(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 November 2008 (06.11.2008)

PCT

(10) International Publication Number
WO 2008/134372 A2

(51) International Patent Classification:
C12N 15/82 (2006.01) ***A23L 1/00*** (2006.01)
A01H 5/00 (2006.01)

(21) International Application Number: PCT/US2008/061301

(22) International Filing Date: 23 April 2008 (23.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/914,279 26 April 2007 (26.04.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
- *without international search report and to be republished upon receipt of that report*
- *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) Title: PRODUCTION OF PROANTHOCYANIDINS TO IMPROVE FORAGE QUALITY

(57) Abstract: The invention provides method and compositions for the modulation of anthocyanin and proanthocyanidin production in plants. The methods of the invention allow creation of plants having novel phenotypes. Increased expression of anthocyanins and proanthocyanidins in plants may be used to increase the nutritional value of food plants for both human and animal consumption. Increased proanthocyanidin content also reduces the potential for bloat in animals fed certain forage plants low in condensed tannin content. The invention may also be used to modify plant pigmentation, and for nutraceutical and food colorant production.

WO 2008/134372 A2

DESCRIPTION

Production of Proanthocyanidins to Improve Forage Quality

GOVERNMENT INTEREST

The government may own rights in this invention pursuant to Grant No. 0416833 of the National Science Foundation- Molecular Biochemistry Program.

BACKGROUND OF THE INVENTION

This application claims the priority of U.S. Provisional application Serial No. 60/914,279, filed April 26, 2007, the entire disclosure of which is incorporated herein by reference.

1. Field of the Invention

The present invention generally relates to plant genetics. More specifically, the invention relates to genes involved in the biosynthesis of anthocyanins, proanthocyanidins, and tannins in alfalfa (*Medicago* sp.), and methods for use thereof.

2. Description of the Related Art

Proanthocyanidins (PAs), also known as condensed tannins (CTs), are polymers of flavonoid (flavan-3-ol) units. Their name reflects the fact that, on acid hydrolysis, the extension units are converted to colored anthocyanidins, and this forms the basis of the classical assay for these compounds (Porter 1989). Anthocyanins and proanthocyanidins are found in many plant species. Anthocyanins contribute to the coloration of plant tissues, may act as attractants to pollinators (Schemske and Bradshaw, 1999), and provide UV protection (Reddy *et al.*, 1994). PAs provide plants with protection against insects, herbivores and fungal infection (Schultz and Baldwin, 1982; Bending and Read, 1996). Recently, realization of the beneficial qualities of dietary PAs for human health has increased the interest in these compounds (Bagchi *et al.*, 2000; Dufresne and Farnworth, 2001). Simple monomeric and/or oligomeric PAs have been shown to possess anticancer, antioxidant and antimicrobial activities (Dixon *et al.*, 2005).

Modest amounts of PAs in forages promote increased dietary protein nitrogen utilization and reduced occurrence of pasture bloat in ruminant animals such as cattle and sheep (Li *et al.*, 1996; Aerts *et al.*, 1999; Barry and McNabb, 1999). Pasture bloat occurs in ruminants when they are fed with a high protein diet such as alfalfa (lucerne; *Medicago sativa*) or clover (*Trifolium* spp), species that lack PAs in their aerial portions. The combination of excessive protein and methane released in the rumen from fermentation of the forage results in formation of a thick foam leading to bloating which, in severe cases, can be fatal. To combat pasture bloat, a common practice is to supplement the ruminant diet with surfactants, which break down the protein foams (Hall *et al.*, 1994). Another remedy involves mixing high protein forage with forage known to contain moderate levels of PAs (Li *et al.*, 1996). Both of these are costly options for the farmer or rancher, resulting in a reluctance to take advantage of the otherwise excellent nutritional qualities of alfalfa and clovers.

The building blocks of most PAs are (+)-catechin and (-)-epicatechin. (-)-Epicatechin has 2,3-*cis* stereochemistry and (+)-catechin has 2,3-*trans*-stereochemistry. These stereochemical differences are of major importance in PA biosynthesis, since all chiral intermediates in the flavonoid pathway up to and including leucoanthocyanidin are of the 2,3-*trans* stereochemistry, raising important questions about the origin of the 2,3-*cis* stereochemistry of (-)-epicatechin, the commonest extension unit in proanthocyanidins (Foo and Porter 1980). The most common anthocyanidins produced are cyanidin (leading to procyanidins) and delphinidin (leading to prodelphinidins). PAs may contain from 2 to 50 or more flavonoid units. PA polymers have complex structures because of variations in the flavonoid units and the sites for interflavan bonds. Depending on their chemical structure and degree of polymerization, PAs may or may not be soluble in aqueous organic solvents.

PAs are attracting increasing attention due to their ability to affect the nutritional quality of human and animal food (Bagchi *et al.,* 2000; Barry and McNabb, 1999; Morris and Robbins, 1997). In addition, PAs and anthocyanins from various plants have beneficial effects on cardiac health and immune responses (Pataki *et al.,* 2002; Foo *et al.,* 2000; Lin *et al.,* 2002), and to prevent macular degeneration (*e.g.* Brevetti *et al.,* 1989; Lee *et al.,* 2005). PAs can reversibly bind to proteins and reduce their degradation rate. The presence of moderate amounts of PAs in forage crops reduces the initial rate of microbial digestion of the protein component of forage material in the rumen. The protein-PA complexes then pass to the abomasum where they dissociate at the lower pH, providing "by-pass protein" for utilization by the animal and consequent enhancement of milk and wool production and live weight gain (Barry and McNabb, 1999; Tanner *et al.,* 1995).

In addition, low concentrations of PA can help counter intestinal parasites in lambs, and confer bloat safety, presumably by interacting with both leaf protein and microbial enzymes such that the rate of protein degradation in the rumen is reduced (Aerts *et al.* 1999). These properties of PAs underscore the potential importance of methods of engineering PA synthesis in crops, including forage crops in particular.

In addition, it has been shown that the presence of PAs in forage crops significantly reduces emission of the greenhouse gas methane by farm animals. Farm animals have been shown to produce large amounts of methane (~ 80 kg/yr/cow). Furthermore, PAs also preserve proteins during the ensiling process, increasing the feed value of silage and reducing the amount of nitrogen that is lost to the environment as feedlot waste (Albrecht and Muck, 1991; Reed, 1995). In laboratory studies, treatment of feed proteins with modest amounts of PAs (around 2-4% of dry matter) reduced proteolysis during both ensiling and rumen fermentation. In studies performed with sheep in New Zealand, increasing dietary PAs from trace amounts to 4% of dry matter increased by-pass protein, and a diet containing only 2% PAs strongly increased absorption of essential amino acids by the small intestine by up to 60% (Douglas *et al.* 1999).

An attractive alternative for forage improvement lies in genetically transferring the capability to synthesize PAs to non PA-accumulators (*e.g.* see WO 06/010096 or US Publication 2006/0123508). Since the precursors for PAs are the same as those for the production of anthocyanins, one approach is to transform plants with a transcription factor which, when ectopically expressed, induces anthocyanin production. Co-expression of one or more PA-specific biosynthetic enzymes such as anthocyanidin reductase (ANR), which converts cyanidin to the flavan-3-ol (-)-epicatechin, a building block of PAs (FIGs. 1-2) (Dixon *et al.*, 2005), may then lead to PA accumulation (Xie *et al.*, 2006).

However, there are several technical problems with this approach. First, apart from enzymes converting anthocyanin pathway precursors to catechin and epicatechin (US Publication 20040191787; Tanner *et al.*, 2003; Xie *et al.*, 2003; US Publication 20060123508), two potential transporters (Debeaujon *et al.*, 2001; Kitamura *et al.*, 2004), and an oxidase that likely acts on polymerized products (Pourcel *et al.*, 2005), little is known of the proteins necessary for polymerization of tannins and their ultimate accumulation in vacuoles or cell walls (Dixon *et al.*, 2005; Xie and Dixon, 2005). Second, transcription factors controlling anthocyanin production appear to be species-specific. Whereas the *Arabidopsis thaliana* producer of anthocyanin pigmentation (AtPAP1) MYB transcription factor (GenBank Accession AF325123) effectively induces anthocyanin production in *Arabidopsis* and tobacco (Borevitz *et al.*, 2000), it does not function in alfalfa or white clover (see below). Similarly, expression of the maize *Lc* gene in alfalfa only resulted in anthocyanin production if the plants were exposed to strong abiotic stress (Ray *et al.*, 2003). Expression of maize Lc Myc in conjunction with other transcription factors in *Arabidopsis* could lead to premature necrosis and death of the plants (Sharma and Dixon, 2005). Finally, even if anthocyanin production and downstream enzymes (for PA synthesis) are expressed, tannins have not necessarily accumulated, as seen in *Arabidopsis* expressing multiple flavonoid-pathway transcription factors (Sharma and Dixon, 2005).

The foregoing studies have provided a further understanding of the mechanisms and manipulation of plant secondary metabolism. However, the prior art has failed to provide techniques for the application of this understanding to the creation of plants having valuable new characteristics. What are thus needed are practical techniques for the production of novel plants with improved phenotypes and methods for the use thereof. Such techniques may allow the creation and use of plants with improved nutritional quality, thereby benefiting both human and animal health and representing a substantial benefit in the art.

SUMMARY OF THE INVENTION

In one aspect, the invention provides an isolated nucleic acid sequence that encodes a polypeptide that activates anthocyanin or proanthocyanidin biosynthesis, or its complement. In certain embodiments, the nucleic acid sequence is operably linked to a heterologous promoter. In one embodiment, the nucleic acid sequence is further defined as selected from the group consisting of: (a) a nucleic acid sequence encoding the polypeptide sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10; (b) a nucleic acid sequence comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9; (c) a nucleic acid sequence that hybridizes to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9 under conditions of 1X SSC and 65°C, or higher stringency, and encodes a polypeptide that activates anthocyanin or proanthocyanidin biosynthesis; (d) a nucleic acid sequence encoding a polypeptide with at least 85% amino acid identity to any of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10 that activates anthocyanin or proanthocyanidin biosynthesis; (e) a nucleic acid sequence with at least 85% identity to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9; and (f) a complement of a sequence of (a)-(e). In certain embodiments, the isolated nucleic acid sequence may be operably linked to a heterologous promoter.

A recombinant vector comprising such an isolated nucleic acid sequence is also an embodiment of the invention, and may further comprise at least one additional sequence chosen from the group consisting of: a regulatory sequence, a selectable marker, a leader sequence and a terminator. In certain embodiments, the additional sequence is a heterologous sequence encoding an anthocyanin or proanthocyanidin biosynthesis activity, for instance selected from the group consisting of: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), an anthocyanidin glycosyltransferase (GT), or AtPAP1 (production of anthocyanin pigment).

The recombinant vector may comprise a promoter, wherein the promoter is a plant developmentally-regulated, organelle-specific, inducible, tissue-specific, constitutive, or cell-specific promoter. In certain embodiments, the recombinant vector may be defined as an isolated expression cassette.

In another aspect, the invention provides an isolated polypeptide having at least 85% amino acid identity to the amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10, or a fragment thereof, having anthocyanin or proanthocyanidin biosynthesis activity. In particular embodiments, the isolated polypeptide comprising the amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10, or a fragment thereof, having anthocyanin or proanthocyanidin biosynthesis activity.

In yet another aspect, the invention provides a transgenic cell, including a bacterial cell, fungal cell, or plant cell transformed with a nucleic acid selected from the group consisting of: (a) a nucleic acid sequence encoding the polypeptide sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID

NO:10; (b) a nucleic acid sequence comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9; (c) a nucleic acid sequence that hybridizes to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9 under conditions of 1X SSC and 65°C, or higher stringency, and encodes a polypeptide that activates anthocyanin or proanthocyanidin biosynthesis; (d) a nucleic acid sequence encoding a polypeptide with at least 85% amino acid identity to any of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10 that activates anthocyanin or proanthocyanidin biosynthesis; (e) a nucleic acid sequence with at least 85% identity to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9; and (f) a complement of a sequence of (a)-(e). In certain embodiments, the invention provides a plant, or a plant part such as a foliar portion of a plant, a root, or a plant seed, comprising such a nucleic acid sequence. The isolated nucleic acid sequence may be operably linked to a heterologous promoter functional in a plant cell. In certain embodiments, the plant is a legume plant. In particular embodiments, the plant is a *Medicago sp.* plant, such as an alfalfa plant.

The transgenic plant, such as a *Medicago* plant, may express the selected nucleic acid and exhibit increased proanthocyanidin and/or anthocyaninbiosynthesis in selected tissues relative to those tissues in a second plant that differs from the transgenic plant only in that the selected nucleic acid is absent. In certain embodiments, the transgenic plant may be defined as transformed with a selected DNA encoding a LAP1, LAP2, LAP3, or LAP4 polypeptide selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or a fragment thereof, having anthocyanin or proanthocyanidin biosynthesis activity.

In other embodiments, the transgenic plant may further be defined as transformed with a selected DNA sequence complementary to a sequence encoding a glycosyltransferase active in anthocyanin biosynthesis. The transgenic plant may comprise a selected DNA sequence comprising the complement of SEQ ID NO:10, or a fragment thereof. In another embodiment, the transgenic plant may be further defined as transformed with a DNA sequence encoding the polypeptide of SEQ ID NO:1.

In certain embodiments, the transgenic plant of is further defined as a crop plant, including a forage crop plant. In particular embodiments, the transgenic plant may be a forage legume, such as alfalfa (*Medicago sativa*), barrel medic (*Medicago* *truncatula*), sweetclover (*Melilotus sp.*), white clover, red clover, alsike clover, milkvetch, crownvetch, birdsfoot trefoil, pea (*Pisum sativum*), lentil (*Lens culinaris*), or soybean, among others.

In other embodiments, the plant may further be defined as comprising a transgenic coding sequence that encodes an anthocyanin or proanthocyanidin biosynthesis activity selected from the group consisting of: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), an anthocyanidin glucosyltransferase (GT), or AtPAP1 (production of anthocyanin pigment). In particular embodiments, the transgenic coding sequence may encode an anthocyanidin reductase (ANR) polypeptide, for instance selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, and SEQ ID NO:46.

The transgenic plant may further comprise a transgenic sequence that down-regulates expression of a glycosyltransferase active in the synthesis of an anthocyanin. In a particular embodiment, the transgenic sequence may down-regulate expression of UGT78G1, for instance comprising the complement of SEQ ID NO:9 or a fragment thereof.

The transgenic plant may be a fertile R_0 transgenic plant, or a progeny plant of any generation of a fertile R_0 transgenic plant, wherein the transgenic plant comprises the selected DNA. A seed of such a transgenic plant comprising a nucleic acid selected from the group consisting of: (a) a nucleic acid sequence encoding the polypeptide sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10; (b) a nucleic acid sequence comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9; (c) a nucleic acid sequence that hybridizes to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9 under conditions of 1X SSC, and 65°C, and encodes a polypeptide that activates anthocyanin or proanthocyanidin biosynthesis; (d) a nucleic acid sequence encoding a polypeptide with at least 85% amino acid identity to any of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10 that activates anthocyanin or proanthocyanidin biosynthesis; (e) a nucleic acid sequence with at least 85% identity to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID

NO:9; and (f) a complement of a sequence of (a)-(e), is also provided, as are cells of such a plant.

Another aspect of the invention provides a method of producing a plant with increased proanthocyanidin biosynthesis, comprising introducing into the plant a selected DNA encoding a sequence that promotes anthocyanin biosynthesis, such as a LAP polypeptide, wherein the coding sequence is operably linked to a promoter functional in the plant and wherein the plant comprises increased anthocyanin biosynthesis in aerial portions of the plant relative to a second plant that differs from the plant only in that the selected DNA is absent in the second plant. The DNA may be selected from the group consisting of: (a) a nucleic acid sequence encoding the polypeptide sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8; (b) a nucleic acid sequence comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4; (c) a nucleic acid sequence that hybridizes to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4 under conditions of 1X SSC, and 65°C, and encodes a polypeptide that activates anthocyanin or proanthocyanidin biosynthesis; (d) a nucleic acid sequence encoding a polypeptide with at least 85% amino acid identity to any of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8 that activates anthocyanin or proanthocyanidin biosynthesis; (e) a nucleic acid sequence with at least 85% identity to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, or SEQ ID NO:4; and (f) a complement of a sequence of (a)-(e).

The selected DNA may be introduced into the plant by plant breeding. Alternatively, the selected DNA may be introduced into the plant by genetic transformation of the plant. The plant may be a legume, such as a forage legume, including alfalfa, and the method may further comprise preparing a transgenic progeny plant of any generation of the plant, wherein the progeny plant comprises the selected DNA.

In another aspect, the present invention provides a method of making food or feed for human or animal consumption, and comprises (a) obtaining a transgenic plant comprising a selected transgene DNA, wherein the selected DNA enhances proanthocyanidin or anthocyanin synthesis; (b) growing the plant under plant growth conditions to produce plant tissue from the plant; and (c) preparing food or feed for human or animal consumption from the plant tissue. Preparing food may comprise harvesting the plant tissue, and the food may be, for instance, hay, silage, starch, protein, meal, seed, flour or grain.

A nutraceutical prepared by the method of: (a) obtaining a transgenic plant comprising a selected transgene DNA, wherein the selected DNA enhances proanthocyanidin or anthocyanin synthesis; (b) growing the plant under plant growth conditions to produce plant tissue from the plant; and (c) preparing a nutraceutical for human or animal consumption from the plant tissue; is also an aspect of the invention.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:

FIG. 1: Biosynthetic pathways leading to the synthesis of anthocyanins and proanthocyanidins. PAL, L-phenylalanine ammonia-lyase (E.C 4.3.1.5); C4H, cinnamate-4-hydroxylase (E.C 1.14.13.11); 4CL, 4-coumarate: CoA ligase (E.C. 6.2.1.12); CHS, chalcone synthase (E.C. 2.3.1.74); F3H, flavanone 3-hydroxylase (E.C. 1.14.11.9); DFR, dihydroflavonol reductase (E.C. 1.1.1.219); LAR, leucoanthocyanidin reductase (E.C. 1.17.1.3); ANS, anthocyanidin synthase (E.C. 1.14.11.19); ANR, anthocyanidin reductase (E.C. 1.3.1.77); AtPAP1 (production of anthocyanin pigment; MYB-type transcription factor from *Arabidopsis*); GT, anthocyanidin specific glycosyltransferase.

