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17 January 2003, Volume 299, pp. 396-399

Role of Anthocyanidin Reductase, Encoded by *BANYULS* in Plant Flavonoid Biosynthesis

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Condensed tannins (CTs) are flavonoid oligomers, many of which have beneficial effects on animal and human health. The flavanol (–)-epicatechin is a component of many CTs and contributes to flavor and astringency in tea and wine. We show that the *BANYULS* (*BAN*) genes from *Arabidopsis thaliana* and *Medicago truncatula* encode anthocyanidin reductase, which converts anthocyanidins to their corresponding 2,3-*cis*-flavan-3-ols. Ectopic expression of BAN in tobacco flower petals and *Arabidopsis* leaves results in loss of anthocyanins and accumulation of CTs.

The presence of condensed tannins (CTs, also known as proanthocyanidins) in the leaves of forage plants protects ruminant animals against pasture bloat (*1*, *2*) and improves their nitrogen nutrition by increasing the amount of bypass protein (dietary protein exiting the rumen) (*1*, *3*, *4*). CTs are also powerful antioxidants with beneficial effects on human cardiac health (*5*) and immunity (*6*), and particular interest is being shown in the CTs from fruits such as cranberry and grape (*7*, *8*). Although CTs occur in the fruits and seeds of many plants (*9*), they are absent from the leaves of certain forage crops such as alfalfa (*Medicago sativa*) (*9*).

CTs are synthesized by a branch of the flavonoid pathway (Fig. 1). In *Arabidopsis thaliana,* mutations in the *BANYULS* (*BAN*) gene (named after a French red wine) result in a transparent testa (tt) that is associated with precocious accumulation of red anthocyanins and loss of CTs in the seed coat (*10*). On the basis of this and of the amino acid sequence similarity of BAN to dihydroflavonol reductase (DFR), it has been suggested that *BAN* encodes leucoanthocyanidin reductase (LAR) (*10*), an enzyme proposed to convert flavan-3,4-diols (leucoanthocyanidins) to 2,3-*trans*-flavan-3-ols such as (+)-catechin (*11*), a "starter unit" for tannin condensation (*11*, *12*) (Fig. 1). However, no biochemical evidence exists to support this hypothesis, and *BAN* therefore represents the only flavonoid pathway gene currently known for which no function has been proven.

Random sequencing of a cDNA library (*13*) from young developing seeds of the legume *Medicago truncatula* led to the identification of a full-length 1.164-kb cDNA (MtBAN, GenBank accession number AY184243) with 59% amino acid sequence identity to *Arabidopsis* BAN (AtBAN, GenBank accession number AF092912) (*10*) (fig. S1A) and 38% identity to *A. thaliana* DFR (GenBank accession number NM_123645). MtBAN was strongly expressed in young seeds, weakly expressed in open flowers and flower buds, and very weakly expressed in leaf tissues of *M. truncatula* (fig. S1B). *BAN* exists as a single copy in the *M. truncatula* genome (fig. S2).

AtBAN and MtBAN were expressed in *Escherichia coli*, yielding high levels of soluble 38-kD BAN protein. Lysates were assayed either directly, with ^{3}H-leucocyanidin as the substrate, or indirectly, in coupled reactions with recombinant *M. truncatula* DFR and the dihydroflavonol dihydroquercetin (Fig. 1) as the substrate, for LAR activity dependent on the reduced forms of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). In all cases, no formation of (+)-catechin, or any other product, was shown.

To functionally characterize BAN, we generated transgenic plants of tobacco (*Nicotiana tabacum*) (36 independent lines) and *Arabidopsis* (20 independent lines) that constitutively expressed MtBAN or AtBAN, respectively, under control of the cauliflower mosaic virus 35S promoter (fig. S3, A and B). Several lines exhibited high levels of ectopic *BAN* transcript expression in leaves (Fig. 2, A and B). Tobacco lines expressing MtBAN lost the pink flower pigmentation characteristic of wild-type and empty vector control plants (Fig. 2C). Extraction of petals in ethanolic HCl fol-

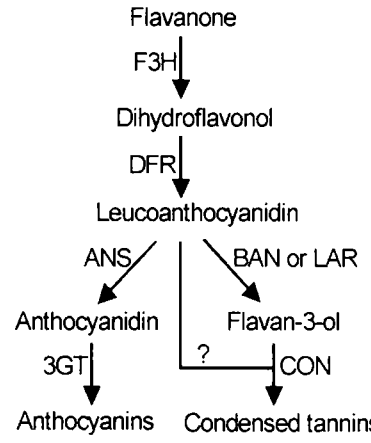

Fig. 1. Proposed relation between the biosynthesis of anthocyanins and CTs. Enzymes are flavanone 3-hydroxylase (F3H), DFR, ANS, LAR, anthocyanidin 3-glucosyltransferase (3GT), and an unknown condensing enzyme(s) (CON).

