCAGTCTGTGGGGG-3’ (forward) and 5’-GCTGGTTAGAATGACACCCCT-3’ (reverse), to give a 508 base pair product. Primer pairs for PAP1 were 5’-GGCAAATGGCCACACATTTTCTT-3’ (forward) and 5’-GCTGGTTAGAATGACACCCCT-3’ (reverse) to give a 560 base pair product. The PCR conditions were 94°C for 2 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1.5 min; followed by a final extension of 72°C for 10 min. Products of PCR amplification were analyzed by electrophoreses of 15 µl aliquots of reactions on 0.8% agarose gels in Tris-acetic acid-EDTA buffer, and visualized with ethidium bromide.

Collection of plant tissues

Leaf tissue was from 4–10 cm long developing young leaves or 15–20 cm long mature leaves, harvested from 30 to 40 cm tall greenhouse grown plants. Corolla tissues from pooled open flowers were divided into upper (naturally pigmented) and lower parts. Root tissues were washed in tap water to remove soil, and dried with paper towels. Tissue samples were ground into a fine powder in liquid nitrogen and stored at −80°C prior to extraction.
**Extraction and measurement of anthocyanins**

Two hundred milligram batches of powdered tissue were suspended in 5 ml 0.5% HCl in methanol in 15 ml screw-cap polypropylene tubes. After vigorously vortexing for 30 sec, tubes were shaken for 1 h at 120 rpm, then centrifuged for 15 min at 2630 g at approximately 20°C. The extraction step was repeated twice, and supernatants pooled and adjusted to 15 ml. A 5 ml aliquot was dried under nitrogen at approximately 20°C, and resuspended in methanol–HCl to a final concentration of 1 mg FW material μl⁻¹. Fifty microliters of this solution was added to 450 ml butanol:HCl (95.5, v/v) and boiled for 1 h to release anthocyanin aglycones. The butanol was removed under nitrogen, and the residue suspended in 50 μl 0.1% HCl in methanol for HPLC and TLC analysis.

For determination of total anthocyanin content by measurement of absorbance at 524 nm, 1 ml of methanolic HCl extract was mixed with 1 ml of water and 1 ml of chloroform. After vigorous vortexing for 10 sec, the tubes were centrifuged for 15 min at 2630 g at approximately 20°C. The chloroform phase containing chlorophyll was separated from the methanol–water phase containing anthocyanin. One milliliter of the upper methanol–water phase was used to measure anthocyanin absorbance.

**Extraction of PAs and flavan-3-ols**

One gram of powdered leaf or stem tissue, or 0.25–0.5 g of powdered corolla tissue, was extracted using 15 volumes (ml g⁻¹) of ethyl acetate in 50 ml polypropylene screw-cap tubes. Samples were sonicated for 10 min, extracted at 120 rpm for 2 h at approximately 20°C, centrifuged for 20 min at 2630 g, and the supernatants transferred to new tubes. The extraction was repeated twice, and the ethyl acetate extracts pooled and dried under nitrogen at approximately 20°C. The residues were suspended in 1 ml water by vortexing. One milliliter of chloroform was added to each tube to remove chlorophyll and other lipids; samples were vigorously vortexed and then centrifuged for 15 min at 2630 g, after which the lower phase containing chlorophyll was removed. The chloroform extraction was repeated twice. The upper phase and interface containing PAs and other flavonoids was dried under nitrogen at 37°C. The residues were dissolved in methanol (1 ml g⁻¹ FW). The methanol extracts were transferred to 1.5 ml polypropylene microcentrifuge tubes, centrifuged for 15 min at 10 000 g to remove insoluble residues, and stored at 4°C prior to estimation of PAs and analysis by HPLC and TLC.

**Estimation of PAs**

Chlorophyll-free PA extract (50 μl) was added to 950 μl butanol:HCl (95: 5, v/v), and the absorbance measured at 550 nm. The sample was then boiled for 1 h and, after cooling to approximately 20°C, the absorbance was rerecorded, and the first value subtracted from the second. Absorbance values were converted into μg PA equivalents using a standard curve (2.5, 5, 10, 20 and 40 μg) of procyanidin B2 (Indofine, Chemical Co., Inc, Hillsborough, NJ, USA) similarly hydroylated with butanol:HCl. Values for corresponding tissue from wild-type plants were subtracted to reduce effects from non-specific background contaminants; plants which did not form PAs gave zero or small positive or negative values.

Hydrolyzates were subjected to TLC and HPLC to determine the nature of the extension units. One milliliter of butanol:HCl-treated sample was dried under nitrogen at room temperature, and residues dissolved in 50 μl 0.1% HCl in methanol for TLC or HPLC assay.

**TLC analysis of PAs, flavan-3-ols and anthocyanins**

For TLC analysis of PAs and monomeric flavan-3-ols extracted in ethyl acetate, methanolic samples (1 mg FW material μl⁻¹ methanol) were concentrated to 5 or 10 mg FW material μl⁻¹ methanol. Samples (10 μl) were spotted on aluminum-backed silica Kieselgel 60 F254 TLC sheets (0.2 mm layer thickness; EM Sciences, Bursling Ridge, NJ, USA) which were developed in water:formic acid:ethyl acetate (1:1:8, v/v), and then sprayed with 0.1% (w/v) DMACA freshly prepared in 6% HCl ethanol (1:1, v/v). The PAs and monomeric flavan-3-ols immediately gave a yellowish blue color which then changed to a stable blue color. The PAs extracted from D. uncinatum, procyanidin B1 and B2 and monomeric flavan-3-ols were used as standards.