FIG. 2: Pathway to PAs, showing (+)-catechin and (-)-epicatechin monomers

FIG. 3: Visible phenotypes of transgenic plants ectopically expressing anthocyanin pathway regulatory genes. (**A**) *Medicago sativa* expressing AtPAP1 (right) or vector control (left); (**B**) a tobacco seedling expressing MtLAP1; (**C**) flowers from two independent MtLAP1-expressing tobacco lines (left, right) and a non-transformed control (center); (**D**) *M. truncatula* plants expressing GUS (left) or MtLAP1 (right); (**E**) higher magnification of leaves of *M. truncatula* plants expressing GUS (left) or MtLAP1 (right), showing seed pods; (**F**) *M. sativa* expressing MtLAP1 and MtANR; (**G**) *M. sativa* expressing MtANR and GUS; (**H**) *Trifolium repens* expressing MtLAP1; (**I**) untransformed *T. repens* control

FIG. 4: Sequence comparisons of anthocyanin-regulatory transcription factors. (**A**) Multiple sequence alignment of published transcription factors involved in anthocyanin production and deduced amino acid sequences for *Medicago truncatula* LAP1 homologs or other sequences (SEQ ID NOs:5-8; SEQ ID NOs:95-96; SEQ ID NO:97 (*i.e.* the peptide encoded by SEQ ID NO:18)). R3/R2 conserved Myb domains are underlined. An2, Petunia An2 MYB regulator of anthocyanin production (SEQ ID NO:96 encoded within SEQ ID NO:94; Quattrochio *et al.*, 1999;); Antho1, tomato Anthocyanin 1 MYB regulator of anthocyanin production (SEQ ID NO:95; encoded within SEQ ID NO:93; Mathews *et al.*, 2003); (**B**) phylogenetic tree of selected anthocyanin regulatory transcription factors. Distances are nucleotide substitutions

FIG. 5: Pigmentation of *M. sativa* leaf transformed with *MtLAP2* (on right) compared to control non-transgenic leaf on left.

FIG. 6: Anthocyanin production in *M. truncatula* ectopically expressing MtLAP1. **(A)** Levels of total anthocyanins in leaves of independent transgenic *M. truncatula* plants expressing MtLAP1 or GUS (control), or co-expressing MtLAP1 with MtANR or GUS (control); **(B)** HPLC analysis of methanolic extracts from *M. truncatula* leaves expressing MtLAP1 (upper trace) and GUS control (lower trace); **(C)** HPLC trace of acid hydrolyzed anthocyanin extract from *M. truncatula* leaves expressing MtLAP1 or GUS; **(D)** HPLC trace of acid-hydrolyzed anthocyanin extract from *M. sativa* leaves co-expressing MtLAP1 and MtANR, or GUS and MtANR. The major peak at 33 min is cyanidin.

FIG. 7: **(A)** LC-MS analysis of anthocyanins from leaf tissue of alfalfa co-expressing MtLAP1 and MtANR. Insets show UV-Visible spectrum for the major peak eluting at 15.8 min (**B**), and the MS/MS fragmentation pattern (**C**), showing ions

at M/z 449 and 287 derived from the parent ion of M/z 625 **(D).** The compound consists of a cyanidin backbone conjugated with a hexose and a glucuronic acid residue (tentative structure shown).

FIG. 8: RT-PCR analysis of expression levels of anthocyanin/PA biosynthetic genes in *M. truncatula* and alfalfa (*M. sativa*) leaf tissues. Plants were transformed with GUS (control), MtLAP (*M. truncatula*) or MtLAP and MtANR (*M. sativa*). PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI chalcone isomerase; F3'H, flavanone 3'-hydroxylase; F3H, flavanone 3-hydroxylase; DFR-1, dihydroflavonol reductase-1; DFR-2 dihydroflavanol reductase-2; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase, ANR, anthocyanidin reductase; LAP1, Legume anthocyanin production, Act, actin (loading control).

FIG. 9: HPLC analysis of substrates and products from incubations of (iso)flavonoids with UGT78G1 in the presence of different sugar donors. Reactions containing substrate (250 μM), UDP-sugar (2.5 μM), and UGT78G1 (1.25 μg) were incubated for 3 h at 30 °C. (**A**) apigenin as acceptor substrate; (**B**) biochanin A; (**C**) formononetin; (**D**) genistein; (**E**) kaempferol; (**F**) quercetin; (**G**) pelargonidin. S, substrate; P, product. Dotted lines, reactions incubated without enzyme; solid black lines, reactions incubated in the presence of UGT78G1 and UDPG; dashed-dotted lines, reactions incubated in the presence of UGT78G1 and UDP-galactose; dashed lines, reactions incubated in the presence of UGT78G1 and UDP-glucuronic acid.

FIG. 10: Michaelis–Menten hyperbola and Lineweaver-Burk plot for UGT78G1 with kaempferol as substrate. Substrate concentration (S) and velocity (v) are given in mM and μM/min, respectively. Donor was UDPG.

FIG. 11: Transcript levels of UGT78G1 in *M. truncatula* tissues and cell cultures quantified by qRT-PCR. Cell cultures were elicited with yeast elicitor (E) or methyl jasmonate (MeJA) for 2 h and 8 h, respectively. Roots, leaves and buds were harvested from unchallenged plants. Leaves were also wounded (C), and then inoculated (I) with a suspension of *Phoma medicaginis* for 72 h (E). Stems were sprayed with a solution of 0.1% Tween 20 (C) or with a spore suspension of *P.medicaginis* in Tween 20 (I). cDNAs obtained from RT-PCR were quantified relative to the abundance of actin. Inset shows transcript levels in cell suspension cultures or inoculated leaves in cases where they were significantly lower than in the other organs.

FIG. 12: Production of oligomeric PAs in leaf tissues of plants expressing MtLAP1. **(A)** Normal phase HPLC chromatogram showing DMACA-reactive PA monomer and oligomer peaks (post-column derivatization) from leaves of *Desmodium uncinatum* (upper trace) and from alfalfa expressing AtTT2 (lower trace). (**B**) Normal phase HPLC chromatogram showing DMACA-reactive PA monomer and oligomer peaks (post-column derivatization) from *M. truncatula* leaves expressing MtLAP1 (upper trace) or GUS (control, lower trace). (**C**) Normal phase HPLC chromatogram showing DMACA-reactive PA monomer and oligomer peaks (post-column derivatization) from alfalfa leaves co-expressing MtLAP1 and MtANR (upper trace), or GUS and MtANR (control, lower trace). **(D)** PA levels in leaves of independent transformants of *M. truncatula* expressing MtLAP1 or GUS (control) and alfalfa (*M. sativa*) co-expressing MtLAP1 and MtANR, or GUS and MtANR (control). Values, determined by microplate assay with DMACA reagent, are the means and SDs of triplicate independent samples from each line.

FIG. 13: Insoluble proanthocyanidin levels in transgenic alfalfa plants expressing β-glucuronidase (GUS, control), *Medicago* LAP1 alone, or *Medicago* LAP1 and *Medicago* ANR. Each bar represents an independent transformants, indicating mean and standard deviation from 3 analytical replicates.

DETAILED DESCRIPTION OF THE INVENTION

The invention overcomes the limitations of the prior art by providing methods and compositions for the modification of anthocyanin and proanthocyanidin (PA) metabolism in plants, specifically in legumes. The invention has numerous important applications to agriculture. The invention allows, for the first time, the production of anthocyanin or PA in legume plants or plant tissues that otherwise lack significant anthocyanin or PA content, including, for example, aerial portions of alfalfa plants. By introduction of a transgene encoding an anthocyanin transcription factor functional in legumes such as a *LAP* (legume anthocyanin production) gene, *e.g.* encoding LAP1, LAP2, LAP3, or LAP4 (nucleotide and amino acid sequences shown in SEQ ID NOs:1-8), into a plant otherwise lacking the gene, the production and accumulation of PA can be induced.

It is shown herein that constitutive expression of the *Medicago truncatula* LAP1 transcription factor in alfalfa surprisingly results in accumulation of significant levels of polymeric proanthocyanidins in aerial portions of alfalfa including foliage that do not normally express PAs (*e.g.* FIGs. 3, 5). This is unexpected given that constitutive expression of the TT2 transcription factor in alfalfa only leads to very low levels of PA accumulation. Therefore, the effects of LAP1 over-expression on PA accumulation in *Medicago sativa* could not have been predicted based on studies in *Arabidopsis*.

Alfalfa lacks significant levels of PAs in the aerial portions, although high levels are found in the seed coat (Koupai-Abyazani *et al.*, 1993), and DMACA-reactive material that may represent PAs is also present in trichomes of glandular haired varieties (Aziz *et al.*, 2005). To date, classical breeding approaches have failed to introduce PAs into alfalfa foliage, and it has been accepted that this problem will likely require a biotechnological solution (Lees, 1992). As the anthocyanin precursors of PAs are also essentially absent from unstressed alfalfa foliage, introducing the PA trait requires increasing, or introducing *de novo*, the activities of at least ten known biosynthetic enzymes, plus a requirement for several additional functions associated with transport and sequestration of intermediates and products.

Although ectopic expression of certain transcription factors can lead to anthocyanin production, this strategy has been problematical in alfalfa. As described in Example 1, the *Arabidopsis* PAP1 transcription factor, which induces anthocyanin levels of up to 1.34 mg per g fresh weight in transgenic tobacco leaves, completely fails to induce anthocyanins in alfalfa. A previous attempt to engineer PAs in alfalfa utilized expression of the maize Lc MYC transcription factor to induce anthocyanin production, in the absence of additional transgenes specific to the PA pathway (Ray *et al.*, 2003). Levels of anthocyanins in *Lc*-expressing alfalfa lines were reported to reach up to 158 μg/g fresh weight (Ray *et al.*, 2003), but these values were not stable or reproducible, and anthocyanin production appeared to require exposure of the plants to abiotic stress, including continuous light for 36 to 48 h (Ray *et al.*, 2003). Expression of the maize B-Peru *MYC* gene in white clover led to anthocyanin production restricted to the white crescent area of the leaf (deMajnik *et al.*, 2000); however, expression of B-Peru in alfalfa failed to induce anthocyanin formation (Ray *et al.*, 2003).

Transformation of alfalfa, *M. truncatula* or clover with a *Medicago LAP* gene as described herein results in stable, high-level anthocyanin production. For instance, the *MtLAP1* gene was expressed under control of the 35S promoter, which is an inefficient constitutive promoter in legumes (Xiao *et al.*, 2005). Thus, relatively low level expression of *LAP1* (as seen from RT-PCR analysis) produces a strong anthocyanin phenotype in legumes associated with greatly increased transcription of anthocyanin biosynthetic genes. Anthocyanin levels in *M. truncatula* and alfalfa were approximately 10-fold higher than those reported in *Lc*-expressing alfalfa, and similar to those reported in tobacco expressing AtPAP1 (Xie *et al.*, 2006). PA biosynthesis utilizes two intermediates of the anthocyanin pathway, leucoanthocyanidin for formation of (+)-catechin via leucoanthocyanidin reductase (LAR; Tanner *et al.*, 2003), and anthocyanidin for formation of (-)-epicatechin via ANR (anthocyanidin reductase) (Xie *et al.*, 2003). Both of these intermediates are unstable and do not accumulate to measurable levels in plants. Presumably, the over-expression of the anthocyanin pathway provides sufficient levels of these compounds to feed the LAR and/or ANR pathways for subsequent formation of PAs. Although LAR is not induced by LAP1 expression in alfalfa, it is interesting that small amounts of free catechin, as well as epicatechin, were observed in alfalfa leaves co-expressing LAP1 and ANR. PAs accumulated in leaves of alfalfa co-expressing LAP1 and ANR to levels of between 150-250 μg catechin equivalents per g fresh weight, well within the range for bloat protection. Similar PA levels were observed in tobacco plants co-expressing PAP1 and MtANR (Xie *et al.*, 2006).

The studies described herein further show that PA levels of between 1-5 mg per g dry weight (approximately 100-500 μg/g fresh weight) can reduce rumenal methane production and thereby confer bloat reduction for ruminant animals fed alfalfa forage (Li *et al.*, 1996). As cattle are a major source of methane release to the environment, successful engineering of a bloat-safe alfalfa is advantageous for both animal health and the environment. Higher levels of PAs (from 20-40 mg/g dry weight) are required to significantly increase by-pass protein levels (the protein that exits the rumen), with associated improvements in animal performance and reduction in nitrogen excretion (Aerts *et al.*, 1999).

The present studies thus demonstrate the biotechnological development of a stable, bloat-resistant phenotype in alfalfa. They also suggest additional strategies for fine-tuning and improving the trait. To achieve higher levels of tannins beneficial for ruminant nitrogen nutrition, an increase in the anthocyanidin pool for synthesis of (-)-epicatechin may be achieved. Anthocyanidin may then accumulate as determined by the relative rates of reduction to epicatechin or glycosylation to anthocyanin. The microarray analysis results also identify UGT78G1 as being strongly up-regulated by LAP1 expression and therefore potentially involved in anthocyanin glycosylation. Down-regulation of UGT78G1 expression in LAP1:MtANR transgenics may therefore increase the pool of anthocyanidin available for PA formation.

Many forage crops are low in PAs, including *Medicago* spp such as alfalfa (*Medicago sativa*) and annual medics, white clover, ball clover, Persian clover, red clover, crimson clover, berseem clover, arrowleaf clover, alsike clover, subterranean clovers, fenugreek, and sweetclover (*Melilotus* spp.). Similarly, bloat can be caused by grazing of wheat pastures and other lush foliage such as fast-growing monocots. "Feedlot bloat" also occurs in cattle fed high-grain rations that may or may not contain legume forage, green-chopped legumes, or other finely ground feed. In these cases, direct engineering of PA accumulation in the forage plant may be used in accordance with the invention to prevent bloat. Further, PA modification could be engineered into feed components that are blended or added to bloat-causing components to reduce the bloat incidence in animals consuming the mixed feed.

One application of the invention is thus the modification of PA biosynthesis in plants with low PA content, resulting in plants, plant parts, or products such as silage or hay, with enhanced value. Alfalfa is one such plant. PAs are made in alfalfa (*Medicago sativa*), as in *Arabidopsis*, in the seed coat, but do not accumulate in the leaves (Koupai-Abyazani *et al.,* 1993; Skadhauge *et al.,* 1997). Nonetheless, alfalfa is the world's major forage legume. Therefore, introducing PA biosynthesis to the leaves or other tissues of alfalfa or other low PA plants would substantially improve the utility of this crop for feed by reduction of its potential for causing pasture bloat. Forage crops that accumulate PAs in leaves have low bloating potential; these include *Lotus corniculatus, Leucaena leucocephala, Hedysarum sulfurescens* and *Robinia* spp, among others.

Technology that could result in constitutive expression of PAs in high protein forage crops would also greatly improve the agronomic value of crops in addition to alfalfa. In addition, the potential importance of anthocyanins and PAs in human health makes methods for their facile production in plants necessary for the full development of their therapeutic potential, for instance allowing their production and use as nutraceuticals or as food colorants.

Over 100 genes are up-regulated in *M. truncatula* leaves in response to constitutive expression of LAP1, most of which are apparently involved in anthocyanin biosynthesis. Thus co-expressing LAP1 with anthocyanidin reductase (ANR), renders it possible to produce alfalfa foliage containing levels of oligomeric proanthocyanidins previously shown to be sufficient for pasture bloat prevention. The present invention provides methods and compositions for increasing PA production comprising introducing transgenic LAP coding sequences, *e.g. LAP1.* In certain aspects, this may be provided in combination with anthocyanidin reductase (ANR) coding sequences provided herein, which functions to direct precursors from the anthocyanin pathway into the formation of proanthocyanidins.

Further, as noted, microarray analysis of genes up-regulated by LAP1 also allowed for identification of a *Medicago* anthocyanidin glycosyltransferase. One glycosyltransferase gene, UGT78G1 (SEQ ID NO:9), that was strongly up-regulated by LAP1 in *M. truncatula* was shown to be active with anthocyanidins *in vitro* and is most probably the major *Medicago* anthocyanidin glycosyltransferase. Such glucosyltransferase genes, like the one encoding UGT78G1, thus are a target, in one aspect of the invention, for down-regulation to increase tannin levels in alfalfa co-expressing LAP1 and ANR.

When ANR from the model legume *Medicago truncatula,* a species closely related to alfalfa, is ectopically expressed in *M. truncatula* A17, the red pigmentation found naturally in the center of the leaves is reduced, accompanied by the production of modest amounts of oligomeric PAs (Xie *et al.*, 2006). Thus, *Medicago* leaf tissue has the potential to accumulate PAs if provided with a supply of anthocyanidin precursor and at least one enzyme for production of flavan-3-ol monomers. The identification of a family of MYB transcription factor genes from *Medicago truncatula* which induce constitutive anthocyanin production when expressed in alfalfa, *M. truncatula* or white clover allows for production of anthocyanin and PAs in parts of alfalfa plants and at levels not previously reported. These *LAP* genes, in combination with ANR, may be used to engineer PAs in alfalfa to levels previously demonstrated to reduce pasture bloat.

I. Application of the Invention

As indicated above, one application of the invention is the introduction or increase of PA biosynthesis in plants. Such applications may result in forage improvement and nutritional improvement of foods. In accordance with the invention this may be carried out by introduction of LAP1 alone or in combination with other PA biosynthesis genes. The invention may be used to improve the nutritional quality of plants. Catechins and similar flavonoids have been reported to behave as strong antioxidants and have other properties which may make their consumption beneficial to human and animal health. Also, such compounds are generally antimicrobial, and their presence may improve food quality by preventing pre- and post-harvest damage. Accordingly, increases in PA biosynthesis may be used to achieve the associated health benefits.

Another use of the invention comprises the alteration of pigmentation in plant parts, including, but not limited to, flower color, seed coat color and leaf color. This can be achieved, for example, by increasing anthocyanin content via over-expression of legume anthocyanin production (LAP) genes, thereby allowing anthocyanin accumulation and the associated pigmentation of plant tissue. Accumulation of anthocyanins may simultaneously improve the nutritional, disease resistance, or herbivore resistance of the plant products. When expressed from a plant gene promoter that responds to a particular nutrient starvation (*e.g.* phosphate, nitrogen or sulfur depletion), LAP genes may serve as sensors for nutrient status, leading to increased coloration under nurtrient stress which can be documented by remote sensing in the field.

In addition to providing the LAP1-LAP4 genes, other genes may be used in conjunction with any of LAP1- LAP4 to enhance the accumulation of proanthocyanidins, for instance by providing a gene encoding ANR, or other enzyme in the anthocyanidin or PA synthesis pathways. An ANR gene may be isolated by PCR, for instance by utilizing a nucleotide primer such as a BAN primer (*e.g.* SEQ ID NO:13) or other BAN primer for instance as found in U.S. Patent Publn. 2004/0093632. Thus, an ANR (BAN) homolog, for instance from *Medicago truncatula* (*e.g.* SEQ ID NO:43) may be utilized. Other anthocyanin synthetic enzyme activities as shown in FIGs. 1-2 may also be utilized in conjunction with the LAP genes, such as dihydroflavonol reductase (DFR) coding sequences (SEQ ID NOs:11-12). The genes may thus find use as part of a combination of genes to introduce or increase condensed tannin biosynthesis in numerous species, for forage improvement and nutritional improvement of foods. PA expression could also be modulated using a transgenic chalcone isomerase coding sequence (*e.g.* McKhann and Hirsch, 1994;; Liu *et al.*, 2002; (*e.g.* SEQ ID NOs:14-17)).

