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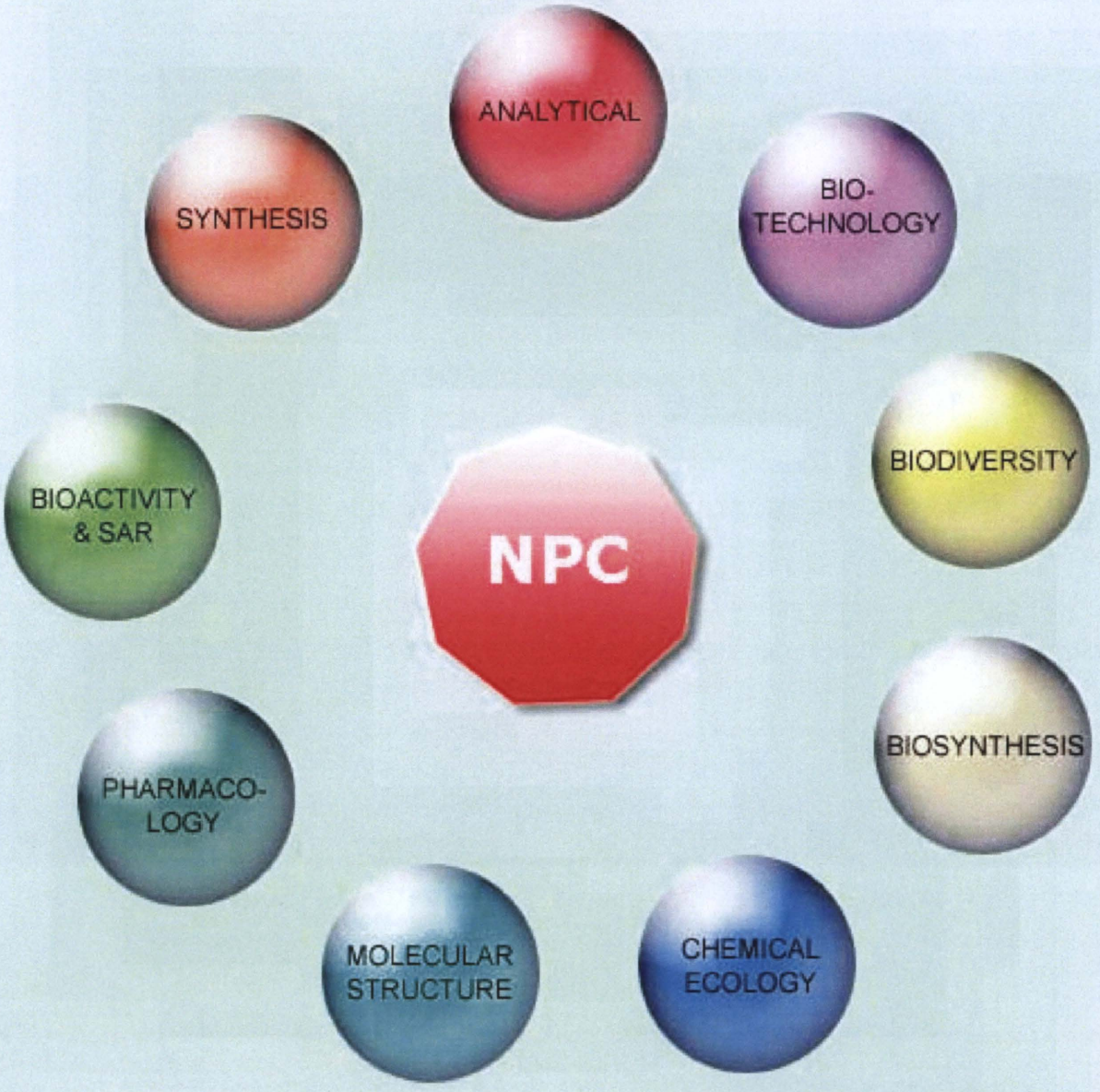

Volume 2. Issue 10. Pages 1009-1014. 2007
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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Natural Product Communications
2007
Volume 2, Number 10

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NPC Natural Product Communications

2007
Vol. 2
No. 10
1009 - 1014

Detection and Quantification of Engineered Proanthocyanidins in Transgenic Plants

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Received: April 12th, 2007; Accepted: May 25th, 2007

This paper is dedicated to Professor Tom J. Mabry for his 75th birthday.