For analysis of anthocyanin core molecules derived from butanol:HCl hydrolyzed anthocyanins or PAs, cellulose F-250 μm analytical TLC plates (J. T. Baker, Phillipsburg, NJ, USA) were developed in concentrated HCl:acetic acid:water (3:30:10, v/v). Two microliters of methanol stock (2 mg μl⁻¹) were loaded for anthocyanins and 10 μl for PAs. One hundred and fifty nanograms of cyanidin, delphinidin or pelargonidin chloride (Indofine, Chemical Co. Inc, Hillsborough, NJ, USA) prepared in 0.1% HCl in methanol were loaded as reference standards.

**HPLC analysis of anthocyanidins and flavan-3-ols**

The HPLC analysis was carried out using an HP 1100 series system (Agilent Technologies, Palo Alto, CA, USA) on a 250 x 4.6 mm C18 reversed phase column (Waters Spherisorb 5 μm ODS2, Metachem Technologies Inc., Palo Alto, CA, USA) with UV detection. Solvents were 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B). For analysis of anthocyanidins and (-)-epicatechin, the gradient program consisted of ratios of solvent A to B of 95:5 (0–5 min), 95:5 to 90:10 (5–10 min), 90:10 to 83:17 (10–25 min), 83:17 to 77:23 (25–30 min), 77:23 to 50:50 (30–65 min) and 50:50 to 0:100 (65–69 min) at a flow rate of 1 ml min⁻¹. Anthocyanidins were monitored at 530 nm and epicatechin at 280 nm. For assay of anthocyanidins produced from hydrolyzed anthocyanins, methanol–HCl stocks were diluted three times, and 10 μl of diluted sample was injected. For assay of anthocyanidins produced from hydrolyzed PAs, the injection volume was 20 or 30 μl. For assay of epicatechin, 5 or 10 μl of methanol stocks from ethyl acetate extractions were injected. Products were identified by retention times and UV spectra, and quantified by reference to standard curves made with authentic pelargonidin, cyanidin and delphinidin chlorides dissolved in HCl:methanol (0.1%, v/v) or (−)-epicatechin (Sigma, St Louis, MO, USA) dissolved in methanol.

For analysis of (−)-galloカテchin, the gradient consisted of ratios of solvent A to B of 95:5 (0–5 min), 95:5 to 90:10 (5–10 min), 90:10 to 84:16 (10–25 min), 84:16 to 66:33:5 (25–75 min), 66:33:5 to 60:40 (75–78 min) and 60:40 to 0:100 (78–79 min), at a flow rate of 1 ml min⁻¹. Ten or 5 μl of methanol stock was injected, absorbance was recorded at 230 nm, and quantification achieved by comparison with a standard curve constructed with authentic (−)-gallocatechin (Sigma).

**Histological localization of anthocyanins and PAs**

Epidermis was peeled from leaf and flower tissues using tip forceps. Cross-sections of fresh leaf and flower tissues were made by hand sectioning. Samples were transferred to glass slides and observed under a Nikon (Optiphot-2) microscope with a SPOT RT COLOR camera (Diagnostic Instruments, Sterling Heights, MI, USA). Freshly prepared 0.1% DMACA reagent was used to stain tissues to localize PA accumulation as described previously (Xie et al., 2003).
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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. RT-PCR analysis of transcript levels in leaf tissue of transgenic plants. Gene specific primers were used for amplification of (a) MtANR and (b) PAP1 transcripts. The line number is shown above the gel images, the genotype below. PAP1 + MtANR, progeny from cross between PAP1 and MtANR transgenic plants; MtANR, parental line transformed with MtANR alone; PAP1, parental lines transformed with Arabidopsis PAP1 alone; PAP1 + 121, progeny from a cross between PAP1 and pBI121 empty vector transformants; WT, wild-type control. Right lanes, molecular size markers.

Figure S2. Anthocyanin levels in flowers of wild-type tobacco and transgenic plants expressing PAP1 with or without expression of MtANR. WT, wild-type plant; B-21-8, MtANR transgenic plant; PAP1 + MtANR; various progeny lines of crosses between MtANR and PAP1 transgensics; PAP1 + 121, progeny of lines of the cross between PAP1 and empty vector lines. Anthocyanins were measured by extraction in methanolic HCl, removal of chlorophyll, and determination of absorbance at 524 nm.

Figure S3. HPLC analyses of flavan-3-ol monomers in wild-type and transgenic tobacco leaf tissues. (a) shows analysis of (−)-gallocatechin and (b) shows analysis of (−)-epicatechin. (c) shows analysis of (−)-epicatechin. (d) shows analysis of (−)-gallocatechin.

Figure S4. (a) RNA gel blot analysis of MtANR transcript levels in wild-type and 13 lines of 35S:MtANR transgenic M. truncatula. (b) DMSA-staining of leaf of wild-type plant showing the trichomes (staining blue-black). Bar, 100 μ.

Table S1 PA levels in control and MtANR-expressing transgenic tobacco plants

Table S2 Levels of anthocyanins and PAs in leaves of wild-type and four independent lines of MtANR-expressing transgenic M. truncatula

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References


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