The invention also relates to feed products containing one or more of the sequences of the present invention. Such products produced from a recombinant plant or seed containing one or more of the nucleotide sequences of the present invention are specifically contemplated as embodiments of the present invention. A feed product containing one or more of the sequences of the present invention is intended to include, but not be limited to, feed, harvested hay, silage, crushed or whole grains or seeds of a recombinant plant or seed containing one or more of the sequences of the present invention.

Over-expression of *Medicago* chalcone isomerase may increase flavonoid biosynthesis in *Arabidopsis* (*e.g.* Liu *et al.*, 2002). This could thus be used in combination with any of LAP1- LAP4 and/or ANR (BAN) to produce more PA. An *Arabidopsis* or other PAP-1 gene could also be used to increase flux into the pathway (Borevitz, 2000; *e.g.* SEQ ID NO:18). LAP1- LAP4 could also be used in conjunction with any one or more other regulatory genes such as TTG1 (GenBank Accession No. AJ133743; SEQ ID NO: 21, SEQ ID NO:22), TT1 (GenBank Accession No. AF190298; SEQ ID NO:23, SEQ ID NO:24), and TT8 (GenBank Accession No. AJ277509; SEQ ID NO: 25, SEQ ID NO:26). Benefit may also be obtained from use of any of LAP1-LAP4 in conjunction with TT12 (GenBank Accession No. AJ294464; SEQ ID NO: 19, SEQ ID NO:20) for transport of PA to the vacuole. Any combination of the foregoing sequences may therefore be used with the invention.

A LAP sequence may be used in conjunction with another sequence encoding an ANR (BAN) homolog, for example, from barley (SEQ ID NO:27, SEQ ID NO:29, SEQ ID NO:31 and SEQ ID NO:33), *Brassica napus* (SEQ ID NO:35), cotton (SEQ ID NO:37) and grape (SEQ ID NO:39). The corresponding encoded polypeptides are given in SEQ ID NO:28, SEQ ID NO:SEQ ID NO:30, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40. Other ANR sequences which may be utilized include those from *M. truncatula* (*e.g.* SEQ ID NO:43) or *A. thaliana* (*e.g.* SEQ ID NO:45). The corresponding encoded peptides are given in SEQ ID NO:44 and SEQ ID NO:46. One aspect of the invention thus provides a LAP-encoding sequence, such as LAP1 –LAP4 (SEQ ID NOs:1-4), used in conjunction with another PA biosynthesis sequence. Also provided are nucleic acids hybridizing to any of the foregoing nucleic acid sequences and encoding a polypeptide conferring a LAP phenotype.

As indicated above, a modulation of the phenotype of a gene may be obtained in accordance with the invention by introduction of recombinant nucleic acids comprising a LAP coding sequence. Other aspects of the invention are sequences that hybridize to the LAP1- LAP4 coding sequences provided herein under high stringency conditions. As used herein, "hybridization" or "hybridizes" is understood to mean the forming of a double or triple stranded molecule or a molecule with partial double or triple stranded nature. As used herein "stringent condition(s)" or "high stringency" are those conditions that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences.

Stringent conditions tolerate little mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity. Medium stringent conditions may comprise relatively low salt and/or relatively high temperature conditions, such as provided by about 1X SSC, and 65°C. High stringency may be defined as 0.02M to 0.10M NaCl and 50°C to 70°C. Specific examples of such conditions include 0.02M NaCl and 50°C; 0.02M NaCl and 60°C; and 0.02M NaCl and 70°C.

Alterations of the native amino acid sequence to produce variant polypeptides can be prepared by a variety of means known to those ordinarily skilled in the art. For instance, amino acid substitutions can be conveniently introduced into the polypeptides by changing the sequence of the nucleic acid molecule at the time of synthesis. Site-specific mutations can also be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the modified sequence. Alternately, oligonucleotide-directed, site-specific mutagenesis procedures can be used, such as disclosed in Walder *et al.* (1986); and U.S. Patents 4,518,584 and 4,737,462.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (*e.g.* Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid may be assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics. These are, for instance: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5). It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within .+-.2 is preferred, those within .+-.1 are more preferred, and those within .+-.0.5 are most preferred.

It is also understood in the art that the substitution of like amino acids may be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101 states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0 .+-.1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 .+-.1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4).

It is understood that an amino acid may be substituted by another amino acid having a similar hydrophilicity score and still result in a protein with similar biological activity, i.e., still obtain a biologically functional protein. In making such changes, the substitution of amino acids whose hydropathic indices are within .+-.2 is preferred, those within .+-.1 are more preferred, and those within .+-.0.5 are most preferred.

As outlined above, amino acid substitutions are therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine, and isoleucine.

It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleobase content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence or concentration of formamide, tetramethylammonium chloride or other solvent(s) in a hybridization mixture. It is also understood that compositions and conditions for hybridization are mentioned by way of non-limiting examples only, and that the desired stringency for a particular hybridization reaction in a plant cell is often determined empirically by comparison to one or more positive or negative controls. Depending on the application envisioned it is preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity of a nucleic acid towards a target sequence. Thus, nucleotide sequences displaying 95%, 98%, 99%, or greater similarity over the length of their coding regions to the LAP1-LAP4 coding sequences (SEQ ID NOs:1-4) provided herein, and that encode a functional LAP protein, are also an aspect of the invention, as is a LAP protein encoded by such a gene.

II. Plant Transformation Constructs

Certain embodiments of the current invention concern plant transformation constructs. For example, one aspect of the current invention is a plant transformation vector comprising a LAP1, LAP2, LAP3, or LAP4 coding sequence alone, or in combination with one or more PA biosynthesis gene(s). Examples of PA biosynthesis genes include BAN (*i.e.* ANR), PAP-1, TTG1 TTG2, TT1, and/or TT8. Exemplary PA biosynthesis coding sequences for use with the invention also include the *Arabidopsis* TT2 coding sequence (SEQ ID NO:41), which encodes the polypeptide sequence of SEQ ID NO:42, as well as a *Medicago truncatula* or *A. thaliana BAN* DNA sequence or encoded BAN polypeptide (*e.g.* SEQ ID NOs:43-46). Such LAP coding sequences may encode a polypeptide of any of SEQ ID NOs:5-8, for instance comprising the nucleotide sequence of any of SEQ ID NOs:1-4. Such coding sequences may be present in one or more plant expression cassettes and/or transformation vectors for introduction to a plant cell.

In certain embodiments of the invention, coding sequences are provided operably linked to a heterologous promoter, in either sense or antisense orientation.

Expression constructs are also provided comprising these sequences, as are plants and plant cells transformed with the sequences.

The construction of vectors which may be employed in conjunction with plant transformation techniques using these or other sequences according to the invention will be known to those of skill of the art in light of the present disclosure (see, for example, Sambrook *et al.,* 1989; Gelvin *et al.,* 1990). The techniques of the current invention are thus not limited to any particular nucleic acid sequences.

One important use of the sequences provided by the invention will be in the alteration of plant phenotypes by genetic transformation with sense or antisense PA biosynthesis genes. The PA biosynthesis gene may be provided with other sequences. Where an expressible coding region that is not necessarily a marker coding region is employed in combination with a marker coding region, one may employ the separate coding regions on either the same or different DNA segments for transformation. In the latter case, the different vectors are delivered concurrently to recipient cells to maximize cotransformation.

The choice of any additional elements used in conjunction with the PA biosynthesis coding sequences will often depend on the purpose of the transformation. One of the major purposes of transformation of crop plants is to add commercially desirable, agronomically important traits to the plant. As PAs are known to confer many beneficial effects on health, one such trait is increased biosynthesis of tannins. Alternatively, plants may be engineered to decrease synthesis of PA and increase anthocyanin content, for instance to promote production of a food colorant. Identification and engineered expression of LAP coding sequences as well as sequences from additional anthocyanin and PA biosynthesis- related functions allows for rational manipulation of the biosynthetic flux through these pathways.

Particularly useful for transformation are expression cassettes which have been isolated from such vectors. DNA segments used for transforming plant cells will, of course, generally comprise the cDNA, gene or genes which one desires to introduce into and have expressed in the host cells. These DNA segments can further include structures such as promoters, enhancers, polylinkers, or even regulatory genes as desired. The DNA segment or gene chosen for cellular introduction will often encode a protein which will be expressed in the resultant recombinant cells resulting in a screenable or selectable trait and/or which will impart an improved phenotype to the resulting transgenic plant. However, this may not always be the case, and the present invention also encompasses transgenic plants incorporating non-expressed transgenes. Preferred components likely to be included with vectors used in the current invention are as follows.

A. Regulatory Elements

Exemplary promoters for expression of a nucleic acid sequence include plant promoter such as the CaMV 35S promoter (Odell *et al.,* 1985), or others such as CaMV 19S (Lawton *et al.,* 1987), *nos* (Ebert *et al.,* 1987), Adh (Walker *et al.,* 1987), sucrose synthase (Yang and Russell, 1990), a-tubulin, actin (Wang *et al.,* 1992), *cab* (Sullivan *et al.,* 1989), PEPCase (Hudspeth and Grula, 1989) or those associated with the R gene complex (Chandler *et al.,* 1989). Tissue specific promoters such as root cell promoters (Conkling *et al.,* 1990) and tissue specific enhancers (Fromm *et al.,* 1986) are also contemplated to be particularly useful, as are inducible promoters such as ABA- and turgor-inducible promoters. In certain embodiments of the invention, the native promoter of a PA biosynthesis gene may be used.

The DNA sequence between the transcription initiation site and the start of the coding sequence, *i.e.*, the untranslated leader sequence, can also influence gene expression. One may thus wish to employ a particular leader sequence with a transformation construct of the invention. Preferred leader sequences are contemplated to include those which comprise sequences predicted to direct optimum expression of the attached gene, *i.e.*, to include a preferred consensus leader sequence which may increase or maintain mRNA stability and prevent inappropriate initiation of translation. The choice of such sequences will be known to those of skill in the art in light of the present disclosure. Sequences that are derived from genes that are highly expressed in plants will typically be preferred.

It is specifically envisioned that PA biosynthesis coding sequences may be introduced under the control of novel promoters or enhancers, *etc.*, or homologous or tissue specific promoters or control elements. Vectors for use in tissue-specific targeting of genes in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure. These include, for example, the *rbcS* promoter, specific for green tissue; the *ocs*, *nos* and *mas* promoters which have higher activity in roots or wounded leaf tissue; a truncated (-90 to +8) 35S promoter which directs enhanced expression in roots, and an α-tubulin gene that also directs expression in roots.

B. Terminators

Transformation constructs prepared in accordance with the invention will typically include a 3′ end DNA sequence that acts as a signal to terminate transcription and allow for the poly-adenylation of the mRNA produced by coding sequences operably linked to a PA biosynthesis gene. In one embodiment of the invention, the native terminator of a PA biosynthesis gene is used. Alternatively, a heterologous 3′ end may enhance the expression of sense or antisense PA biosynthesis genes. Terminators which are deemed to be particularly useful in this context include those from the nopaline synthase gene of *Agrobacterium tumefaciens* (nos 3′ end) (Bevan *et al.,* 1983), the terminator for the T7 transcript from the octopine synthase gene of *Agrobacterium tumefaciens,* and the 3′ end of the protease inhibitor I or II genes from potato or tomato. Regulatory elements such as an Adh intron (Callis *et al.,* 1987), sucrose synthase intron (Vasil *et al.,* 1989) or TMV omega element (Gallie *et al.,* 1989), may further be included where desired.

C. Transit or Signal Peptides

Sequences that are joined to the coding sequence of an expressed gene, which are removed post-translationally from the initial translation product and which facilitate the transport of the protein into or through intracellular or extracellular membranes, are termed transit (usually into vacuoles, vesicles, plastids and other intracellular organelles) and signal sequences (usually to the endoplasmic reticulum, golgi apparatus and outside of the cellular membrane). By facilitating the transport of the protein into compartments inside and outside the cell, these sequences may increase the accumulation of gene product protecting them from proteolytic degradation. These sequences also allow for additional mRNA sequences from highly expressed genes to be attached to the coding sequence of the genes. Since mRNA being translated by ribosomes is more stable than naked mRNA, the presence of translatable mRNA in front of the gene may increase the overall stability of the mRNA transcript from the gene and thereby increase synthesis of the gene product. Since transit and signal sequences are usually post-translationally removed from the initial translation product, the use of these sequences allows for the addition of extra translated sequences that may not appear on the final polypeptide. It further is contemplated that targeting of certain proteins may be desirable in order to enhance the stability of the protein (U.S. Patent No. 5,545,818, incorporated herein by reference in its entirety).

Additionally, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This generally will be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular, or extracellular destination, respectively, and will then be post-translationally removed.

D. Marker Genes

By employing a selectable or screenable marker protein, one can provide or enhance the ability to identify transformants. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker protein and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can "select" for by chemical means, *i.e.*, through the use of a selective agent (*e.g.*, a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, *i.e.*, by "screening" (*e.g.*, the green fluorescent protein). Of course, many examples of suitable marker proteins are known to the art and can be employed in the practice of the invention.

Included within the terms "selectable" or "screenable markers" also are genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which are secretable antigens that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, *e.g.*, by ELISA; small active enzymes detectable in extracellular solution (*e.g.*, α-amylase, β-lactamase, phosphinothricin acetyltransferase); and proteins that are inserted or trapped in the cell wall (*e.g.*, proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is considered to be particularly advantageous. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

Many selectable marker coding regions are known and could be used with the present invention including, but not limited to, *neo* (Potrykus *et al.,* 1985), which provides kanamycin resistance and can be selected for using kanamycin, G418, paromomycin, *etc.*; *bar*, which confers bialaphos or phosphinothricin resistance; a mutant EPSP synthase protein (Hinchee *et al.,* 1988) conferring glyphosate resistance; a nitrilase such as *bxn* from *Klebsiella ozaenae* which confers resistance to bromoxynil (Stalker *et al.,* 1988); a mutant acetolactate synthase (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate resistant DHFR (Thillet *et al.,* 1988), a dalapon dehalogenase that confers resistance to the herbicide dalapon; or a mutated anthranilate synthase that confers resistance to 5-methyl tryptophan.

An illustrative embodiment of selectable marker capable of being used in systems to select transformants are those that encode the enzyme phosphinothricin acetyltransferase, such as the *bar* gene from *Streptomyces hygroscopicus* or the *pat* gene from *Streptomyces viridochromogenes.* The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami *et al.,* 1986; Twell *et al.,* 1989) causing rapid accumulation of ammonia and cell death.

Screenable markers that may be employed include a β-glucuronidase (GUS) or *uid*A gene which encodes an enzyme for which various chromogenic substrates are known; an R-locus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta *et al.,* 1988); a β-lactamase gene (Sutcliffe, 1978), which encodes an enzyme for which various chromogenic substrates are known (*e.g.*, PADAC, a chromogenic cephalosporin); a *xyl*E gene (Zukowsky *et al.,* 1983) which encodes a catechol dioxygenase that can convert chromogenic catechols; an α-amylase gene (Ikuta *et al.,* 1990); a tyrosinase gene (Katz *et al.,* 1983) which encodes an enzyme capable of oxidizing tyrosine to

DOPA and dopaquinone which in turn condenses to form the easily-detectable compound melanin; a β-galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (*lux*) gene (Ow *et al.,* 1986), which allows for bioluminescence detection; an aequorin gene (Prasher *et al.,* 1985) which may be employed in calcium-sensitive bioluminescence detection; or a gene encoding for green fluorescent protein (Sheen *et al.,* 1995; Haseloff *et al.,* 1997; Reichel *et al.,* 1996; Tian *et al.,* 1997; WO 97/41228).

Another screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the *lux* gene. The presence of the *lux* gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It also is envisioned that this system may be developed for populational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening. The gene which encodes green fluorescent protein (GFP) is also contemplated as a particularly useful reporter gene (Sheen *et al.,* 1995; Haseloff *et al.,* 1997; Reichel *et al.,* 1996; Tian *et al.,* 1997; WO 97/41228). Expression of green fluorescent protein may be visualized in a cell or plant as fluorescence following illumination by particular wavelengths of light.

III. Antisense and RNAi Constructs

Antisense treatments represent one way of altering PA biosynthesis in accordance with the invention. In this manner, the accumulation of PA precursors, including anthocyanidins, could also be achieved. As such, antisense technology may be used to "knock-out" the function of an anthocyanin biosynthesis gene or homologous sequences thereof, such as UGT78G1, to increase the pool of anthocyanidin available for PA formation.

Antisense methodology takes advantage of the fact that nucleic acids tend to pair with "complementary" sequences. By complementary, it is meant that polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of an intron-exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

As stated above, “complementary” or “antisense” means polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have complementary nucleotides at thirteen or fourteen positions. Naturally, sequences which are completely complementary will be sequences which are entirely complementary throughout their entire length and have no base mismatches. Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, ribozyme; see above) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

It may be advantageous to combine portions of genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

RNA interference (RNAi) is a process utilizing endogenous cellular pathways whereby a double stranded RNA (dsRNA) specific target gene results in the degradation of the mRNA of interest. In recent years, RNAi has been used to perform gene “knockdown” in a number of species and experimental systems, from the nematode *C. elegans*, to plants, to insect embryos and cells in tissue culture (Fire *et al.*, 1998; Martinez *et al.*, 2002; McManus and Sharp, 2002). RNAi works through an endogenous pathway including the Dicer protein complex that generates ~21-nucleotide small interfering RNAs (siRNAs) from the original dsRNA and the RNA-induced silencing complex (RISC) that uses siRNA guides to recognize and degrade the corresponding mRNAs. Only transcripts complementary to the siRNA are cleaved and degraded, and thus the knock-down of mRNA expression is usually sequence specific. One of skill in the art would routinely be able to identify portions of, for instance, the UGT78G1 sequence, as targets for RNAi-mediated gene suppression to increase proanthocyanidin levels in alfalfa.

IV. Tissue Cultures

Tissue cultures may be used in certain transformation techniques for the preparation of cells for transformation and for the regeneration of plants therefrom. Maintenance of tissue cultures requires use of media and controlled environments. "Media" refers to the numerous nutrient mixtures that are used to grow cells *in vitro*, that is, outside of the intact living organism. The medium usually is a suspension of various categories of ingredients (salts, amino acids, growth regulators, sugars, buffers) that are required for growth of most cell types. However, each specific cell type requires a specific range of ingredient proportions for growth, and an even more specific range of formulas for optimum growth. Rate of cell growth also will vary among cultures initiated with the array of media that permit growth of that cell type.