Proanthocyanidins (PAs) are oligomeric plant natural products mostly derived from epicatechin and/or catechin monomers. In studies aimed at engineering PAs into plant tissues that do not naturally make these compounds, we have expressed PA biosynthetic and regulatory genes in tobacco, alfalfa (*Medicago sativa*) and the model legume *Medicago truncatula*. Because engineered tannins may be produced in small quantities and it is often necessary to screen many independent plant lines, we have developed an improved, highly sensitive method to quantify and determine the composition of oligomeric PAs in plant extracts. The method involves normal-phase HPLC separation of semi-purified PAs followed by post-column reaction with the PA-specific reagent DMACA (dimethylaminocinnamaldehyde). This procedure allows for accurate and sensitive quantification of individual oligomeric PAs and, unlike currently used methods, does not require exhaustive sample preparation and clean-up. Compositional data are shown for genetically engineered PAs in tobacco and alfalfa.

Keywords: Condensed tannins, normal-phase high performance liquid chromatography, metabolic engineering.

Proanthocyanidins (PAs), also known as condensed tannins, are polymeric derivatives of catechins, gallocatechins, or afzelechins, which in turn are derived from anthocyanidins or their immediate precursors (Figure 1). PAs have many beneficial attributes including strong antioxidant activity [1] and flavor enhancement [2], and moderate levels of PAs decrease the occurrence of pasture bloat in ruminant animals [3]. Bloat protection occurs when PAs bind to proteins and reduce the rate of their digestion in the rumen; "bloating" forages such as alfalfa (*Medicago sativa*) have high leaf protein content and do not contain foliar PAs. There is, therefore, considerable interest in introducing PAs into plant tissues that do not naturally produce them.

In previous studies, we have generated a range of transgenic plants that ectopically express genes encoding various transcription factors and biosynthetic enzymes involved in PA biosynthesis, and have reported that these plants produce PAs in their aerial organs [4,5]. However, these studies relied on histochemical staining for initial screening of tannin-containing tissues, use of the acid-butanol method for determining PA levels [6], and thin-layer chromatography for determination of oligomer size [7]. HPLC-MS can be used to detect and quantify small oligomers of PA, but they need to be relatively pure [8]. These methods are relatively insensitive, and the acid-butanol method is particularly susceptible to interference from endogenous anthocyanins. This is a serious problem since it is first necessary to engineer anthocyanins into plants to provide the substrates for PA formation [9]. To get around this problem, samples are extracted with ethyl acetate; this leaves the majority of the anthocyanins in the tissues, but only solubilizes a fraction of the total PA content of the tissue, predominantly the smaller oligomer classes (G.J. Peel, unpublished results). Thus, earlier estimates of the PA content of engineered tobacco and Arabidopsis tissues [10,11] are likely to be underestimates.