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lowed by spectrophotometric determination at 528 nm confirmed a reduction in anthocyanin pigmentation in plants expressing MtBAN (Fig. 2D). Staining of transgenic tobacco petals with dimethylaminocinnamaldehyde (DMACA) reagent resulted in a blue coloration in BAN-expressing lines, indicative of the presence of CTs (*12*) (Fig. 2E). Light microscopy revealed individual blue epidermal cells (Fig. 2F). No blue coloration was observed when we stained petals from wild-type or empty vector control plants. Extraction with butanol-HCl reagent (*14*) confirmed the presence of CTs in tobacco petals, at levels of 7.7 to 42.7 μg of cyanidin equivalents per g of fresh weight, whereas CTs were absent from control petals (Fig. 2G; fig. S4, A to C). Similarly, *Arabidopsis* leaves constitutively expressing high levels of BAN contained 12.9 to 44.3 μg cyanidin equivalents of CTs per g of fresh weight (Fig. 2H), similar to levels in the tannin-accumulating leaves of the forage legume *Lotus corniculatus*.

In the pathway shown in Fig. 1, BAN competes with anthocyanidin synthase (ANS), a 2-oxoglutarate–dependent dioxyge-

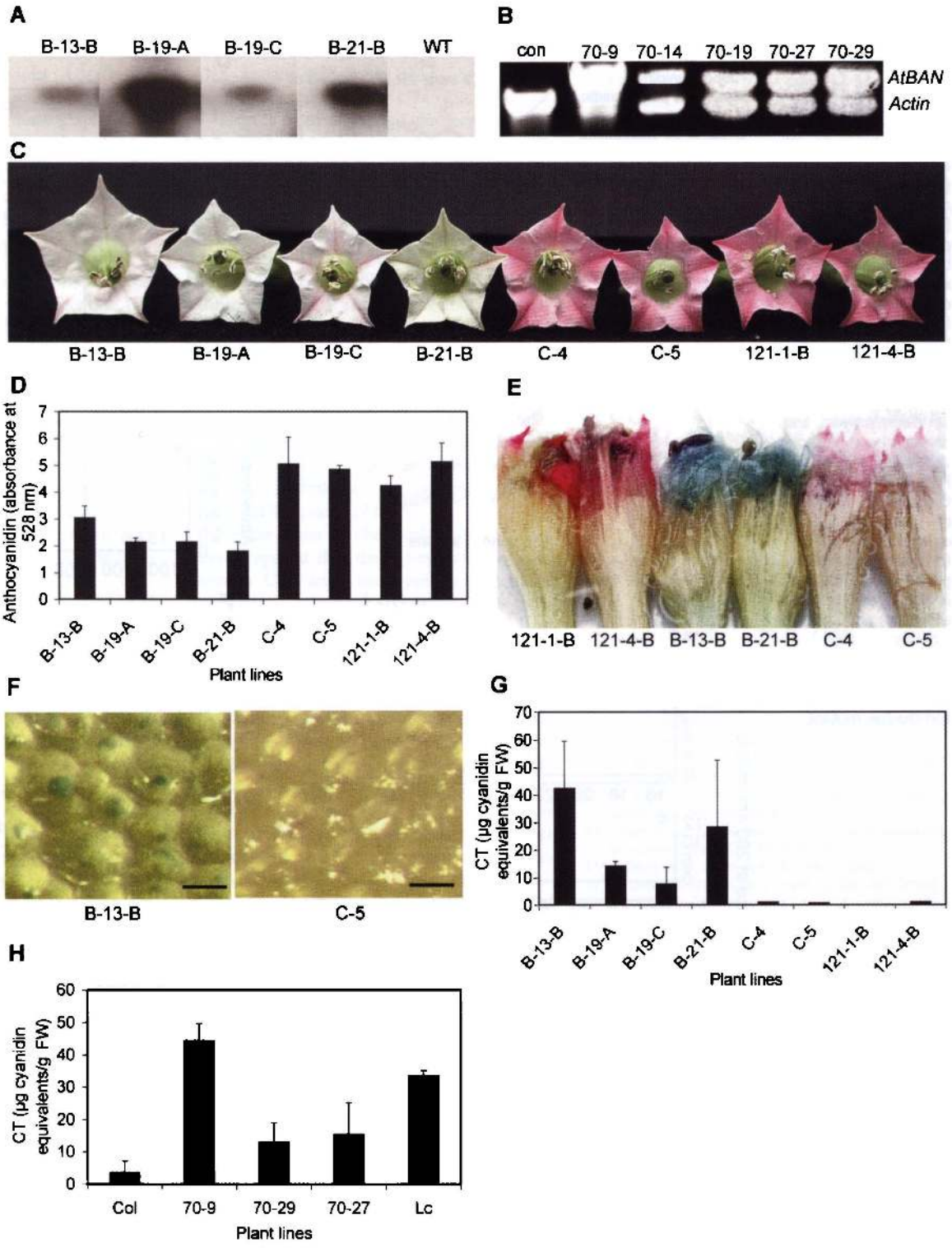

Fig. 2. Ectopic expression of BAN leads to reduction in anthocyanin levels and accumulation of CTs. (**A**) RNA gel blot analysis of total RNA from leaves of wild-type (WT) and transgenic tobacco expressing MtBAN (four independent lines). (**B**) Analysis by reverse transcriptase–polymerase chain reaction of AtBAN and actin (internal control) transcripts in total RNA from leaves of an empty vector control (con) and from transgenic *Arabidopsis* ectopically expressing AtBAN. (**C**) Pigmentation of flower petals from MtBAN transgenic (B designations), WT (C4 and C5), and empty vector control (121-1-B and 121-4-B) tobacco plants. (**D**) Anthocyanin levels in flower petals of the plants shown in (C). (**E**) DMACA staining of petals from empty vector control (121-1-B and 121-4-B), BAN transgenic (B-13-B and B-21-B), and WT (C4 and C5) tobacco. (**F**) DMACA staining showing CT localization in petal epidermal cells of MtBAN transgenic B-13-B, compared with staining of WT control C-5. Scale bar, 25 μm. (**G**) CT levels in flower petals of the same tobacco lines analyzed in (C) and (D). FW, fresh weight. (**H**) CT levels in leaves of wild-type (Col) and transgenic *Arabidopsis* ectopically expressing AtBAN and in leaves of *Lotus comiculatus* (Lc).