Nutrient media is prepared as a liquid, but this may be solidified by adding the liquid to materials capable of providing a solid support. Agar is most commonly used for this purpose. Bacto™ agar (Difco-BD, Franklin Lakes, NJ), Hazelton agar (Hazleton, Lenexa, KS, USA), Gelrite® (Sigma, St. Louis, MO), PHYTAGEL (Sigma-Aldrich, St. Louis, MO), and GELGRO (ICN-MP Biochemicals, Irvine, CA, USA) are specific types of solid support that are suitable for growth of plant cells in tissue culture.

Some cell types will grow and divide either in liquid suspension or on solid media. As disclosed herein, plant cells will grow in suspension or on solid medium, but regeneration of plants from suspension cultures typically requires transfer from liquid to solid media at some point in development. The type and extent of differentiation of cells in culture will be affected not only by the type of media used and by the environment, for example, pH, but also by whether media is solid or liquid.

Tissue that can be grown in a culture includes meristem cells, callus, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. Callus may be initiated from tissue sources including, but not limited to, immature embryos, seedling apical meristems, root, leaf, microspores and the like. Those cells which are capable of proliferating as callus also are candidate recipient cells for genetic transformation.

Somatic cells are of various types. Embryogenic cells are one example of somatic cells which may be induced to regenerate a plant through embryo formation. Non-embryogenic cells are those which typically will not respond in such a fashion. Certain techniques may be used that enrich recipient cells within a cell population, for example by manual selection and culture of friable, embryogenic tissue. Manual selection techniques which can be employed to select target cells may include, *e.g.*, assessing cell morphology and differentiation, or may use various physical or biological means. Cryopreservation also is a possible method of selecting for recipient cells.

Where employed, cultured cells may be grown either on solid supports or in the form of liquid suspensions. In either instance, nutrients may be provided to the cells in the form of media, and environmental conditions controlled. There are many types of tissue culture media comprised of various amino acids, salts, sugars, growth regulators and vitamins. Most of the media employed in the practice of the invention will have some similar components, but may differ in the composition and proportions of their ingredients depending on the particular application envisioned. For example, various cell types usually grow in more than one type of media, but will exhibit different growth rates and different morphologies, depending on the growth media. In some media, cells survive but do not divide. Various types of media suitable for culture of plant cells previously have been described. Examples of these media include, but are not limited to, the N6 medium described by Chu *et al.*, (1975) and MS media (Murashige and Skoog, 1962).

V. Methods for Genetic Transformation

Suitable methods for transformation of plant or other cells for use with the current invention are believed to include virtually any method by which DNA can be introduced into a cell, such as by direct delivery of DNA such as by PEG-mediated transformation of protoplasts (Omirulleh *et al.,* 1993), by desiccation/inhibition-mediated DNA uptake (Potrykus *et al.,* 1985), by electroporation (U.S. Patent No. 5,384,253, specifically incorporated herein by reference in its entirety), by agitation with silicon carbide fibers (Kaeppler *et al.,* 1990; U.S. Patent No. 5,302,523, specifically incorporated herein by reference in its entirety; and U.S. Patent No. 5,464,765, specifically incorporated herein by reference in its entirety), by *Agrobacterium*-mediated transformation (U.S. Patent No. 5,591,616 and U.S. Patent No. 5,563,055; both specifically incorporated herein by reference) and by acceleration of DNA coated particles (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,877; and U.S. Patent No. 5,538,880; each specifically incorporated herein by reference in its entirety), *etc.* Through the application of techniques such as these, the cells of virtually any plant species may be stably transformed, and these cells developed into transgenic plants.

A. *Agrobacterium*-mediated Transformation

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because the DNA can be introduced into whole plant tissues, thereby bypassing the need for regeneration of an intact plant from a protoplast. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art. See, for example, the methods described by Fraley *et al.,* (1985), Rogers *et al.,* (1987) and U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety.

Agrobacterium-mediated transformation is most efficient in dicotyledonous plants and is the preferable method for transformation of dicots, including *Arabidopsis,* tobacco, tomato, alfalfa and potato. Indeed, while *Agrobacterium*-mediated transformation has been routinely used with dicotyledonous plants for a number of years, it has only recently become applicable to monocotyledonous plants. Advances in *Agrobacterium*-mediated transformation techniques have now made the technique applicable to nearly all monocotyledonous plants. For example, *Agrobacterium*-mediated transformation techniques have now been applied to rice

(Hiei *et al.,* 1997; U.S. Patent No. 5,591,616), wheat (McCormac *et al.,* 1998), barley (Tingay *et al.,* 1997; McCormac *et al.,* 1998), alfalfa (*e.g.* Thomas *et al.,* 1990; McKersie *et al.,* 1993) and maize (Ishida *et al.,* 1996).

Modern *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium,* allowing for convenient manipulations as described (Klee *et al.,* 1985). Moreover, recent technological advances in vectors for *Agrobacterium*-mediated gene transfer have improved the arrangement of genes and restriction sites in the vectors to facilitate the construction of vectors capable of expressing various polypeptide coding genes. The vectors described (Rogers *et al.,* 1987) have convenient multi-linker regions flanked by a promoter and a polyadenylation site for direct expression of inserted polypeptide coding genes and are suitable for present purposes. In addition, *Agrobacterium* containing both armed and disarmed Ti genes can be used for the transformations. In those plant strains where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer.

B. Electroporation

To effect transformation by electroporation, one may employ either friable tissues, such as a suspension culture of cells or embryogenic callus or alternatively one may transform immature embryos or other organized tissue directly. In this technique, one would partially degrade the cell walls of the chosen cells by exposing them to pectin-degrading enzymes (pectolyases) or mechanically wounding in a controlled manner. Examples of some species which have been transformed by electroporation of intact cells include maize (U.S. Patent No. 5,384,253; Rhodes *et al.,* 1995; D'Halluin *et al.,* 1992), wheat (Zhou *et al.,* 1993), tomato (Hou and Lin, 1996), soybean (Christou *et al.,* 1987) and tobacco (Lee *et al.,* 1989).

One also may employ protoplasts for electroporation transformation of plants (Bates, 1994; Lazzeri, 1995). For example, the generation of transgenic soybean plants by electroporation of cotyledon-derived protoplasts is described by Dhir and Widholm in Intl. Patent Appl. Publ. No. WO 9217598 (specifically incorporated herein by reference). Other examples of species for which protoplast transformation has been described include barley (Lazerri, 1995), sorghum (Battraw *et al.,* 1991), maize (Bhattacharjee *et al.,* 1997), wheat (He *et al.,* 1994) and tomato (Tsukada, 1989).

C. Microprojectile Bombardment

Another method for delivering transforming DNA segments to plant cells in accordance with the invention is microprojectile bombardment (U.S. Patent No. 5,550,318; U.S. Patent No. 5,538,880; U.S. Patent No. 5,610,042; and PCT Application WO 94/09699; each of which is specifically incorporated herein by reference in its entirety). In this method, particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, platinum, and preferably, gold. It is contemplated that in some instances DNA precipitation onto metal particles would not be necessary for DNA delivery to a recipient cell using microprojectile bombardment. However, it is contemplated that particles may contain DNA rather than be coated with DNA. Hence, it is proposed that DNA-coated particles may increase the level of DNA delivery via particle bombardment but are not, in and of themselves, necessary.

For the bombardment, cells in suspension are concentrated on filters or solid culture medium. Alternatively, immature embryos or other target cells may be arranged on solid culture medium. The cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate.

An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is the Biolistics® Particle Delivery System (Dupont), which can be used to propel particles coated with DNA or cells through a screen, such as a stainless steel or nylon screen (*e.g.* NYTEX screen; Sefar America, Depew, NY USA), onto a filter surface covered with plant cells cultured in suspension. The screen disperses the particles so that they are not delivered to the recipient cells in large aggregates. Microprojectile bombardment techniques are widely applicable, and may be used to transform virtually any plant species. Examples of species for which have been transformed by microprojectile bombardment include monocot species such as maize (PCT Application WO 95/06128), barley (Ritala *et al.*, 1994), wheat (U.S. Patent No. 5,563,055), and sorghum (Casa *et al.*, 1993); as well as a number of dicots including tobacco (Tomes *et al.*, 1990; Buising and Benbow, 1994), soybean (U.S. Patent No. 5,322,783), sunflower (Knittel *et al.*, 1994), peanut (Singsit *et al.*, 1997), cotton (McCabe and Martinell, 1993), tomato (VanEck *et al.*, 1995), and legumes in general (U.S. Patent No. 5,563,055, specifically incorporated herein by reference in its entirety).

D. Other Transformation Methods

Transformation of protoplasts can be achieved using methods based on calcium phosphate precipitation, polyethylene glycol treatment, electroporation, and combinations of these treatments (see, *e.g.*, Potrykus *et al.*, 1985; Lorz *et al.*, 1985; Omirulleh *et al.*, 1993; Fromm *et al.*, 1986; Uchimiya *et al.*, 1986; Callis *et al.*, 1987; Marcotte *et al.*, 1988).

Application of these systems to different plant strains depends upon the ability to regenerate that particular plant strain from protoplasts. Illustrative methods for the regeneration of plants from protoplasts have been described (Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Abdullah *et al.*, 1986; Omirulleh *et al.*, 1993 and U.S. Patent No. 5,508,184). Examples of the use of direct uptake transformation of protoplasts include transformation of rice (Ghosh-Biswas *et al.*, 1994), sorghum (Battraw and Hall, 1991), barley (Lazerri, 1995), oat (Zheng and Edwards, 1990) and maize (Omirulleh *et al.*, 1993).

To transform plant strains that cannot be successfully regenerated from protoplasts, other ways to introduce DNA into intact cells or tissues can be utilized. For example, regeneration of cereals from immature embryos or explants can be effected as described (Vasil, 1989). Also, silicon carbide fiber-mediated transformation may be used with or without protoplasting (Kaeppler, 1990; Kaeppler *et al.*, 1992; U.S. Patent No. 5,563,055). Transformation with this technique is accomplished by agitating silicon carbide fibers together with cells in a DNA solution. DNA passively enters as the cells are punctured. This technique has been used successfully with, for example, the monocot cereals maize (PCT Application WO 95/06128; (Thompson, 1995) and rice (Nagatani, 1997).

VI. Production and Characterization of Stably Transformed Plants

After effecting delivery of exogenous DNA to recipient cells, the next steps generally concern identifying the transformed cells for further culturing and plant regeneration. In order to improve the ability to identify transformants, one may desire to employ a selectable or screenable marker gene with a transformation vector prepared in accordance with the invention. In this case, one would then generally assay the potentially transformed cell population by exposing the cells to a selective agent or agents, or one would screen the cells for the desired marker gene trait.

A. Selection

It is believed that DNA is introduced into only a small percentage of target cells in any one experiment. In order to provide an efficient system for identification of those cells receiving DNA and integrating it into their genomes one may employ a means for selecting those cells that are stably transformed. One exemplary embodiment of such a method is to introduce into the host cell, a marker gene which confers resistance to some normally inhibitory agent, such as an antibiotic or herbicide. Examples of antibiotics which may be used include the aminoglycoside antibiotics neomycin, kanamycin and paromomycin, or the antibiotic hygromycin. Resistance to the aminoglycoside antibiotics is conferred by aminoglycoside phosphostransferase enzymes such as neomycin phosphotransferase II (NPT II) or NPT I, whereas resistance to hygromycin is conferred by hygromycin phosphotransferase.

Potentially transformed cells then are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene has been integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA.

One herbicide which constitutes a desirable selection agent is the broad spectrum herbicide bialaphos. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus* and is composed of phosphinothricin (PPT), an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of the L-alanine residues by intracellular peptidases, the PPT is released and is a potent inhibitor of glutamine synthetase (GS), a pivotal enzyme involved in ammonia assimilation and nitrogen metabolism (Ogawa *et al.*, 1973). Synthetic PPT, the active ingredient in the herbicide Liberty™ also is effective as a selection agent. Inhibition of GS in plants by PPT causes the rapid accumulation of ammonia and death of the plant cells.

The organism producing bialaphos and other species of the genus *Streptomyces* also synthesizes an enzyme phosphinothricin acetyl transferase (PAT) which is encoded by the *bar* gene in *Streptomyces hygroscopicus* and the *pat* gene in *Streptomyces viridochromogenes*. The use of the herbicide resistance gene encoding phosphinothricin acetyl transferase (PAT) is referred to in DE 3642 829 A, wherein the gene is isolated from *Streptomyces viridochromogenes*. In the bacterial source organism, this enzyme acetylates the free amino group of PPT preventing auto-toxicity (Thompson *et al.,* 1987). The *bar* gene has been cloned (Murakami *et al.,* 1986; Thompson *et al.,* 1987) and expressed in transgenic tobacco, tomato, potato (De Block *et al.,* 1987) *Brassica* (De Block *et al.,* 1989) and maize (U.S. Patent No. 5,550,318). In previous reports, some transgenic plants which expressed the resistance gene were completely resistant to commercial formulations of PPT and bialaphos in greenhouses.

Another example of a herbicide which is useful for selection of transformed cell lines in the practice of the invention is the broad spectrum herbicide glyphosate. Glyphosate inhibits the action of the enzyme EPSPS which is active in the aromatic amino acid biosynthetic pathway. Inhibition of this enzyme leads to starvation for the amino acids phenylalanine, tyrosine, and tryptophan and secondary metabolites derived thereof. U.S. Patent No. 4,535,060 describes the isolation of EPSPS mutations which confer glyphosate resistance on the *Salmonella typhimurium* gene for EPSPS, aroA. The EPSPS gene was cloned from *Zea mays* and mutations similar to those found in a glyphosate resistant aroA gene were introduced *in vitro.* Mutant genes encoding glyphosate resistant EPSPS enzymes are described in, for example, International Patent WO 97/4103. The best characterized mutant EPSPS gene conferring glyphosate resistance comprises amino acid changes at residues 102 and 106, although it is anticipated that other mutations will also be useful (PCT/WO97/4103).

To use the *bar*-bialaphos or the EPSPS-glyphosate selective system, transformed tissue is cultured for 0 - 28 days on nonselective medium and subsequently transferred to medium containing from 1-3 mg/l bialaphos or 1-3 mM glyphosate as appropriate. While ranges of 1-3 mg/l bialaphos or 1-3 mM glyphosate will typically be preferred, it is proposed that ranges of 0.1-50 mg/l bialaphos or 0.1-50 mM glyphosate will find utility.

It further is contemplated that the herbicide DALAPON, 2,2-dichloropropionic acid, may be useful for identification of transformed cells. The enzyme 2,2-dichloropropionic acid dehalogenase (*deh*) inactivates the herbicidal activity of 2,2-dichloropropionic acid and therefore confers herbicidal resistance on cells or plants expressing a gene encoding the dehalogenase enzyme (Buchanan-Wollaston *et al.,* 1992; U.S. Patent No. 5,508,468).

Alternatively, a gene encoding anthranilate synthase, which confers resistance to certain amino acid analogs, *e.g.*, 5-methyltryptophan or 6-methyl anthranilate, may be useful as a selectable marker gene. The use of an anthranilate synthase gene as a selectable marker was described in U.S. Patent No. 5,508,468.

An example of a screenable marker trait is the enzyme luciferase. In the presence of the substrate luciferin, cells expressing luciferase emit light which can be detected on photographic or x-ray film, in a luminometer (or liquid scintillation counter), by devices that enhance night vision, or by a highly light sensitive video camera, such as a photon counting camera. These assays are nondestructive and transformed cells may be cultured further following identification. The photon counting camera is especially valuable as it allows one to identify specific cells or groups of cells which are expressing luciferase and manipulate those in real time. Another screenable marker which may be used in a similar fashion is the gene coding for green fluorescent protein.

It further is contemplated that combinations of screenable and selectable markers will be useful for identification of transformed cells. In some cell or tissue types a selection agent, such as bialaphos or glyphosate, may either not provide enough killing activity to clearly recognize transformed cells or may cause substantial nonselective inhibition of transformants and nontransformants alike, thus causing the selection technique to not be effective. It is proposed that selection with a growth inhibiting compound, such as bialaphos or glyphosate at concentrations below those that cause 100% inhibition followed by screening of growing tissue for expression of a screenable marker gene such as luciferase would allow one to recover transformants from cell or tissue types that are not amenable to selection alone. It is proposed that combinations of selection and screening may enable one to identify transformants in a wider variety of cell and tissue types. This may be efficiently achieved using a gene fusion between a selectable marker gene and a screenable marker gene, for example, between an NPTII gene and a GFP gene.

B. Regeneration and Seed Production

Cells that survive the exposure to the selective agent, or cells that have been scored positive in a screening assay, may be cultured in media that supports regeneration of plants. In an exemplary embodiment, MS and N6 media may be modified by including further substances such as growth regulators. One such growth regulator is dicamba or 2,4-D. However, other growth regulators may be employed, including NAA, NAA + 2,4-D or picloram. Media improvement in these and like ways has been found to facilitate the growth of cells at specific developmental stages. Tissue may be maintained on a basic media with growth regulators until sufficient tissue is available to begin plant regeneration efforts, or following repeated rounds of manual selection, until the morphology of the tissue is suitable for regeneration, at least 2 wk, then transferred to media conducive to maturation of embryoids. Cultures are transferred every 2 wk on this medium. Shoot development will signal the time to transfer to medium lacking growth regulators.

The transformed cells, identified by selection or screening and cultured in an appropriate medium that supports regeneration, will then be allowed to mature into plants. Developing plantlets are transferred to soiless plant growth mix, and hardened, *e.g.*, in an environmentally controlled chamber, for example, at about 85% relative humidity, 600 ppm CO_2, and 25-250 microeinsteins m^{-2} s^{-1} of light. Plants are preferably matured either in a growth chamber or greenhouse. Plants can be regenerated from about 6 wk to 10 months after a transformant is identified, depending on the initial tissue. During regeneration, cells are grown on solid media in tissue culture vessels. Illustrative embodiments of such vessels are petri dishes and Plantcon™ containers (MP-ICN Biomedicals, Solon, OH, USA). Regenerating plants are preferably grown at about 19 to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Seeds on transformed plants may occasionally require embryo rescue due to cessation of seed development and premature senescence of plants. To rescue developing embryos, they are excised from surface-disinfected seeds 10-20 days post-pollination and cultured. An embodiment of media used for culture at this stage comprises MS salts, 2% sucrose, and 5.5 g/l agarose. In embryo rescue, large embryos (defined as greater than 3 mm in length) are germinated directly on an appropriate media. Embryos smaller than that may be cultured for 1 wk on media containing the above ingredients along with 10^{-5} M abscisic acid and then transferred to growth regulator-free medium for germination.