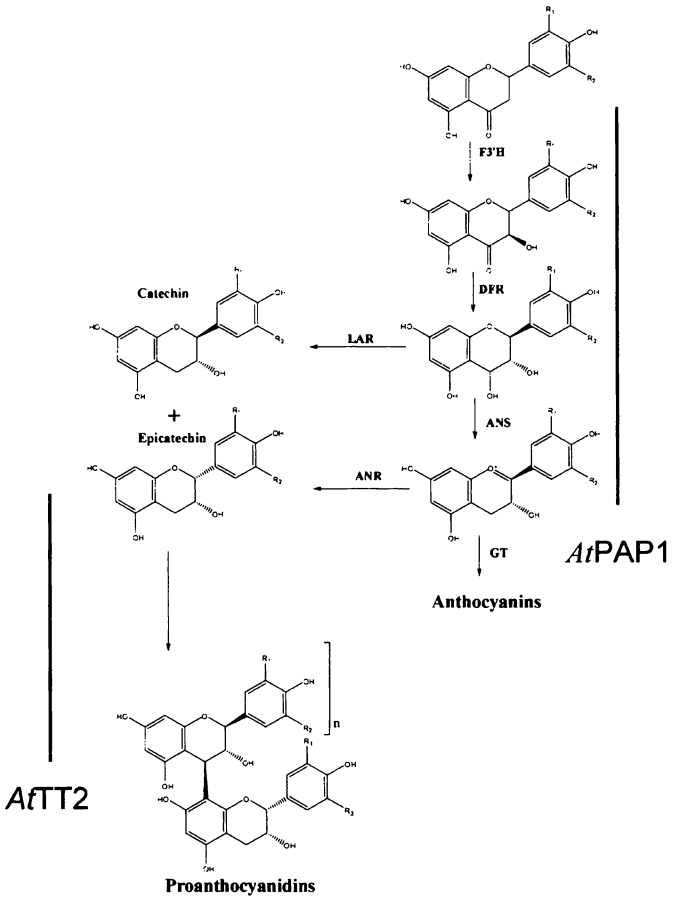

Figure 1: Biosynthetic pathways leading to anthocyanidin and PA synthesis in plants. Steps regulated by *At*PAP1 and *At*TT2 are shown (TT2 also induces ANR). Enzymes are: F3'H, flavanone 3'-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase, GT, glycosyltransferase.

High sensitivity and high throughput are important for genetic engineering studies, since many plant lines may need to be screened, and it is important to be able to distinguish between low PA production and no PA production in order to evaluate which transgenic approaches show promise, but require optimization. DMACA (dimethlyaminocinnamaldehyde) reagent provides a sensitive assay for detection and quantification of PAs present in plant samples [12]. DMACA is quite specific for flavan-3-ols, and has been used for post-column-reaction-detection (PCRD) of PAs in plant and food samples [13, 14]; however, these studies have utilized reverse phase HPLC and have been limited to resolution of small oligomers of no more than seven units. Normal phase HPLC (NP-HPLC) is used widely to identify and characterize higher oligomeric PAs from plants [15-18]; however, the PAs under study must be exhaustively purified and obtained in sufficient quantity to allow detection based on absorbance or fluorescence methods. We here report the combination of NP-HPLC with DMACA-PCRD to provide a sensitive method for PA profiling of transgenic plants.

Leaf tissues of the legume *Desmodium uncinatum* produce high levels of PAs of varying degree of polymerization (DP), and this material was, therefore, chosen as a standard with which to develop the new analytical method. Partially purified leaf extracts (no Sephadex LH-20 clean-up, see Methods) were separated and analyzed by the NP-HPLC-PCRD method. Well-separated and clearly defined peaks of DMACA-reactive compounds were observed, followed by a large unresolved peak corresponding to a mixture of higher polymeric PAs (Figure 2). To calibrate the separation system, *Desmodium* PAs were purified by both Sephadex-LH20 chromatography (to remove sugars and non-PA phenolics) and preparative HPLC. Individual PA fractions were subjected in parallel to phloroglucinolysis (which determines DP in terms of the proportion of extender to starter units [19]) and to analysis by the NP-HPLC-PCRD system to correlate mean DP with retention time. This relationship appeared exponential (Figure 3). Importantly, the method could reliably detect quite large oligomers present in small amounts of a crude mixture; NP-HPLC analyses that rely on either absorbance or fluorescence can effectively resolve polymers of DP 10 or less [15-18, 20], but the samples examined must be exhaustively purified and be in significantly larger amounts than required for detection with DMACA.

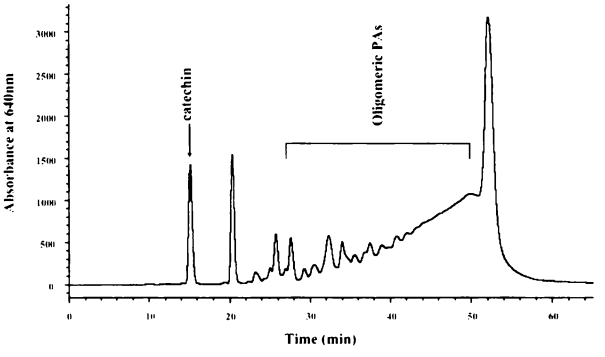

Figure 2: Separation of a crude *Desmodium* PA preparation by normal phase HPLC coupled to post-column derivatization with DMACA reagent. All absorbance values are x 10^3.