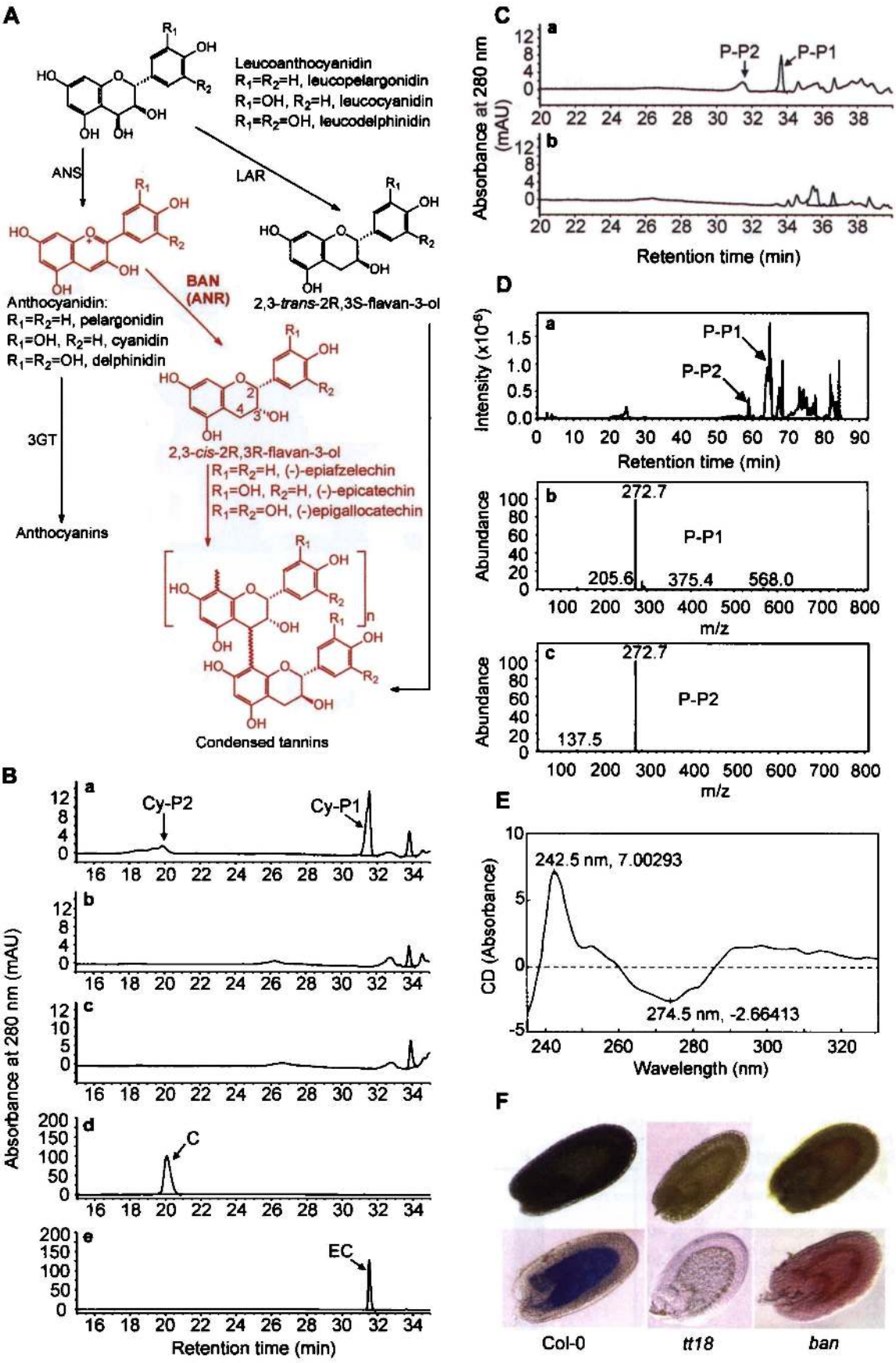

Fig. 3. BAN encodes anthocyanidin reductase. (**A**) Pathway for CT biosynthesis placing BAN immediately downstream of ANS. (**B**) HPLC analysis of products from (a) the incubation of recombinant MtBAN with cyanidin and NADPH and from control incubations with (b) active BAN in the absence of NADPH or (c) extract from an empty vector control plus NADPH. Panels (d) and (e) show chromatography of authentic (±)-catechin (C) and (−)-epicatechin (EC), respectively. mAU, milli-absorption units. (**C**) HPLC analysis of products from the incubation of pelargonidin and NADPH with (a) recombinant MtBAN and (b) boiled enzyme. (**D**) HPLC-MS of products formed from pelargonidin (P-P1 and P-P2). Panel (a) shows total ion chromatogram, and panels (b) and (c) show the mass spectra of P-P1 and P-P2, respectively. (**E**) CD spectrum of 2,3-*cis*-(2R,3R)-(−)-epiafzelechin (P-P1) formed from pelargonidin by the action of BAN. (**F**) Visible appearance (top) and DMACA staining (bottom) of seeds of *Arabidopsis* Col-0, the *tt18* mutant that lacks ANS activity, and the *ban* mutant.

nase (*15*), for the pool of flavan-3,4-diol (*10*). An alternative model consistent with the biochemical and genetic data places BAN immediately downstream of ANS to convert anthocyanidin to flavan-3-ol (Fig. 3A). To test this model, recombinant *M. truncatula* and *Arabidopsis* BAN proteins were incubated with anthocyanidins (cyanidin, pelargonidin, or delphinidin) and either NADPH or NADH. Incubation of BAN protein from either plant with cyanidin and NADPH resulted in efficient formation of a product (cyanidin product 1, or Cy-P1) identified as epicatechin by high-performance liquid chromatography (HPLC) retention time (Fig. 3B), ultraviolet (UV)–visible spectrophotometry, HPLC–mass spectrometry (HPLC-MS), and gas chromatography–mass spectrometry (GC-MS) after derivatization (figs. S5A and S6). A minor second product (Cy-P2) with a similar HPLC retention time and UV spectrum to (±)-catechin (and therefore possible epimerization product) was also formed (Fig. 3B). Likewise, recombinant BAN converted pelargonidin to a compound (pelargonidin product 1, P-P1) with an identical UV-visible spectrum to its corresponding flavan-3-ol, epiafzelechin (Fig. 3, A and C), and converted delphinidin to a compound (delphinidin product 1, D-P1) putatively identified as epigallocatechin. In both cases, minor potential epimerization products (P-P2 and D-P2) were also formed (Fig. 3C, fig. S5B). HPLC-MS provided molecular mass data supporting the assignment of the structures of epiafzelechin (16 mass units lighter than the catechin standard, Fig. 3D), epigallocatechin, and the tentative epimerization products (fig. S5B).