C. Characterization

To confirm the presence of the exogenous DNA or "transgene(s)" in the regenerating plants, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays, such as Southern and northern blotting and PCR; "biochemical" assays, such as detecting the presence of a protein product, *e.g.*, by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

D. DNA Integration, RNA Expression and Inheritance

Genomic DNA may be isolated from cell lines or any plant parts to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art. Note, that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell. The presence of DNA elements introduced through the methods of this invention may be determined, for example, by polymerase chain reaction (PCR). Using this technique, discreet fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a gene is present in a stable transformant, but does not prove integration of the introduced gene into the host cell genome. It is typically the case, however, that DNA has been integrated into the genome of all transformants that demonstrate the presence of the gene through PCR analysis. In addition, it is not typically possible using PCR™ techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, *i.e.*, whether transformants are of independent origin. It is contemplated that using PCR techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced gene.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridization pattern of a given transformant serves as an identifying characteristic of that transformant. In addition it is possible through Southern hybridization to demonstrate the presence of introduced genes in high molecular weight DNA, *i.e.*, confirm that the introduced gene has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR, *e.g.*, the presence of a gene, but also demonstrates integration into the genome and characterizes each individual transformant.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA will only be expressed in particular cells or tissue types and hence it will be necessary to prepare RNA for analysis from these tissues. PCR techniques also may be used for detection and quantitation of RNA produced from introduced genes. In this application of PCR it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species also can be determined using dot or slot blot northern hybridizations. These techniques are modifications of northern blotting and will only demonstrate the presence or absence of an RNA species.

E. Gene Expression

While Southern blotting and PCR may be used to detect the gene(s) in question, they do not provide information as to whether the corresponding protein is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced genes or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focusing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques.

Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures also may be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabeled acetylated phosphinothricin from phosphinothricin and ^{14}C-acetyl CoA or for anthranilate synthase activity by following loss of fluorescence of anthranilate, to name two.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of genes encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

VII. Breeding Plants of the Invention

In addition to direct transformation of a particular plant genotype with a construct prepared according to the current invention, transgenic plants may be made by crossing a plant having a selected DNA of the invention to a second plant lacking the construct. For example, a selected CT biosynthesis gene can be introduced into a particular plant variety by crossing, without the need for ever directly transforming a plant of that given variety. Therefore, the current invention not only encompasses a plant directly transformed or regenerated from cells which have been transformed in accordance with the current invention, but also the progeny of such plants. As used herein the term “progeny” denotes the offspring of any generation of a parent plant prepared in accordance with the instant invention, wherein the progeny comprises a selected DNA construct prepared in accordance with the invention. "Crossing" a plant to provide a plant line having one or more added transgenes relative to a starting plant line, as disclosed herein, is defined as the techniques that result in a transgene of the invention being introduced into a plant line by crossing a starting line with a donor plant line that comprises a transgene of the invention. To achieve this one could, for example, perform the following steps:

(a) plant seeds of the first (starting line) and second (donor plant line that comprises a transgene of the invention) parent plants;

(b) grow the seeds of the first and second parent plants into plants that bear flowers;

(c) pollinate a flower from the first parent plant with pollen from the second parent plant; and

(d) harvest seeds produced on the parent plant bearing the fertilized flower.

Backcrossing is herein defined as the process including the steps of:

(a) crossing a plant of a first genotype containing a desired gene, DNA sequence or element to a plant of a second genotype lacking the desired gene, DNA sequence or element;

(b) selecting one or more progeny plant containing the desired gene, DNA sequence or element;

(c) crossing the progeny plant to a plant of the second genotype; and

(d) repeating steps (b) and (c) for the purpose of transferring a desired DNA sequence from a plant of a first genotype to a plant of a second genotype.

Introgression of a DNA element into a plant genotype is defined as the result of the process of backcross conversion. A plant genotype into which a DNA sequence has been introgressed may be referred to as a backcross converted genotype, line, inbred, or hybrid. Similarly a plant genotype lacking the desired DNA sequence may be referred to as an unconverted genotype, line, inbred, or hybrid.

VIII. Definitions

Expression: The combination of intracellular processes, including transcription and translation undergone by a coding DNA molecule such as a structural gene to produce a polypeptide.

Genetic Transformation: A process of introducing a DNA sequence or construct (*e.g.*, a vector or expression cassette) into a cell or protoplast in which that exogenous DNA is incorporated into a chromosome or is capable of autonomous replication.

Heterologous: A sequence which is not normally present in a given host genome in the genetic context in which the sequence is currently found In this respect, the sequence may be native to the host genome, but be rearranged with respect to other genetic sequences within the host sequence. For example, a regulatory sequence may be heterologous in that it is linked to a different coding sequence relative to the native regulatory sequence.

Obtaining: When used in conjunction with a transgenic plant cell or transgenic plant, obtaining means either transforming a non-transgenic plant cell or plant to create the transgenic plant cell or plant, or planting transgenic plant seed to produce the transgenic plant cell or plant. Such a transgenic plant seed may be from an R_0 transgenic plant or may be from a progeny of any generation thereof that inherits a given transgenic sequence from a starting transgenic parent plant.

Proanthocyanidin (PA) biosynthesis gene: A gene encoding a polypeptide that catalyzes one or more steps in the biosynthesis of condensed tannins (proanthocyanidins).

Promoter: A recognition site on a DNA sequence or group of DNA sequences that provides an expression control element for a structural gene and to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene.

R_0 transgenic plant: A plant that has been genetically transformed or has been regenerated from a plant cell or cells that have been genetically transformed.

Regeneration: The process of growing a plant from a plant cell (*e.g.*, plant protoplast, callus or explant).

Selected DNA: A DNA segment which one desires to introduce into a plant genome by genetic transformation.

Transformation construct: A chimeric DNA molecule which is designed for introduction into a host genome by genetic transformation. Preferred transformation constructs will comprise all of the genetic elements necessary to direct the expression of one or more exogenous genes. In particular embodiments of the instant invention, it may be desirable to introduce a transformation construct into a host cell in the form of an expression cassette.

Transformed cell: A cell the DNA complement of which has been altered by the introduction of an exogenous DNA molecule into that cell.

Transgene: A segment of DNA which has been incorporated into a host genome or is capable of autonomous replication in a host cell and is capable of causing the expression of one or more coding sequences. Exemplary transgenes will provide the host cell, or plants regenerated therefrom, with a novel phenotype relative to the corresponding non-transformed cell or plant. Transgenes may be directly introduced into a plant by genetic transformation, or may be inherited from a plant of any previous generation which was transformed with the DNA segment.

Transgenic plant: A plant or progeny plant of any subsequent generation derived therefrom, wherein the DNA of the plant or progeny thereof contains an introduced exogenous DNA segment not naturally present in a non-transgenic plant of the same strain. The transgenic plant may additionally contain sequences which are native to the plant being transformed, but wherein the "exogenous" gene has been altered in order to alter the level or pattern of expression of the gene, for example, by use of one or more heterologous regulatory or other elements.

Vector: A DNA molecule capable of replication in a host cell and/or to which another DNA segment can be operatively linked so as to bring about replication of the attached segment. A plasmid is an exemplary vector.

IX. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

EXAMPLE 1

The *Arabidopsis* PAP1 Myb Transcription Factor Does Not Induce Anthocyanin Production in Legumes

The *PAP1* (producer of anthocyanin pigmentation) gene of *Arabidopsis* encodes a MYB transcription factor (Borevitz *et al.,* 2000). The *PAP1* gene of *Arabidopsis* is a global regulator of the anthocyanin biosynthetic pathway, and its ectopic expression in *Arabidopsis* results in a deep purple phenotype associated with accumulation of anthocyanins (Borevitz *et al.*, 2000; Tohge *et al.*, 2005). A similar phenotype is observed when AtPAP1 is expressed in tobacco (Borevitz *et al.*, 2000; Xie *et al.*, 2006), suggesting that AtPAP1 function might not be species-specific. During attempts to introduce anthocyanin as substrate for conversion into PAs in forage legumes, a *35S-AtPAP1* construct was introduced by *Agrobacterium*-mediated transformation into alfalfa, *Medicago truncatula* and white clover. Multiple independent transgenic lines were verified as expressing the *PAP1* transgene, but the plants never demonstrated a purple phenotype (*e.g.* FIG. 3A), and foliar anthocyanins could not be detected by HPLC analysis. Thus, over-expression of PAP1 leads to strong constitutive induction of the complete pathway leading to anthocyanins in *Arabidopsis* and tobacco, but not in leguminous plants.

EXAMPLE 2

Identification of *Medicago* Anthocyanin Pathway Regulatory Genes

To overcome the above limitation in expressing PA's and/or anthocyanins in legumes including alfalfa, a bioinformatics search was undertaken to identify transcription factors from *M. truncatula* (a close relative of alfalfa) which might regulate anthocyanin synthesis in legumes in a similar manner to the effects of expression of AtPAP1 in *Arabidopsis* and tobacco. Since transcription factors are expressed at very low levels they are rarely found in traditional EST collections. Indeed, the *Arabidopsis PAP1* and *PAP2* genes were first identified using an activation tagging screen (Borevitz *et al.*, 2000). Searches of the publicly available *M. truncatula* EST collections (*e.g. Medicago truncatula* Gene Index at Dana Farber

Cancer Institute (www.compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago) led to no *Medicago* sequences apparently homologous to *Arabidopsis* PAP1 or PAP2. The approach subsequently taken was therefore to identify similar genes from other plant species and use them as templates for further BLAST analysis.

There are several PAP1-like *MYB* genes in various species including tomato, grape, strawberry and petunia, all of which have been shown to regulate anthocyanin synthesis in the respective species (Quattrocchio *et al.*, 1999; Aharoni *et al.*, 2001; Mathews *et al.*, 2003; Deluc *et al.*, 2006). However, although all these transcription factors share the conserved R2-R3 MYB domain, the C-terminal regions of the open reading frames appears to exhibit marginal cross-species similarity, and it is not possible to ascribe a function based on overall sequence similarity. Using the AtPAP1 sequence, BLAST analysis (*e.g.* NCBI BLAST found at www.ncbi.nlm.nih.gov/BLAST) was performed against the currently known genomic sequence of *M. truncatula,* and several *MYB*-like sequences were identified. To better identify true anthocyanin-regulatory genes, two functional orthologs of *PAP1*, *AN2* from Petunia and *Anthocyanin 1* from tomato (SEQ ID NOs:95-96), were then compared with the Medicago *MYB* genes. Four *Medicago* genomic sequences with moderate similarity to both AN2 and AtPAP1 were identified and named MtLAP1-MtLAP4 (for Legume Anthocyanin Producer: gi|84662902|gb|AC152405.15|, 100321-101295 bases (SEQ ID NO:89); gi|86604519|emb|CT573509.1|, 52130-53141 bases (SEQ ID NO:90); gi|86361369|gb|AC172742.2|, 29610-32813 bases (SEQ ID NO:91; and gi|86604519|emb|CT573509.1|, 13597-15603 bases (SEQ ID NO:92). These genes were not represented by ESTs in GenBank or the TIGR *M. truncatula* database, indicating that the encoded transcription factors are either expressed at very low levels, or have temporally and or/spatially limited expression patterns.

FIG. 4A shows the predicted open reading frames of *Medicago* LAP1-LAP4 aligned with the *Arabidopsis*, tomato and petunia anthocyanin regulatory transcription factors. All of these proteins share the conserved R2R3 MYB-like domain; however, past this region there is very little similarity. FIG. 4B shows the phylogenetic relationships between the predicted ORFs. MtLAP1 and MtLAP3 were the most similar pair, with close to 73% identity. The other MtLAP members have between 59.5 and 69% identity to one another, whereas AtPAP1 and AN2 proteins are between 36 and 47% identical, with MtLAP1 sharing only 41% amino acid identity to

AtPAP1. This lack of similarity probably explains the lack of an anthocyanin phenotype in alfalfa plants expressing AtPAP1.

Primers were designed to amplify the three exons of MtLAP1 and MtLAP2 separately (Table 1; SEQ ID NOs:47-52), and the PCR products were then combined to amplify the corresponding complete open reading frame which was cloned directionally into the pENTR-D vector (Invitrogen, Carlsbad, California) and transformed into *E. coli* DH5α. After sequencing to confirm that no errors had been introduced, the ORFs were transferred into the binary expression vector pB2GW7. The plasmids (pB2-MtLAP1, pB2-MtLAP2, and pB2-GUS as control) were then re-sequenced to confirm proper orientation and transformed into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991).

Table 1. Primers used to amplify the MtLAP1 ORF from genomic sequences (SEQ ID NOs:47-52).

Primer Name	Sequence(5'-3')
5'MtLAP1Gen-1	CACCATGGAGAATACCGGAGGTGTGAGAAAA
3'MtLAP1Gen-1	TATTCAATCCAGATCTCTGAGGAACTAAATT
5'MtLAP1Gen-2	AGAGATCTGGATTGAATAGATGCAGAAAAAG
3'MtLAP1Gen-2	AATGACCATCTATTTCCTAGTAGTTTGTGTA
5'MtLAP1Gen-3	AGGAAATAGATGGTCATTGATTGCTGGAAGG
3'MtLAP1Gen-3	TCAAGGTAGATCCCAAAGAGAATTCAAATCACAA

EXAMPLE 3

Plant Transformation And Visible Phenotypes Arising From *LAP1* Expression In Transgenic Plants

Constructs harboring MtLAP1, MtLAP2 or GUS (for controls) were transformed into *Medicago truncatula* R108, *Nicotiana tabacum* and white clover (*Trifolium pratense*) using published protocols (Horsch *et al.*, 1985; Thomas *et al.*, 1990; Wright *et al.*, 2006). Transgene DNA was isolated using the Dellaporta method (Dellaporta *et al.*, 1983) and analyzed by qualitative PCR using 35S promoter- and transgene-specific primers. Plants were maintained in the greenhouse and allowed to self pollinate. In the case of the *Medicago truncatula* transformation, a total of 34 independent lines (each derived from a single embryo transformant) were obtained for the MtLAP1 and GUS transgenes, and 15 independent lines for the MtLAP2 transgene. MtLAP1 transformed into clover yielded 20 lines, while transformation of

MtLAP1 into MtANR-expressing alfalfa (see below) yielded 20 independent lines (each line derived from a separate callus).

The open reading frame of MtANR was sub-cloned into the binary vector pBI121 under control of the cauliflower mosaic virus 35S promoter with kanamycin as the plant selectable marker. Alfalfa plants of cultivar R2336 (Forage Genetics International, Nampa, ID) were transformed following published protocols (Thomas *et al.*, 1990) and selected for kanamycin resistance. From this population of primary transformants, a single line designated R15 was selected and used for further transformation with MtLAP1. Plants were transformed as above and plantlets screened for both kanamycin and phosphinothricin resistance.

The open reading frame of MtLAP1 was obtained using primers designed to amplify the three exons of the gene separately (Table 1); the exons were then combined prior to cloning as described in Example 2. LAP1 was initially expressed in transgenic tobacco under control of the 35S-promoter. FIG. 3B shows the mottled anthocyanin phenotype of the leaves, and the clearly enhanced pink phenotype of the flowers is shown in FIG. 3C. Interestingly, the observed phenotype was not as strong or uniform as seen when AtPAP1 is similarly expressed in tobacco (Xie *et al.*, 2006).

The 35S-MtLAP1 construct was then introduced into *M. truncatula* and alfalfa by *Agrobacterium*-mediated transformation. The alfalfa line used for transformation also contained a 35S-promoter driven copy of the *Medicago ANR* gene. An intense, uniform purple phenotype was observed in both species. FIG. 3D and FIG. 3E show the strong pigmentation in *M. truncatula* leaves, stems and seed pods. The roots also appeared to be strongly pigmented (data not shown). Alfalfa plants co-expressing LAP1 and ANR exhibited a strong but more diffuse purple phenotype than the *M. truncatula* lines expressing LAP1 alone (FIG. 3F). Similarly, a significant proportion of the purple pigmentation resulting from AtPAP1 expression is lost in tobacco plants co-expressing AtPAP1 and ANR (Xie *et al.*, 2006). Expression of MtLAP2 also resulted in purple pigmentation of alfalfa foliage (FIG. 5), indicating increased anthocyanin production as well. Transformation of white clover (*Trifolium pratense*) with 35S-LAP1 resulted in strong anthocyanin pigmentation throughout the leaf, except for the central region around the major leaf vein (FIG. 3H), as compared to the untransformed control plant (FIG. 3I).

EXAMPLE 4

Anthocyanin And Proanthocyanidin Content And Composition In *Medicago* Leaf Tissue Expressing LAP1

A. Anthocyanins

Total flavanoids were extracted from 100-500 mg samples (*i.e.* samples of Example 5) using 10 volumes of methanol:HCl (99:1 v/v). The first extraction was overnight at 4°C and the 2nd and 3rd extractions were for 2 h each. The supernatants were combined and dried under a stream of nitrogen or in a Speed Vac (Thermo-Savant, Waltham, MA). The dried samples were resuspended in 1ml of methanol mixed with 500µl of chloroform and 600µl ddH$_2$O. Following centrifugation at 12,000 g for 5 min, the aqueous supernatant was removed and dried. The dried samples were resuspended in methanol (1mg/ml); one aliquot was re-dried in a speed vac, and the absorbance of another aliquot was measured at 535nm (diluting samples where necessary) for total anthocyanin content using cyanidin as a reference standard. The samples were then resuspended in 2N HCl and heated at 90°C for 2 h to hydrolyze glycosidic bonds. Following cooling to room temperature the aglycones were extracted with ethyl acetate (3x 250 µl), dried under nitrogen, and then resuspended in methanol. Samples were analyzed on an HP 1100 series HPLC (Agilent Technologies, Palo Alto, CA) on a 250 x 4.6 mm C18 reversed phase column (Waters Sperisorb 5µ ODS2, Metachem Technologies Inc., Palo Alto, CA) with UV detection. The solvents used in the separation were 0.1% phosphoric acid (A) and acetonitrile (B). The gradient used for analysis was the same as reported previously (Xie *et al.*, 2006). Individual compounds were identified by retention times and absorption spectra by comparison with authentic standards injected separately.

Mass spectra of anthocyanins were acquired using a Bruker Esquire LC equipped with an electrospray ionization source in the positive mode (Bruker Optics, Billerica, MA). Positive ion ESI was performed using a source voltage of 3000 and capillary offset voltage of -70.7 V. Nebulization was achieved using Nitrogen gas at a pressure of 70 psi. Desolvation was aided by using nitrogen at a pressure of 12 psi as counter current gas. The capillary temperature was set at 350°C. Mass spectra were recorded over the range of 50-2200 m/z. The Bruker ion trap was operated under an ion current control of 20,000 with a max acquire time of 100 ms and a trap drive setting of 50. Automated MS/MS was performed by isolating the parent ion between

100-1900 m/z using an isolation width of 2.0, a fragmentation amplitude of 0.9, and a threshold of 1,500 with a max acquire time of 100 ms.