Tobacco plants expressing the Arabidopsis transcription factor PAP1 for production of anthocyanins [10,21], *Medicago* ANR [10] (Figure 1), or progeny of crosses between the two

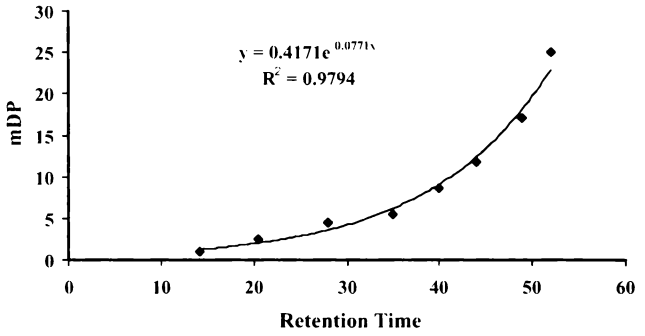

Figure 3: Relationship between retention time on NP-HPLC and the mean degree of polymerization (mDP) of *Desmodium* PA oligomers.

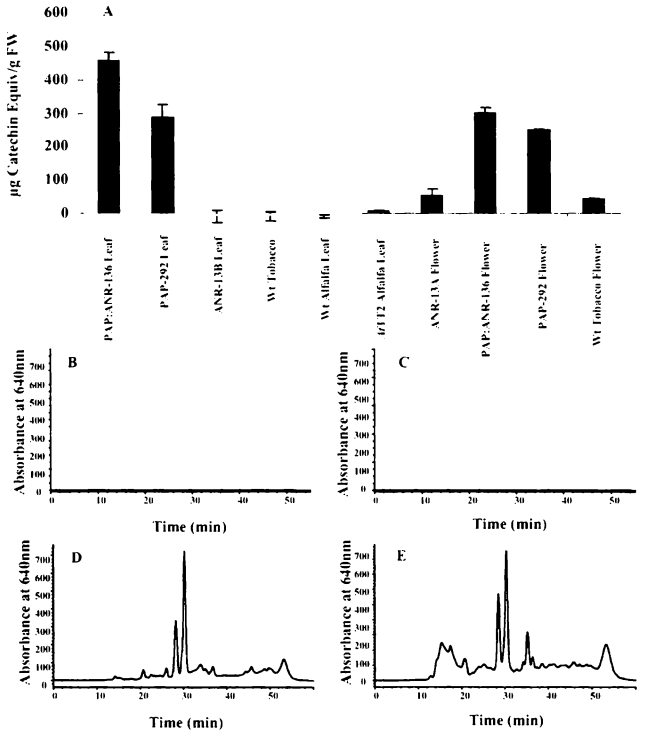

Figure 4: Analysis of PA levels and compositions in transgenic plants. A, Levels of total PAs as determined by DMACA micro-plate assay. Plant tissue designations refer to the PA pathway transgene expressed, the number of the independent transgenic plant line, and the tissue type analyzed (eg PAP1:ANR-136-leaf). All plants were tobacco unless stated. B-E. NP-HPLC-PCRD chromatograms of tobacco leaf extracts. Total PAs were analyzed from leaves of (B) wild-type plants, or plants expressing (C) *Mt*ANR, (D) *At*PAP1, or (E) *At*PAP1 and *Mt*ANR together. All absorbance values are x 10^3.

lines validated to be expressing both transgenes, were first analyzed for total PA levels using the DMACA micro-plate assay. The plants expressing PAP1 alone were believed not to synthesize PAs based on analysis by the acid-butanol method [10]. Utilizing the more sensitive DMACA method, which is not compromised by high background levels of anthocyanins, tobacco leaves expressing PAP1 alone were shown to contain DMACA-positive material (Figure 4A), and co-expression of *Mt*ANR strongly enhanced the level of putative PA production. No