P-P1 showed a specific rotation of −59°, whereas the ^{1}H nuclear magnetic resonance spectrum in acetone-d_6 as solvent was identical to literature data (*16*) and showed the typical spin systems of a 4′,5,7-trihydroxyflavan-3-ol framework, i.e., a two-spin AB-system for the A-ring, a four-spin AA′BB′-system for the B-ring, and a four-spin AMXY-system for the protons of the heterocyclic ring. The 2,3-*cis* relative configuration was evident from the $^3J_{2,3}$ value of ~1.0 Hz for the broadened 2-H resonance at δ 4.91. The circular dichroism (CD) spectrum in methanol exhibited a high-amplitude negative Cotton effect at 274.5 nm for the ^1L_b transition and a positive Cotton effect at 242.5 nm for the ^1L_a transition (Fig. 3E), hence unequivocally indicating a 2*R*,3*R* absolute configuration (*16*–*18*) and confirming the structure of P-P1 as (−)-epiafzelechin (Fig. 3A). The CD spectra of the minor products Cy-P2, D-P2, and P-P2 exhibited positive Cotton effects near 280 nm for the ^1L_b transition, reminiscent of 2*S* absolute configuration. These compounds are thus *ent*-catechin, *ent*-gallocatechin, and *ent*-afzelechin, respectively, and most likely represent artifacts arising from epimerization at C-2 of the thermodynamically less stable 2,3-*cis* diastereoisomers to give the thermodynamically more stable 2,3-*trans* ent forms (*19*).

Identical products were obtained with either MtBAN or AtBAN. BAN was active both with NADPH and NADH, and no activity was observed with boiled enzyme or with non-denatured protein extracts from *E. coli* harboring empty vector (Fig. 3, B and C). Thus, BAN is an anthocyanidin reductase (ANR) involved in CT biosynthesis. This explains why mutations in ANS lead to a transparent seed testa (*20*). Staining of the ANS mutant *tt18* with DMACA indicated greatly reduced levels of CTs (Fig. 3F).

The CT from *Medicago* seed coat consists of 4 → 8 linked (−)-epicatechin residues with a (+)-catechin residue as "starter" (*21*), a common structure among CTs (*12*). Biochemical origins of the 2,3-*cis* stereochemistry of the (−)-epicatechin units have been hypothesized (*11*) but have lacked supporting biochemical data. The discovery of ANR and confirmation of its reaction product now provide a biochemical explanation for the formation of 2,3-*cis*-flavan-3-ols from the corresponding nonchiral anthocyanidins, with LAR a potential step in the formation of (+)-catechin. LAR activity has been demonstrated in crude extracts of Douglas fir (*22*) and ginkgo (*23*) cell cultures, in leaves of the forage legume sanfoin (*21*, *24*), and in developing barley grains (*24*, *25*).

On the basis of chemical models, it has been proposed that the (−)-epicatechin residues in CTs arise from polymerization of carbocation or quinone methide derivatives of flavan-3,4-diol, rather than flavan-3-ol, units with the growing chain (*26*). Our data suggest that other mechanisms of polymerization may operate.

Genetic evidence implicates BAN, several transcription factors (*27*–*29*), and TT12 (a multidrug transporter–like protein) (*30*) as necessary for correct CT accumulation in *Arabidopsis* seeds (*10*, *31*). Perhaps the extra factors are necessary for correct temporal and spatial expression of CTs during seed development, when BAN is expressed in the endothelium at the onset of fertilization and persists only up to the preglobular embryo stage (*10*), but are not required in petals or leaves. Correct biochemical functioning of BAN in vegetative tissues with a supply of flavonoid substrate may make genetic engineering of CTs easier than originally foreseen. Likely products of this technology in agriculture will include bloat-safe alfalfa (*32*), which will substantially reduce greenhouse gas emissions from cattle (*33*), have better silage quality (*34*), and increase the efficiency of alfalfa protein utilization by dairy cows, leading to reduced urine-N losses to the environment and a decreased requirement for feeding of supplemental protein (*35*). Further uses of the *BAN* gene for the generation of fruits and vegetables with health-beneficial properties for humans, or for modification of flavor and astringency in plant products such as tea and wine, can also be envisaged.