B. Proanthocyanidins

PAs were extracted by the method of Gu (Gu *et al.*, 2002) with the following modifications; frozen ground tissues (0.5g) were extracted with 5 ml 70% acetone/0.5% acetic acid first by vortexing then by sonication at 30°C for 30 min. Following centrifugation at 3,000 g, the supernatant was decanted into a 50 ml tube, while the residue was re-extracted again as above. The samples were then treated with 20 ml of chloroform to separate hydrophilic from hydrophobic compounds. Following centrifugation as above, the supernatant was retained and extracted a further two times with an equal volume of chloroform, then three times with hexanes (to remove residual fats). The samples were briefly dried under a stream of nitrogen, then freeze-dried for 48 h. The dried samples were resuspended in extraction (70% acetone/0,5% acetic acid) (3mg/µl initial weight). Total PAs were measured using a modified microplate assay: Samples (2.5µl) were mixed with 197.5 µl DMACA (dimethylaminocinnamaldehyde) reagent (0.2% w/v in methanol: 6N HCl (1/1)) in a well of a microplate. Samples were incubated for 5 min, then the absorbance at 630nm was recorded on a Victor2 microplate reader (Victor2 ™ multilabel counter, Perkin Elmer) equipped with a 630 nm emission filter. Blanks consisted of samples mixed with methanol: 6N HCl (1/1). Amounts are reported as catechin equivalents.

The polymer size of the PAs was examined using a combination of HPLC methods. Samples (5-20 µl) were analyzed on a 250 x 4.6 mm Silica column (Luna 5µ Supleco, St. Louis, MO). The solvents and gradients were similar to those reported previously (Gu *et al.*, 2002) with the following modifications: starting solvent composition was 82% methylene chloride (Solvent B), 14% methanol (Solvent C); 0-30 min 14-28.4 % B; 30-45 min 28.4-39.6 % B, 45-50 min 39.6-96 % B; hold for 10 min then 60-65 min 96-14 % B with 10 min re-equilibration. The individual components were detected by a modification of a post-column reaction system (Treutter, 1989; Treutter *et al.*, 1994; Pascual-Teresa *et al.*, 1998). The effluent from the column was combined with 1% DMACA in methanol (acidified with H_2SO_4, 1.5 N) via a mixing tee connected to an auxiliary pump (Alltech 460; Alltech Associates, Deerfield, IL, USA). The sample passed through 8 m of 0.2mm i.d. PEEK tubing before detection at 640 nm.

C. Summary

To qualitatively and quantitatively determine the metabolic effects of constitutive expression of LAP1 in *M. truncatula*, leaves were extracted in acidic methanol. *M. truncatula* leaves over-expressing MtLAP1 accumulate high levels of anthocyanins (1.0-2.3 mg/g FW, as determined by absorption spectroscopy) (FIG. 6A). When these leaf extracts were analyzed by reverse phase-HPLC, the presence of several cyanidin conjugates was observed as well as increased levels of flavone (luteolin, apigenin) glycosides in the MtLAP1-expressing lines (FIG. 6B). Following acid hydrolysis of the leaf extracts, the majority of the anthocyanidin released was cyanidin, indicating that MtLAP1 expression leads to the accumulation of predominately cyanidin conjugates (glycosides and/or other esters) (FIG. 6C). FIG. 6D shows the hydrolysed anthocyanin extract from MsLAP1:MtANR leaf extracts, showing cyanidin as the major anthocyanin backbone.

Methanolic extracts from leaves of alfalfa plants co-expressing LAP1 and ANR were subjected to LC MS/MS analysis to obtain further information as to the nature of the induced anthocyanins. FIG. 7A shows a typical chromatogram which reveals several cyanidin-containing peaks. All anthocyanins detected were derived from the cyanidin backbone, but differed in the type and degree of glycosylation. Eight major cyanidin derivatives were identified, most of which were multiply conjugated, with glucuronic acid conjugates predominating. UV and MS/MS analysis of the major compound eluting in the chromatograph shown in FIG. 7B revealed cyanidin conjugated with a glucuronic acid and a hexose (presumably glucose; tentative structure in FIG. 7D).

EXAMPLE 5

Genes Regulated By LAP1 In *Medicago truncatula*

RNA was extracted from leaves of *M. truncatula* expressing LAP1, and alfalfa co-expressing LAP1 and MtANR, and subjected to RT-PCR analysis to monitor transcript levels for genes known to be involved in anthocyanin biosynthesis. The primers used are shown in Table 2.

Table 2: Primers used for RT-PCR analysis of anthocyanin and proanthocyanidin pathway gene expression. (**SEQ ID NOs:53-78**)

Primer Name	Sequence(5'-3')
3'MtCHI	GTGTGCCACACAGTTCTCCA
5'MtCHI	ATGGCTGCATCAATCACCGC
3'MtF3'H	ATCTTCCTCCTATACATTTCAG
5'MtF3'H	GGCACTATTACTCTATTGCT
3'MtDFR-1	CATCCCACAAGGGCTTTTGA
5'MtDFR-1	ATGGGTTCTATGGCCGAAACTG
3'MtDFR-2	CATCAATTACAGAATTTTGTTGCTCAG
5'MtDFR-2	ATGGGTTCAGTCTCAGAAACAG
3'MtANS	TCCATAACCTTGAATCTTCC
5' MtANS	CAAGTTCCAACAATAGACCT
3' MtLAR	TGATAGATTTCATGGCTTCC
5' MtLAR	TAACTGAGGCAAGTATTTCC
3'MtActin	TAACCCTCATAGATTGGCAC
5'MtActin	AGTAACTGGGATGACATGGA
3'MtLAP1	TGACAAAGTTATAGGACGAG
5'MtLAP1	AAGTTGTAGATTGAGGTGG
5'MtBAN	TGCAAACAAAACATCTCACCTCATAG
3'MtBAN	AATTTCCACGCAGCCTTTTCAG
5'MtPAL	ACAGGGAGTCATTTGGATGAGGTG
3'MtPAL	GGAACTCCTAATCAACATGTTGACG
5'MtC4H	AGCTAGTGAACCACCAAGGCATCC
3'MtC4H	ACTGTCCTCCTTTCTCAGAAGTGTC
5'MtCHS	AGTCTCAATGGTAAGTCCTGGTCC
3'MtCHS	GGACAAGCACTATTTGGAGATGGAG
5'MtF3H	AGACCAAGTGGGTGGTCTTCAAGC
3'MtF3H	ATCTCTGAGATACACGATCAAGGAC

RNA was also analyzed from leaves of control plants transformed with the β-glucuronidase (GUS) gene. Several flavonoid pathway genes were induced more than 10-fold as a result of LAP1 expression. These included chalcone synthase (*CHS*), flavanone 3'-hydroxylase (*F3'H*), dihydroflavonol reductase 1 (*DFR1*), and anthocyanidin synthase (*ANS*) (FIG. 8). However, neither phenylalanine ammonia-lyase (PAL) nor cinnamate 4-hydroxylase (C4H), the first two enzymes of the phenylpropanoid pathway, were up-regulated, suggesting that MtLAP1 acts only on anthocyanin pathway-specific genes. In contrast, PAL and C4H are strongly up-regulated by AtPAP1 expression in *Arabidopsis* (Borevitz *et al.*, 2000). However, transcripts encoding chalcone isomerase, the second enzyme in the flavonoid branch, were not up-regulated by LAP1, and DFR2 was strongly induced in alfalfa but not in *M. truncatula*. LAP1 did not induce the PA-pathway specific enzymes leucoanthocyanidin reductase (LAR) or ANR.

A more global assessment of transcript induction in response to LAP1 expression was obtained by Affymetrix microarray comparison (Affymetrix Corp., Santa Clara, CA, USA) of transcripts from *M. truncatula* expressing MtLAP1 or GUS. Three independent MtLAP1and GUS control lines were examined.

Plants used for the microarray experiment were grown and maintained in the greenhouse under standard conditions (16hr light/8hr dark, 24°C/18°C). Leaves from MtLAP1- and MtGUS-expressing plants were harvested directly into liquid nitrogen; care was taken to select leaves that were the same age and developmental stage to minimize variations between samples and treatments. Total RNA was isolated using Tri-Reagent™ (Sigma-Aldrich) according to the manufacturer's directions. Total RNA was then further purified, and DNA removed using Qiagen's RNeasy™ MinElute Cleanup Kit. Samples were diluted to 250 ng/ul. RNA from three independent lines from each transformation were subjected to microarray analysis using the Affymetrix Medicago Gene Chip® as described (Deavours *et al.*, 2006; Modolo *et al.*, 2007).

Table 3 provides a summary of the relative transcript levels and annotations of all the genes present on the chip that were induced 1.5-fold or more as a result of LAP1 expression. All the major anthocyanin biosynthetic genes were up-regulated, including 4-coumarate CoA ligase (*4CL*; 4.9-fold), *CHS* (multiple genes, 5-96-fold), *F3'H* (110-fold), *DFR-1* (109-fold), *DFR-2* (6.6-fold) and *ANS* (32-fold). Several uridine diphosphate glycosyltransferases (UGTs) were up-regulated by LAP1 expression. These included a gene with homology to *Arabidopsis* UGT75CI that has previously been ascribed a role in the glycosylation of anthocyanidins (Tohge *et al.*, 2005). Another glucosyltransferase, represented by TC105632, was up-regulated almost 30-fold in MtLAP1 leaf tissues. This enzyme has recently been classified as UGT78G1 (SEQ ID NO:10), and shows activity with anthocyanidins (cyanidin, delphinidin, and pelargonidin) although isoflavones are preferred substrates *in vitro* (Modolo *et al*, 2007).

In addition to biosynthetic enzymes involved in flavonoid biosynthesis, the other groups of induced genes encoded transcription factors, transporters, and a number of proteins of unknown function (Table 3) Two of the MYB transcription factor probes may be hybridizing to the *LAP1* transgene product. It remains to be determined whether the more highly induced transcription factors, transporters and unknown proteins are directly involved in anthocyanin production and accumulation.

Table 3: Genes induced by *LAP1* expression.

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
1	1.500459438	TC108827		ABE94086	VQ (*Medicago truncatula*). VQ motif; pfam05678
1	1.50431511	CX527054		NP_568578	Unknown
1	1.508595402	AJ498860.	AJ498860.		Unknown
1	1.526219104	AC137701	genomic clone bases 104725 106607		Unknown
1	1.533385131	TC103649		NP_201288	unknown protein (*Arabidopsis thaliana*).
1	1.564856352	BI311172		ABE80976	Yeast 73.5kDa hypothetical-related protein
1	1.595350872	AL381833		NP_683304.	unknown protein (*Arabidopsis thaliana*)
1	1.635723584	TC102352			Unknown protein
1	1.645928157	BG588732			Unknown
1	1.701040537	TC94794		ABD32591	conserved hypothetical protein (*Medicago truncatula*)
1	1.730560109	BI263622		BAD43289.	Unknown Protein
1	1.781167825	AC151666.	genomic clone bases 41495 40597	ABE86457.	hypothetical protein like
1	1.968633719	TC111020		ABD32591	conserved hypothetical protein (*Medicago truncatula*)
1	2.123920941	CB892051		AAM63790.	Unknown Protein
1	2.948376671	TC107896		NP_190741	unknown protein (*Arabidopsis thaliana*).saposin B domain-containing protein eukaryotic aspartyl protease
1	3.952930904	AC132565	genomic clone bases 77742 74206	NP_198869	Unknown Protein?
1	4.726130066	BF637940		BF637940	Phsphate starved leaf library clone Mt. unkown
1	5.719636462	AC145330	genomic clone bases 112146 110346	ABE91561	Protein of unknown function DUF341 (*Medicago truncatula*).
1	17.51410973	BG647066		Unknown	EST508685 HOGA *Medicago truncatula* cDNA clone pHOGA-15H19 5' end,RNA sequence
1	56.1393086	AC154034	genomic clone bases 58483 61171	NP_001055680	Eukaryotic protein of unknown function (DUF914);

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
2	1.57632892	TC102174		NP_201474	ZincFinger Protein
2	1.610893163	AC146720	ABE89629.	AC146720	Zinc finger, GATA-type
2	1.659262196	TC105690		AAY30857.	MADS-box transcription factor (*Prunus dulcis*). Involved in flower development?
2	1.707336474	TC102175		NP_201474.	Zinc-Finger like protein *Arabidopsis*
2	3.237013509	AC149079	genomic clone bases 98811 99796	ABE83707.1	transcription factor-like protein - Arabidopsis thaliana (*Medicago truncatula*). AP2 like may be involved with ethylene response
2	5.277956696	AL368418		ABE79293	IQ calmodulin-binding region (*Medicago truncatula*).
2	5.777555021	TC105769		Q9ZUU0	(Q9ZUU0) WRKY transcription factor 44 (WRKY DNA-binding protein 44) (TRANSPARENT TESTA GLABRA 2)
2	6.086416289	TC97762		AAS55706.	WRKY2 [*Nicotiana benthamiana*].Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus *Plant J.* 38 (5), 800-809 (2004)
2	15.32543616	BI312112		AAM97321	Homeodomain protein GhHOX1 involved with development?
2	23.78314984	BQ147051		AAN28286.	Myb like transcription factor similar to severa cotton Mybs but also to a Strawberry Myb that negatively regulates anthocyanin and flavanol accumulation
2	37.40303349	AC135317	genomic clone bases 9174 16609	AAG25927	anthocyanin 1 (Petunia x hybrida). anthocyanin1 of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes *Plant Cell* 12 (9), 1619-1632 (2000)
3	1.516857391	TC101144		CAA93316	nitrite transporter (*Cucumis sativus*).

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
3	1.673305706	CR932966		AAL05427	Vacuolar acid invertase
3	2.271546919	BG450094		ABE85045.	ABC transporter, transmembrane region, type 1 (*Medicago*)
3	2.302502449	CB892086		AAF15946	Cloning and expression of amino acid transporters from broad bean. *Plant Mol. Biol.* 41 (2), 259-268 (1999)
3	2.516468468	BE316901		NP_191829	ATMRP10 [*Arabidopsis thaliana*]. ABC-type multidrug transport system, ATPase and permease components (Defense mechanisms)
3	7.291492933	BQ147947		NP_001053053	Transmembrane amino acid transporter protein; pfam01490 Rice
3	8.871794434	BF639602		AAD01600	LeOPT1 (*Lycopersicon esculentum*). Oligopeptide transporter?
3	17.39794094	CX540228		Q7XYX0	Na+/H+ antiporter NHX6.Molecular characterization of Na+/H+ antiporters (ZmNHX) of maize (*Zea mays* L.) and their expression under salt stress *J. Plant Physiol.* 162 (1), 55-66 (2005)
4	1.511489409	TC109128		AAO22131	quinone oxidoreductase (Fragaria x ananassa).
4	1.514604938	TC688		AAY23356	3-ketoacyl-CoA reductase 3 (*Gossypium hirsutum*).
4	1.5492671	TC107174		Q6VAB3	GT from Stevia making sweet glycosides UDP Glucosyltransferase
4	1.561701368	BI268054.			UDP-glycosyltransferase/ transferase, transferring glycosyl groups
4	1.569246005	BE322778		S39507	glucuronosyl transferase homolog, ripening-related - tomato
4	1.597672972	U01020		U01020	*Medicago sativa* clone MsCHS6-4 chalcone synthase
4	1.61744966	TC102229		gi|92892500|gb|ABE91274.1|	Anthocyanin acytransfeerase
4	1.695040148	AL381856		ABL74480	Molecular cloning of Sweet potato (*Ipomoea batatas* L.) involved in anthocyanin production
4	1.790778397	TC102062		AAR13305	phytochelatin synthetase-like protein

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
					(*Phaseolus vulgaris*).
4	1.882478846	TC105988		BAE72096	*Lactuca sativa* short-chain dehydrogenase/reductase 1. Abscisic acid biosynthetic genes of lettuce
4	1.934971944	AC125473	genomic clone bases 53689 57789	ABE83980.1	probable UDP-glucose,sterol glucosyltransferase
4	2.012678128	AC125473	genomic clone bases 50127 52935	ABE83979.1	Glycosyl transferase, family 28
4	2.06856077	AW684295		ABE90441	Type III polyketide synthase (*Medicago truncatula*).Predicted naringenin-chalcone synthase
4	2.091294444	BE321766		Q9LZJ5	Multidrug resistance-associated protein 10 (Glutathione S-conjugate-transporting ATPase 10) (ATP-energized glutathioneS-conjugate pump 10).
4	2.117167571	AC140035	genomic clone bases 67938 67189	Q9LZJ5	Multidrug resistance-associated protein 10 (GlutathioneS-conjugate-transporting ATPase 10) (ATP-energized glutathioneS-conjugate pump 10).
4	2.204715236	BG450101		BAC78438.	cDNA cloning and expression of isoflavonoid-specific glucosyltransferase from *Glycyrrhiza echinata* cell-suspensioncultures *Planta* 218 (3), 456-459 (2004)
4	2.957758028	TC100292		BAC78656	beta-primeverosidase [Camellia sinensis].glycosyl hydrolase Cloning of beta-primeverosidase from tea leaves, a key enzyme in tea aroma formation *Plant Physiol.* 130 (4), 2164-2176 (2002)
4	3.929191593	TC100294		AAZ31067.	beta-glucosidase (*Medicago sativa*). Molecular analysis of the effect of thidiazuron on morphogenesis of the *Medicago sativa* callus

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
4	4.09441353	AC146789	genomic clone bases 34320 32141	P93149	Cytochrome P450 93B1 (Licodione synthase) ((2S)-flavanone2-hydroxylase) (Flavone synthase II) (CYP GE-5).Two new cytochrome P450 cDNAs from elicitor-induced Licorice (*Glycyrrhiza echinata* L.) cells
4	4.813343338	AC146342	genomic clone bases 5219 6819	AAO22131	Zinc-containing alcohol dehydrogenase superfamily quinone oxidoreductase (Fragaria x ananassa). FaQR, Required for the Biosynthesis of the Strawberry Flavor Compound 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone, Encodes an Enone Oxidoreductase. *Plant Cell* 18 (4), 1023-1037 (2006)
4	4.963582493	TC108579		P31687	4-coumarate--CoA ligase (*Glycine max*).Molecular cloning and expression of 4-coumarate:coenzyme A ligase,an enzyme involved in the resistance response of soybean (Glycine max L.) against pathogen attack. *Plant Physiol.* 102 (4), 1147-1156 (1993)
4	5.308798334	AC146650	genomic clone bases 79531 80861	ABE90076	Naringenin-chalcone synthase Type III polyketide synthase (*Medicago truncatula*).Chalcone and stilbene synthases; plant-specific polyketide synthases (PKS) and related enzymes, also called type III PKSs
4	6.559801163	TC101521		NP 194260	iron ion binding / isopenicillin-N synthase/ oxidoreductase oxidoreductase, 2OG-Fe(II) oxygenase familyprotein, similar to flavonol synthase (Petunia xhybrida)(GI:311658), anthocyanidin synthase (*Torenia*