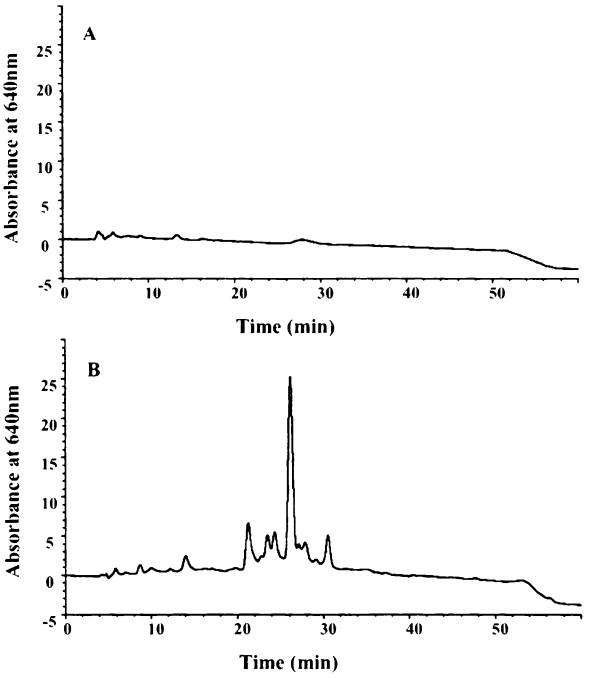

Figure 5: NP-HPLC-PCRD analysis of a crude PA extract from wild type (A) and *At*TT2 (B) expressing alfalfa leaf tissues. All absorbance values are x 10^3.

DMACA-positive material was detected in tobacco leaf tissue expressing ANR alone, since no anthocyanin precursors were available for conversion to epicatechin and beyond (Figure 1). Conversely, tobacco flowers, which naturally contain anthocyanins, produced similar levels of DMACA-reactive material as a result of PAP1 expression with or without expression of ANR.

To test the sensitivity of the method, we analyzed transgenic alfalfa plants expressing the Arabidopsis TT2 transcription factor that regulates the later stages of PA synthesis (Figure 1) [22, 23]. These plants did not contain an engineered supply of anthocyanidin substrate for PA production, and previous examination of the plants with either acid-butanol or tissue staining with DMACA, did not detect any PAs. Analysis of total PA levels by the DMACA micro-plate assay revealed detectable PAs, but at levels of around ~50-fold lower than observed in the tobacco leaves co-expressing *PAP1* and *MtANR* (Figure 4A). Nevertheless, HPLC-PCRD analysis clearly resolved a series of PA oligomers, with DP values from 4-5 (Figure 6B) with as little as 0.6 µg of total PA applied to the column, compared to the wild-type, which lacked any DMACA reactive compounds (Figure 6A). Based on this result and calibration of the method with catechin standards we believe that the lower level of detection per peak is around 10 nmol.