References and Notes

1. R. J. Aerts, T. N. Barry, W. C. McNabb, *Agric. Ecosyst. Environ.* **75**, 1 (1999).
2. L. R. McMahon *et al.*, *Can. J. Plant Sci.* **80**, 469 (2000).
3. T. N. Barry, W. C. McNabb, *Br. J. Nutr.* **81**, 263 (1999).
4. G. B. Douglas, M. Stienezen, G. C. Waghorn, A. G. Foote, R. W. Purchas, *N.Z. J. Agric. Res.* **42**, 55 (1999).
5. D. Bagchi *et al.*, *Toxicology* **148**, 187 (2000).
6. L.-C. Lin, Y.-C. Kuo, C.-J. Chou, *J. Nat. Prod.* **65**, 505 (2002).
7. L. Y. Foo, Y. Lu, A. B. Howell, N. Vorsa, *Phytochemistry* **54**, 173 (2000).
8. T. Pataki *et al.*, *Am. J. Clin. Nutr.* **75**, 894 (2002).
9. B. Skadhauge, M. Y. Gruber, K. K. Thomsen, D. von Wettstein, *Am. J. Bot.* **84**, 494 (1997).
10. M. Devic *et al.*, *Plant J.* **19**, 387 (1999).
11. H. A. Stafford, in *Flavonoid Metabolism*, H. A. Stafford, Ed. (CRC Press, Boca Raton, 1990), pp. 63–99.
12. L. J. Porter, *Meth. Plant Biochem.* **1**, 389 (1989).
13. Materials and methods are available as supporting material on *Science* Online.
14. S. A. Dalzell, G. L. Kerven, *J. Sci. Food Agric.* **78**, 405 (1998).
15. K. Saito, M. Kobayashi, Z. Gong, Y. Tanaka, M. Yamazaki, *Plant J.* **17**, 181 (1999).
16. R. J. J. Nel, H. Van Rensburg, P. S. Van Heerden, D. Ferreira, *J. Chem. Res.* **(S)** 606 (1999).
17. O. Korver, C. K. Wilkins, *Tetrahedron* **27**, 5459 (1971).
18. H. Van Rensburg, P. J. Steynberg, J. F. W. Burger, P. S. Van Heerden, D. Ferreira, *J. Chem. Res.* **(S)** 450 (1999).
19. L. Y. Foo, L. J. Porter, *J. Chem. Soc. Perkin Trans.* **1**, 1535 (1983).
20. B. Winkel-Shirley, *Plant Physiol.* **126**, 485 (2001).
21. S. Singh *et al.*, *Phytochemistry* **44**, 425 (1997).
22. H. A. Stafford, H. H. Lester, *Plant Physiol.* **76**, 184 (1984).
23. ———, *Plant Physiol.* **78**, 791 (1985).
24. G. J. Tanner, K. N. Kristiansen, *Anal. Biochem.* **209**, 274 (1993).
25. K. N. Kristiansen, *Carlsberg Res. Commun.* **51**, 51 (1986).
26. H. A. Stafford, *Phytochemistry* **22**, 2643 (1983).
27. A. R. Walker *et al.*, *Plant Cell* **11**, 1377 (1999).
28. N. Nesi, C. Jond, I. Debeaujon, M. Caboche, L. Lepiniec, *Plant Cell* **13**, 2099 (2001).
29. M. Sagasser, G.-H. Lu, K. Hahlbrock, B. Weisshaar, *Genes Dev.* **16**, 138 (2002).
30. I. Debeaujon, A. J. M. Peeters, K. M. Leon-Kloosterziel, M. Korneef, *Plant Cell* **13**, 853 (2001).
31. N. Nesi *et al.*, *Plant Cell* **12**, 1863 (2000).
32. B. Coulman *et al.*, *Can. J. Plant Sci.* **80**, 487 (2000).
33. J. Lee, AgResearch New Zealand, media release, May 2002.
34. K. A. Albrecht, R. E. Muck, *Crop. Sci* **31**, 464 (1991).
35. G. A. Broderick, *J. Animal Sci.* **73**, 2760 (1995).

36. We thank T. G. van Aardt for synthesis of ^{3}H-leucocyanidin; D. Huhman and F. Chen for assistance with HPLC-MS and GC-MS, respectively; A. Tanaka for tt18 seeds; and S. Temple for critical reading of the manuscript. Supported by Forage Genetics International and the Samuel Roberts Noble Foundation.

Supporting Online Material

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Materials and Methods
Figs. S1 to S6
References and Notes

18 September 2002; accepted 22 November 2002