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
					fournieri)(GI:12583673)
4	6.598831576	TC103465		AAR27015	dihydroflavonal-4-reductase 2 (*Medicago truncatula*).
4	7.348495446	AC146575	genomic clone bases 96668 95373	ABE90441	Naringenin-chalcone synthase Type III polyketide synthase (*Medicago truncatula*).
4	9.76505364	BI311259		ABE89970	chalcone synthase 3 Type III polyketide synthase (*Medicago truncatula*).
4	18.53831328	AC146575	genomic clone bases 92557 91179	ABE90440.	Naringenin-chalcone synthase; Type III polyketide synthase
4	19.59706853	BF635325		AAD10774.	Cytochrome b5 DIF-FA cytochrome b5 is required for full activity of flavonoid 3',5'-hydroxylase, a cytochrome P450 involved in the formation of blue flower colors. *Proc. Natl. Acad. Sci. U.S.A.* 96 (2), 778-783 (1999)
4	22.09700291	AC138453	genomic clone bases 120053 122730	ABE81099.	2OG-Fe(II) oxygenase (*Medicago truncatula*).Similar to Gibberellin and Flavanone hydroxylases
4	29.89064908	TC105632		BAE72453	UDP-glucose: flavonol 3-O-glucosyltransferase (Rosa hybrid) Novel Anthocyanin Synthesis Pathway in *Rosa hybrida* Uses a Single Enzyme for Glucosyltransferase Activity at Two Sites
4	32.00486522	BI311113		AAR26526	anthocyanidin synthase 2 (*Glycine max*).
4	35.25649325	CX526507		AAL07435	Aphid infested shoots of *Medicago truncatula*; similar to Prunasin hydrolase A
4	37.10455896	AC146683	genomic clone bases 54170 52902	ABE89969	Naringenin-chalcone synthase; Type III polyketide synthase

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
4	40.12194709	TC100057		ABE91274	anthocyanin 5-aromatic acyltransferase/benzoyltransferase-like protein/anthocyanin acyltransferase-like protein-related [Medicago
4	45.31027702	AC147472	genomic clone bases 118222 120283	ABA42223	Glutathione S-transferase, C-terminal; Thioredoxin-like fold Similar to Blood Orange GST
4	46.05536469	X80222		X80222	*M.sativa* mRNA for dihydroflavonol-4-reductase
4	64.32267886	TC98548		ABE91274	anthocyanin acyltransferase-like protein-related (*Medicago*)
4	68.35044523	AC146575	genomic clone bases 82667 81204	ABE90437	Naringenin-chalcone synthase; Type III polyketide synthase
4	81.68289535	BM812824		AAR26526	anthocyanidin synthase 2 [*Glycine max*].
4	96.28192087	AC146683	genomic clone bases 50180 48876	P51082	Chalcone synthase 1B (Naringenin-chalcone synthase 1B).
4	100.8305355	BQ147749		BAE71221	Noble est BQ147749 Similar to putative flavonoid 3'-hydroxylase [*Trifolium pratense*].
4	109.6525563	TC102034		Q6TQT1	DFR-1 *Medicago truncatula*
4	110.9283997	BE248436		BAE71221	putative flavonoid 3'-hydroxylase (*Trifolium pratense*).
5	1.507179982	TC109624		NP_001042139	Putative receptor protein kinase
5	1.518332136	TC105110		NP_198442	AMP binding / catalytic
5	1.561081065	TC98122		ABE82689	Tyrosine protein kinase, active site [*Medicago truncatula*
5	1.575299914	CX529437		CX529437	Latent Membrane Protein From Methyl Jasmonate eliceted Roots cultures
5	1.57717807	AJ499132		Q9M011	Light inducible protein
5	1.586842934	TC109710		AAR99376	ring domain containing protein (*Capsicum annuum*).
5	1.689120618	BQ153446		AAN07898	Xyloglucan endotransglycosylase
5	1.692612124	CB893247		NP_198442.	AMP binding / catalytic (*Arabidopsis*

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
					thaliana).
5	1.729894042	AJ499132		CAB82281.	light-inducible protein ATLS1 (*Arabidopsis thaliana*).
5	1.756047217	AC127018	genomic clone bases 39514 34926	ABE92051.	RNA-directed DNA polymerase (Reverse transcriptase);Polynucleotidyl transferase, Ribonuclease H fold [*Medicago truncatula*].
5	1.761465656	TC97484		NP_567636.	TOM1 (TOBAMOVIRUS MULTIPLICATION 1) [*Arabidopsis thaliana*].Transmembrane domain?
5	1.766759229	TC97483		BAE43836	tobamovirus multiplication 1 [Nicotiana tabacum].
5	1.867177685	BF651140		AAQ76706	microtubule-associated protein 1 light chain 3 [*Gossypium hirsutum*].GABA-receptor-associated protein) belongs ot a large family of proteins that mediate intracellular membrane trafficking and/or fusion
5	1.894328458	TC98046		NP_171993	phosphoprotein phosphatase [*Arabidopsis thaliana*].
5	1.935798086	AC134049	genomic clone bases 3231 1339	ABE82689.1	Tyrosine protein kinase, active site
5	2.016545422	AC142222	genomic clone bases 28683 27870	ABL97946	copper ion binding/electron transporter [*Brassica rapa*].Plastocyanin-like domain
5	2.065367484	BQ154698		ABE88271	IMP dehydrogenase/GMP reductase [*Medicago truncatula*].
5	2.113493752	AC146862	genomic clone bases 18999 13099	ABE91459	Protein kinase; NAF [Medicago truncatula]. Serine/Threonine protein kinases, catalytic domain
5	2.246515768	TC109490		ABE88271.	IMP dehydrogenase/GMP reductase [*Medicago truncatula*].
5	2.347021892	TC110695		NP_194589	NPGR2 (NO POLLEN GERMINATION RELATED 2); calmodulin binding [*Arabidopsis thaliana*].
5	2.55133089	AC149637	genomic clone bases 65354 63597	ABE88374	Peptidase C1A, papain

	Fold Increase	TC/Gene	Medicago annotation	Link to homolog/est	Description
5	3.497575404	CO516065	est	CO516065	Glandular trichome clone-ATP citrate lyase b-subunit [*Lupinus albus*].
5	3.980711007	BF634212		Q9FXS7.	EIG-I24 protein TRANSFERASE. Characterization of Elicitor-inducible Tobacco Genes Isolated by Differential Hybridization. *J. Gen. Plant Pathol.* 67:89-96 (2001).
5	4.144032233	BQ147614		CAC24477	GTP binding protein. Characterisation of cDNAs homologous to Rab5-GTP binding protein expressed during early somatic embryogenesis in hickory *Plant Sci.* 163:413-422 (2002)
5	4.411209254	TC112152		AAU14999	MtN19-like protein Treatment of pea pods with Bruchin B results in up-regulation of a gene similar to MtN19 *Plant Physiol. Biochem.* 43 (3), 225-231 (2005)
5	4.871792955	TC525		ABE90151	Cytochrome c oxidase assembly protein CtaG / Cox11, putative [*Medicago truncatula*].
5	4.959858613	CX539005		ABE78861.	Leucine-rich repeats (LRRs), ribonuclease inhibitor *Medicago*, very weak similarity
5	12.42467905	AC146755	genomic clone bases 64745 61928	BAD13141	="myosin heavy chain -related-like
5	17.41195083	BQ147874		CAB77393.	Germin-like protein from Bean
5	22.11465366	AW736653		NP_850317	Fructose-bisphosphate aldolase(7%)/unknown
5	26.2220735	AC127169	genomic clone bases 95538 98294	ABE83405	Protein kinase [*Medicago truncatula*].

EXAMPLE 6

Cloning And Expression Of UGT78G1

The *M. truncatula* EST clone NF083F04ST (GT83F, corresponding to TIGR clone TC105632) was PCR amplified from pBluescript II SK+ (Stratagene, La Jolla, CA, USA) with addition of BamHI and NotI sites (5'-CGGATCCATGTCTACCTTCAAAAATG-3'; (SEQ ID NO:83), upstream primer; 5'-TGCGGCCGCACTAGTGACAATTTG-3', downstream primer) (SEQ ID NO:84). The PCR product was purified, ligated to pGEMTeasy vector (Promega, Madison, WI, USA), sequenced, excised and re-cloned between the BamHI and NotI sites of pET28a(+) (Novagen, Madison, WI, USA) with a hexahistidine tag and a thrombin cleavage site. *E. coli* BL21 (DE3) cells harboring the expression construct were grown to an OD_{600} of 0.4-0.5, and expression was initiated by addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) at a final concentration of 0.2 mM, with further incubation with shaking overnight at 16 °C. The enzyme was purified from *E. coli* lysates by nickel affinity chromatography.

EXAMPLE 7

Analysis Of UGT78G1 Activity

Enzyme reactions were performed with 1-2.5 μg of enzyme in a total volume of 50 or 200 μl containing 50 mM Tris-HCl pH 7.0, 1.0-5.0 mM UDP-glucose, UDP-galactose or UDP-glucuronic acid, and 100-250 μM acceptor substrate at 30 °C. Reactions were stopped with TCA and products analyzed by HPLC as described above.

For kinetic analysis of GT83F, purified enzyme (1.25 μg) was added to reaction mixtures (50 μl final volume) containing 50 mM Tris-HCl pH 7.0, 10 μM UDP-[U-^{14}C]-glucose (0.3 Ci/mmol), 490 μM UDP-glucose (unlabelled), and 0-500 μM acceptor substrate. Reactions were stopped with TCA after 15 min incubation at 30 °C. Samples were extracted with 250 μl of ethyl acetate, and 200 μl were taken for liquid scintillation counting (Beckman LS6500). Data were analyzed using Hyper32 software for the analysis of enzyme-kinetic data (J. S. Easterby, University of Liverpool, Liverpool, UK; available at www.liv.ac.uk/~jse/software.html).

EXAMPLE 8

qRT-PCR Experiments to Determine the Expression Pattern of UGT78G1

PCR primers (Table 5) were designed using Primer3 software (Rozen and Skaletsky, 2000; www.primer3.sourceforge.net/). Design criteria were Tm values of 60 °C ± 1 °C, PCR amplicon lengths of 60 to 150 bp, and 18 to 24 nucleotide primers with GC content of 40% to 60%. Primer quality was checked using NetPrimer software (PREMIER Biosoft, Palo Alto, CA). The specificity of the primer pair sequences was checked against the *Medicago truncatula* transcript database using nucleotide-nucleotide BLAST (*e.g.* Altschul *et al.*, 1990).

Total RNA was isolated from tissues or cultured cells using TRIreagent (Molecular Research Center, Inc, Cincinnati, OH, USA) according to the manufacturer's instructions. RNA samples were treated with TURBO DNA-free DNase I (Ambion) according to the manufacturer's instructions, and checked for genomic DNA contamination by PCR using the primers 5'-GTCCTCTAAGGTTTAATGAACCGG-3' (upstream) (SEQ ID NO:85) and 5'-GAAAGACACAGCCAAGTTGCAC-3' (downstream) (SEQ ID NO:86) designed to amplify an intron sequence of the *M. truncatula* ubiquitin gene (TC# 102473), and the primers 5'-ATTGCCTGCCCAAGAGTGTAAG-3' (upstream) (SEQ ID NO:87) and 5'-CAGCCAAGTTGCACAAAACAAC-3' (downstream) (SEQ ID NO:88) designed to amplify an intron/exon fragment of the same gene. RNA integrity was evaluated with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) using RNA nano chips. RT reactions were done using the DNase I-treated RNAs and SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions.

PCR reactions were performed in an optical 384-well plate with an ABI PRISM 7900 HTsequence detection system (Applied Biosystems, Foster City, CA, USA), using SYBR Green to monitor dsDNA synthesis. Reactions contained 2.5 μl of SYBR Green Master Mix reagent (Applied Biosystems), 0.5 μl of cDNA, and 200 nM of each gene-specific primer in a final volume of 5 μl. PCR reactions were performed as described elsewhere (Czechowski *et al*, 2005). Data were analyzed using the SDS 2.2.1 software (Applied Biosystems). PCR efficiency (E) was estimated using the LinRegPCR software (Ramakers *et al*, 2003) and the transcript levels were determined by relative quantification (Pfaffl, 2001) using the actin gene (TC# 107326) as a reference.

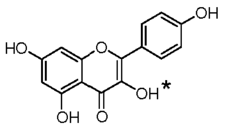
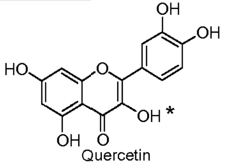
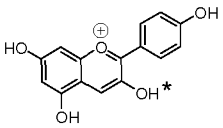
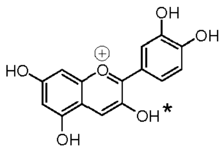
EXAMPLE 9

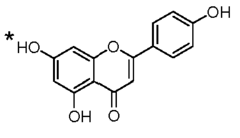
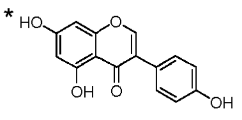
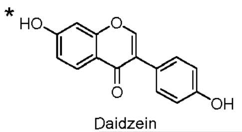
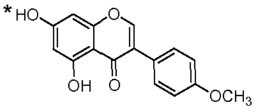
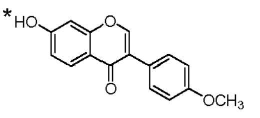
Properties Of UGT78G1

A. UGT78G1 Substrate Specificity and Products

Glucosyltransferase UGT78G1 (SEQ ID NO:10) was shown, in a preliminary screen, to exhibit activity with a wide range of flavonoid substrates (FIG. 9), and these compounds were therefore selected for in-depth kinetic analysis of substrate specificity. UGT78G1 displayed typical Michaelis-Menten kinetics for the various acceptor substrates FIG. 10, Table 4). A comparison of substrate structure-activity relationships revealed that the carbonyl group on ring C of the (iso)flavonoid acceptor is not critical, since the enzyme glycosylates anthocyanidins, which lack this group (Table 4). Substitution of the B-ring hydroxyl group at C4' with a methyl group increases the catalytic specificity (K_{cat}/K_m ratio) for the substrate when no hydroxyl group is present at C5 of the A ring. Thus, UGT78G1 prefers formononetin to daidzein by about 2-fold. Although the K_m values for biochanin A (4'-methoxy genistein) and genistein are different (11.7 and 36.7 μM, respectively), UGT78G1 exhibits similar overall specificity for these compounds under the experimental conditions used. Likewise, only slight differences in specificity were observed between genistein and daidzein, even though the K_m for the former is 23-fold higher than for the latter.

Table 4: Kinetic parameters for UGT78G1 toward different (iso)flavonoids. * indicates the position of glycosylation.

Substrate	V_{max} (μmol/min)	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})
 Kaempferol	1.5830	89.8	5.3×10^{-2}	589.5
 Quercetin	0.8557	28.7	2.9×10^{-2}	997.0
 Pelargonidin	0.2025	52.0	6.8×10^{-3}	130.2
 Cyanidin	0.1698	71.8	5.7×10^{-3}	79.1

 Apigenin	0.0688	1.5	2.3×10^{-3}	1,534.2
 Genistein	1.2050	36.7	4.0×10^{-3}	1,098.0
 Daidzein	0.0530	1.6	1.8×10^{-3}	1,107.7
 Biochanin A	0.3666	11.7	1.2×10^{-2}	1,047.8
 Formononetin	0.0879	1.5	2.9×10^{-3}	1,959.8

The position of the aromatic B ring only slightly affects substrate binding/turnover (Table 4). Thus, the K_{cat}/K_m ratio for apigenin (a flavone) is 1,534 whereas the K_{cat}/K_m ratio is 1,098 for its corresponding isoflavone (genistein). UGT78G1 glycosylates apigenin, genistein, biochanin A, daidzein, and formononetin at the 7-*O*- position (Table 4). However, the V_{max} of UGT78G1 for the flavonols kaempferol and quercetin, both of which have OH groups at C7 and C3, is much higher when compared with that for most of the other substrates, and the flavonols are glycosylated at the 3-position. However, anthocyanidins (cyanidin and pelargonidin), which also possess a 3-OH group, were relatively poor substrates for UGT78G1 in vitro. Nevertheless, UGT78G1 was the only enzyme, from among eight tested *Medicago* UGTs active with flavonoids, that could glycosylate anthocyanidins (data not shown).

B. Tissue-specific expression patterns of *Medicago* UGT78G1

The *in vitro* substrate specificity of a UGT does not necessarily reflect the enzyme's *in vivo* function (Achnine *et al.*, 2005). The strong induction of UGT78G1 as a result of ectopic expression of LAP1 in *M. truncatula* suggests that this particular UGT might be involved in anthocyanin glycosylation *in vivo*. To further address potential *in vivo* functions of UGT78G1, the expression pattern of this gene was evaluated by real time quantitative RT-PCR (qRT-PCR) using gene-specific primers (FIG. 11, Table 5). Stem, flower, root, leaf, and bud were the tissues examined, and root-derived cell suspension cultures also included in these analyses. Leaves were also wounded and inoculated with spores of the leaf spot pathogen *Phoma medicaginis*, a treatment that induces accumulation of isoflavonoid aglycones (He and Dixon, 2000; Deavours and Dixon, 2005). Cell suspension cultures were treated with yeast extract (YE), an inducer of isoflavonoid biosynthesis, or MeJA (methyl jasmonate), an inducer of triterpene saponin biosynthesis (Achnine *et al.*, 2005; Suzuki *et al.*, 2005).

Table 5: Primers used for the quantification of the transcript levels of *M. truncatula* (iso)flavonoid glycosyltransferase genes by qRT-PCR. (SEQ ID NOs:79-82).

Gene	Primer
Actin (upstream)	5'-TCAATGTGCCTGCCATGTATGT-3'
Actin (downstream)	5'-ACTCACACCGTCACCAGAATCC-3'
UGT78G1(upstream)	5'-GGCAGAGACAGGGAAGAACA-3'
UGT78G1 (downstream)	5'-TAAATCCGCACCAAACCAA-3'

UGT78G1 was expressed in all plant organs examined by qRT-PCR, but was most strongly expressed in buds. Its expression was strongly down-regulated by wounding of leaves, and also by application of MeJA to cell cultures. Expression in buds is consistent with involvement in anthocyanin synthesis.