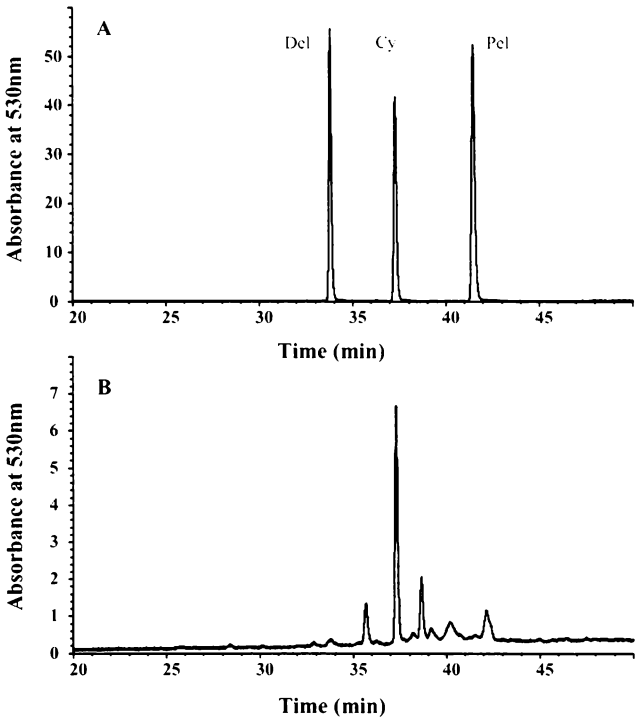

Figure 6: HPLC analysis of butanolysis products of oligomeric PAs from tobacco plants expressing AtPAP1 and MtANR. A, authentic standards of delphinidin (Del), cyanidin (Cy) and pelargonidin (Pel). B, butanolysis products of the PA fraction eluting from 32-38 min (see Figure 4E) showing cyanidin as the major product. All absorbance values are x 10^3.

To examine the nature of the DMACA-positive material synthesized by these plants, samples corresponding to 60 mg FW were analyzed by NP-HPLC coupled with DMACA detection. This confirmed that *At*PAP1 expression leads to synthesis of a range of oligomeric DMACA-reactive products, with the majority eluting around 26-30 min and corresponding to PA oligomers with a mean DP of approximately 4.0 (Figure 4D). Leaves expressing both *At*PAP1 and *Mt*ANR had higher levels of both free epicatechin and oligomers, with additional oligomer sizes (Figure 4E) as compared to plants expressing PAP1 alone.

To confirm that the DMACA-positive oligomers were indeed PAs, leaf samples from *AtPAP1*: *MtANR* co-expressing transgenic tobacco plants were run through the NP-HPLC system without post-column DMACA reaction, and the fractions corresponding to the peaks eluting at 32-38 min were collected, dried, and subjected to acid-butanol analysis. Although the collected material was colorless, the samples turned deep red following butanolysis, and HPLC analysis revealed the production of cyanidin (Figure 5), confirming that the material consisted of PA oligomers made from either epicatechin or catechin units.

The present method is ideal for profiling PAs in screens for transgenes with potential utility for PA engineering. In the case of *AtTT2*, use of the existing methods of analysis would have led us to conclude that this gene was ineffective in alfalfa foliage, whereas the increased sensitivity of the HPLC-PCRD method revealed that PAs were in fact made as a result of *TT2* expression, and provision of anthocyanin substrate could potentially improve their yield. Furthermore, expression of the PAP1 transcription factor alone can result in formation of oligomeric PAs in tobacco leaf and flower tissue; these compounds were missed in previous analyses because of the high background anthocyanin production. Most important, the new method can provide preliminary compositional analysis on less than 1 μg of total PA, without the need for prior purification.

Experimental

Sample extraction: Plant material was harvested from greenhouse stocks and frozen in liquid nitrogen. Tissues were ground in liquid nitrogen, and 0.5-0.75 g batches were then extracted with 10 volumes of 70% acetone: 0.5% acetic acid (v/v) by vortexing followed by sonication at 37°C for 30 min. After centrifugation at 2,500 *g* for 10 min, the residues were re-extracted as above. Pooled supernatants were extracted with 30 mL of chloroform, re-centrifuged, and the aqueous supernatant extracted twice with chloroform and twice with *n*-hexane to remove residual fats. Samples were dried briefly under nitrogen, then frozen and freeze-dried to yield crude PA powders, which were resuspended in extraction solution to a concentration equivalent to 3 g of original tissue sample per mL. The samples were spun briefly and aliquots taken for analysis of total PA levels and/or subjected to HPLC analysis.

Purification of PA fractions from Desmodium: To provide a positive control and PA size standards for comparative purposes, mature leaves of the PA-rich legume *Desmodium uncinatum* were harvested and ground to a fine powder in liquid nitrogen followed by lyophilization. Ten g of powdered leaf tissue were extracted three times with 100 mL 70% acetone: 0.5% acetic acid with sonication for 20 min at room temperature. Chlorophyll was removed from the pooled extracts with three chloroform extractions followed by three *n*-hexane extractions. After drying to remove *n*-hexane and chloroform, the sample was made to 20% with ethanol (v/v) and applied to a 5.5 x

7 cm column of Sephadex LH-20 conditioned with 20% ethanol (v/v). The column was then washed with five volumes of 20% ethanol to remove sugars and non-PA phenolics. The total PA fraction was collected by elution with 70% aqueous acetone. The acetone was removed by rotary evaporation and the red-brown liquid lyophilized for 4 days, yielding approximately 6 g of powder. The purified powder was subjected to a modified preparative HPLC separation [15] to isolate the various sizes of PA oligomers. A Beckman HPLC system (System Gold) equipped with preparative pump, a 1 mL loading loop and a 2.5 x 21.2 cm silica column (Supelco Supelcosil™ LC-Si) was used with the following solvent system: Solvent A, methylene chloride: acetic acid: water (96:2:2, v/v/v); solvent B, methanol: acetic acid: water (96:2:2); starting at 8.5% B in A for 5 min then increasing to 22% B in A over 60 min, 22-86% B in A over 30 min and holding for 10 min. Fractions (10-14, 15-19, 20-24, 25-29, 30-34, 35-39, 40-50, 51-60, 61-70, 71-80 and 81-100 min) from 4 separate runs were collected and pooled prior to rotary evaporation and lyophilization.

To determine the mean DP of the purified *Desmodium* PA fractions, phloroglucinolysis and HPLC analyses were performed on samples (100-300 µg) according to previous methods [19].

PA quantification by micro-plate and normal-phase HPLC-PCRD assays: Total PA levels were determined spectrophotometrically by micro-plate assay using DMACA reagent with catechin standards, as reported by Abrahams [23]. In brief, aliquots of either samples or standards (2.5µL) were mixed with 197.5 µL of DMACA reagent (0.2% w/v DMACA in methanol-3M HCl) in micro-plate wells; for blanks, the sample was replaced with an additional 2.5 µL of methanol-3M HCl. Samples, standards and blanks were read within 15 min on a Wallac ViPAor2 plate reader equipped with a 630 nm emission filter. Blanks were subtracted from samples and PA levels calculated as catechin equivalents.

NP-HPLC analyses of partially purified PA extracts (acetone extraction without Sephadex LH-20 clean up) were performed using an HP 1100 system equipped with a diode-array-detector. Samples were separated using the solvent system and conditions reported by Gu [27], except that the flow rate was reduced to 0.8 mL/min and the rinse and re-equilibration between runs extended to 15 min. Samples (10-20 µL, corresponding to 30-60 mg fresh weight of plant material) were separated on a 250 x 4.6 mm Luna 5µ silica column (Phenomonex, Torrance, CA). Post-column derivatization was accomplished using a separate HPLC pump (Alltech model 426) to deliver the DMACA reagent (1% DMACA in 1.5 M H_2SO_4 in methanol) at 0.8 mL/min to a mixing tee where effluent from the column and the reagent combined and passed through an 8 m coil of 0.2 mm i.d. PEEK tubing (Alltech, Holland, MI) prior to detection at 640 nm [12, 13].

Analysis of PA composition with acid-butanol reagent: HPLC column fractions that had not been subjected to reaction with DMACA were analyzed by the acid-butanol method. Collected fractions were pooled from several runs and dried under nitrogen. Samples were resuspended in 1 mL of *n*-butanol-HCl (95:5, v/v), and the absorbance was then measured before and after 1 h at 95°C. The released anthocyanidins were analyzed by reverse-phase HPLC using published protocols [26]. An authentic standard of procyanidin B1 (Sigma Aldrich) was also taken through the hydrolysis/separation procedures.

Plant materials: Transgenic tobacco plants expressing the *Medicago truncatula* anthocyanidin reductase (*MtANR*) and *Arabidopsis* Production of Anthocyanin Pigmentation 1 (*PAP1*) genes, either singly or in combination, have been described previously [10]. For generation of transgenic alfalfa expressing the *TT2* MYB transcription factor gene [22], a construct containing the full length open reading frame of Arabidopsis *TT2* under control of the cauliflower mosaic virus 35 S promoter [11] was transformed into *Medicago sativa* cv Regen SY, according to previously described protocols [27].

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