EXAMPLE 10

Production Of PAs In Transgenic Alfalfa Co-Expressing LAP1 And ANR

To identify PAs in the transgenic tissues, a modified post column reaction HPLC analysis was performed (*e.g.* Peel and Dixon, 2007). This protocol allows for the detection of monomeric as well as simple oligomeric PAs. When leaf extracts from the tannin-rich legume *Desmodium uncinatum* are run under these conditions, monomeric PA units (catechin and epicatechin) as well as various oligomers of increasing size are separated easily (FIG. 12A). Extracts from leaves of LAP1-expressing *M. truncatula* plants were prepared with aqueous acidic acetone and analyzed in the same way for oligomeric PAs by normal phase HPLC followed by post-column derivatization with DMACA reagent (FIG. 12B). Control plants expressing GUS were analyzed in parallel, and shown to yield no detectable DMACA-positive material. In contrast, plants expressing MtLAP1 produced (-)-epicatechin and small amounts of oligomeric PAs. Since

LAP1 expression did not increase expression of transcripts encoding ANR or LAR (FIG. 8), it is likely that the massively elevated pool of anthocyanins in MtLAP1-expressing plants simply leaks towards formation of epicatechin and oligomeric PAs through low basal levels of endogenous ANR and/or LAR activity.

The above observation suggests that co-expression of ANR with MtLAP1 should lead to high levels of PA synthesis, as previously reported for co-expression of AtPAP1 and MtANR in tobacco (Xie *et al*., 2006). To test this hypothesis, leaves of transgenic alfalfa co-expressing MtLAP1 and MtANR were analyzed. It is already known that alfalfa foliage contains no detectable PA levels (Jackson and Barry, 1996), and this was confirmed by normal phase HPLC analysis of extracts from alfalfa plants co-expressing MtANR and GUS (FIG. 12C). Anthocyanidin supply is presumably limiting for PA production in such plants. However, in alfalfa plants co-expressing MtLAP1 and MtANR, small amounts of free epicatechin and catechin, plus significant levels of a range of oligomeric PAs, were observed (FIG. 12C). PA production was considerably higher than observed in *M. truncatula* plants expressing LAP1 alone (FIG. 12A). Overall levels of PAs in three independent lines of *M. truncatula* expressing LAP1, and alfalfa co-expressing LAP1 and MtANR, are shown in FIG. 12D. Importantly, the PA levels achieved in the alfalfa plants were within the range described as necessary for pasture bloat reduction (Li *et al*., 1996).

EXAMPLE 11

Production Of Insoluble PAs In Transgenic Alfalfa Co-Expressing LAP1 And ANR

Leaf extracts from transgenic alfalfa plants at 3 weeks after cut-back were analyzed for insoluble PA polymers using the butanol-HCl method described below.

For analysis of insoluble PAs, 0.5g of ground samples were extracted with 5 ml of 70% acetone/0.5% acetic acid (extraction solution) by vortexing, and then sonicated at room temperature for 1 hour. Following centrifugation at 2,500g for 10 min, the residues were re-extracted twice as above. The pooled supernatants were then extracted three times with chloroform and three times with hexane, and the supernatants (containing soluble PAs) and residues (containing insoluble PAs) from each sample were freeze dried separately. For quantification of insoluble PAs, 2 ml of butanol-HCl (95: 5, v/v) reagent was added to the dried residues and the mixtures sonicated at room temperature for 1 h, followed by centrifugation at

2,500g for 10 min. The absorption of the supernatants was measured at 550 nm; the samples were then boiled for 1 h, cooled to room temperature, and the absorbance at 550 nm recorded again, with the first value being subtracted from the second. Absorbance values were converted into PA equivalents using a standard curve of procyanidin B1 (Indofine, NJ, USA). Transgenic alfalfa plants co-expressing LAP1 and ANR produced at least four times more insoluble PAs than plants expressing LAP1 alone (FIG. 13). Control plants expressing GUS alone had very low levels of insoluble tannins.

EXAMPLE 12

Effects of ectopic expression of *Arabidopsis* TT2 in alfalfa

TT2 is a MYB transcription factor that controls the downstream, PA-specific branch of flavonoid biosynthesis in the *Arabidopsis* seed coat. Ectopic expression of AtTT2 in *Medicago truncatula* hairy roots leads to accumulation of PAs which when propagated normally accumulate large levels of anthocyanins. The same construct has been transformed into *Medicago sativa* plants, which do not accumulate significant levels of anthocyanins under normal conditions; however analysis of leaf tissues was able to show small amounts of oligomeric PA accumulation (FIG. 12A). Thus it appears that without an adequate supply of anthocyanidins, no accumulation of PAs can occur regardless of which downstream PA specific genes are expressed. It follows that co-expression of LAP1 and TT2 represents another strategy for introducing tannins to combat pasture bloat in alfalfa.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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The references listed below are incorporated herein by reference to the extent that they supplement, explain, provide a background for, or teach methodology, techniques, and/or compositions employed herein.

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CLAIMS

1. An isolated nucleic acid sequence selected from the group consisting of:
   (a) a nucleic acid sequence encoding the polypeptide sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10;
   (b) a nucleic acid sequence comprising a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9
   (c) a nucleic acid sequence that hybridizes to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, under conditions of 1X SSC, and 65°C and encodes a polypeptide that activates anthocyanin or proanthocyanidin biosynthesis;
   (d) a nucleic acid sequence encoding a polypeptide with at least 85% amino acid identity to any of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, that activates anthocyanin or proanthocyanidin biosynthesis;
   (e) a nucleic acid sequence with at least 85% identity to any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:9; and
   (f) a complement of a sequence of (a)-(e).

2. The isolated nucleic acid sequence of claim 1, wherein the nucleic acid sequence is operably linked to a heterologous promoter.

3. A recombinant vector comprising the isolated nucleic acid sequence of claim 1.

4. The recombinant vector of claim 3, further comprising at least one additional sequence chosen from the group consisting of: a regulatory sequence, a selectable marker, a leader sequence and a terminator.

5. The recombinant vector of claim 4, wherein the additional sequence is a heterologous sequence.

6. The recombinant vector of claim 5, wherein the heterologous sequence encodes an anthocyanin or proanthocyanidin biosynthesis activity selected from the group consisting of:

phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), anthocyanidin reductase (ANR), a proanthocyanidin or anthocyanidin glucosyltransferase (GT), or AtPAP1 (production of anthocyanin pigment).

7. The recombinant vector of claim 3, wherein the promoter is a plant developmentally-regulated, organelle-specific, inducible, tissue-specific, constitutive, or cell-specific promoter.

8. The recombinant vector of claim 3, defined as an isolated expression cassette.

9. An isolated polypeptide having at least 85% amino acid identity to the amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10, or a fragment thereof, having anthocyanin or proanthocyanidin biosynthesis activity.

10. The isolated polypeptide of claim 9, comprising the amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:10, or a fragment thereof, having anthocyanin or proanthocyanidin biosynthesis activity.

11. A transgenic plant transformed with the nucleic acid of claim 1.

12. The transgenic plant of claim 11, wherein the plant is a *Medicago* plant.

13. The transgenic *Medicago* plant of claim 12, wherein the plant expresses the selected DNA and exhibits increased proanthocyanidin biosynthesis in selected tissues relative to those tissues in a second plant that differs from the transgenic plant only in that the selected DNA is absent.

14. The transgenic plant of claim 11, further defined as transformed with a selected DNA encoding a LAP1, LAP2, LAP3, or LAP4 polypeptide selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or a fragment thereof, having anthocyanin or proanthocyanidin biosynthesis activity.

15. The transgenic plant of claim 11, further defined as transformed with a selected DNA sequence complementary to a sequence encoding a glycosyltransferase active in proanthocyanidin biosynthesis.

16. The transgenic plant of claim 15, wherein the selected DNA sequence comprises the complement of SEQ ID NO:10, or a fragment thereof.

17. The transgenic plant of claim 11, further defined as transformed with a DNA sequence encoding the polypeptide of SEQ ID NO:1.

18. The transgenic plant of claim 11, further defined as a forage crop.

19. The transgenic plant of claim 18, wherein the plant is a forage legume.

20. The transgenic plant of claim 19, wherein the forage legume is alfalfa (*Medicago sativa*).

21. The transgenic plant of claim 11, wherein the plant is further defined as comprising a transgenic coding sequence encoding an anthocyanin reductase polypeptide selected from the group consisting of: SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, and SEQ ID NO:46.

22. The transgenic plant of claim 11, wherein the plant is further defined as transformed with the recombinant vector of claim 6.

23. The transgenic plant of claim 11, further defined as a fertile R_0 transgenic plant.

24. The transgenic plant of claim 11, further defined as a progeny plant of any generation of a fertile R_0 transgenic plant, wherein the transgenic plant comprises the selected DNA.

25. The transgenic plant of claim 11, wherein the plant is further defined as comprising a transgenic sequence that down-regulates UGT78G1 expression.

26. A seed of the transgenic plant of claim 11, comprising the nucleic acid of claim 1.

27. A cell transformed with the nucleic acid of claim 1.

28. A method of producing a plant with increased proanthocyanidin biosynthesis, comprising introducing into the plant a selected DNA encoding a LAP polypeptide, wherein the coding sequence is operably linked to a promoter functional in the plant and wherein the plant comprises increased proanthocyanidin biosynthesis in aerial portions of the plant relative to a second plant that differs from the plant only in that the selected DNA is absent in the second plant.

29. The method of claim 28, wherein the selected DNA encoding a LAP polypeptide comprises the nucleic acid sequence of claim 1.

30. The method of claim 28, wherein the plant further comprises the recombinant vector of claim 6.

31. The method of claim 28, wherein the selected DNA is introduced into the plant by plant breeding.

32. The method of claim 28, wherein the selected DNA is introduced into the plant by genetic transformation of the plant.

33. The method of claim 28, wherein the promoter is a constitutive or tissue specific promoter.

34. The method of claim 28, wherein the plant is further defined as a forage crop.

35. The method of claim 28, wherein the plant is a forage legume.

36. The method of claim 28, wherein the plant is alfalfa.

37. The method of claim 28, further comprising preparing a transgenic progeny plant of any generation of the plant, wherein the progeny plant comprises the selected DNA.

38. A plant prepared by the method of claim 28.

39. A plant part prepared by the method of claim 28.

40. A method of making food or feed for human or animal consumption comprising:
    (a) obtaining the plant of claim 11;
    (b) growing the plant under plant growth conditions to produce plant tissue from the plant; and
    (c) preparing food or feed for human or animal consumption from the plant tissue.

41. The method of claim 40, wherein preparing food comprises harvesting the plant tissue.

42. The method of claim 40, wherein the food is hay, silage, starch, protein, meal, flour or grain.

43. A nutraceutical prepared by the method of:
    (a) obtaining the plant of claim 11;
    (b) growing the plant under plant growth conditions to produce plant tissue from the plant; and

(c) preparing a nutraceutical for human or animal consumption from the plant tissue.

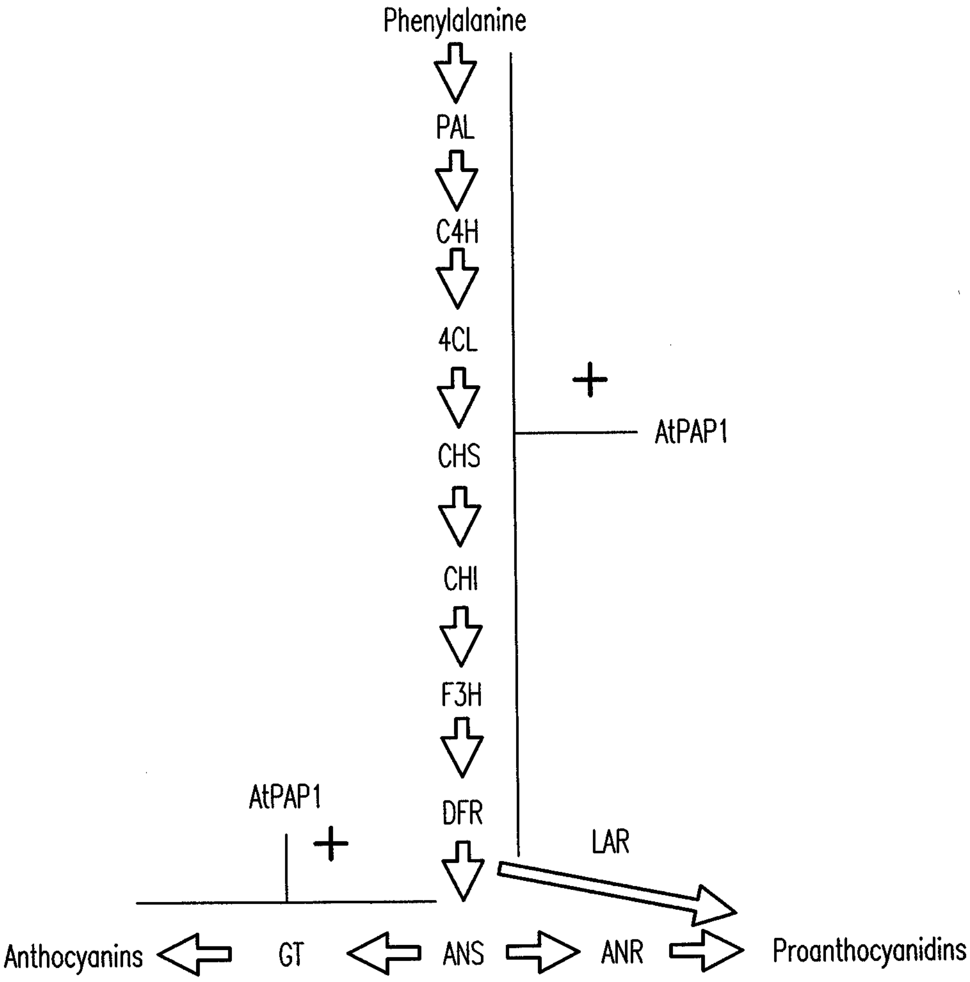
Phenylalanine
PAL
C4H
4CL
+
AtPAP1
CHS
CHI
F3H
AtPAP1
+
DFR
LAR
Anthocyanins
GT
ANS
ANR
Proanthocyanidins

FIG.1

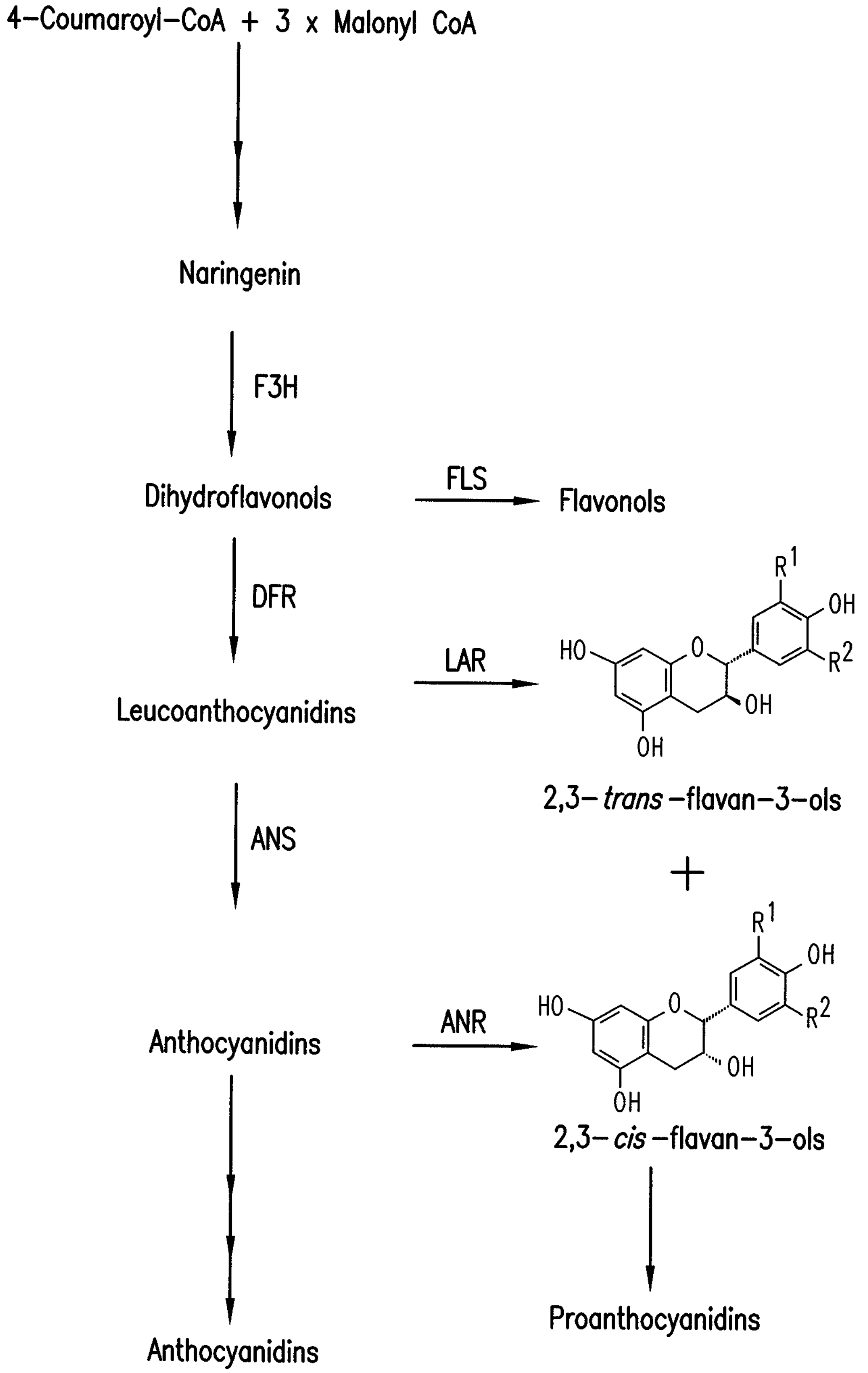
4-Coumaroyl-CoA + 3 x Malonyl CoA
Naringenin
F3H
Dihydroflavonols
FLS
Flavonols
DFR
LAR
R1
OH
HO
O
R2
OH
OH
Leucoanthocyanidins
2,3-*trans*-flavan-3-ols
+
ANS
R1
OH
HO
O
R2
OH
OH
Anthocyanidins
ANR
2,3-*cis*-flavan-3-ols
Proanthocyanidins
Anthocyanidins

FIG.2

FIG.3A

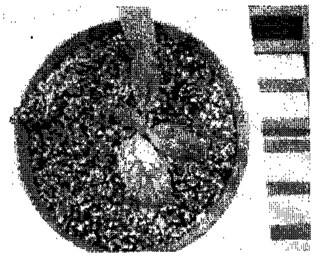

FIG.3B

FIG.3C

FIG.3D

FIG.3E

FIG.3F

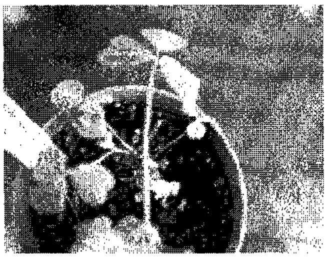

FIG.3G

FIG.3H

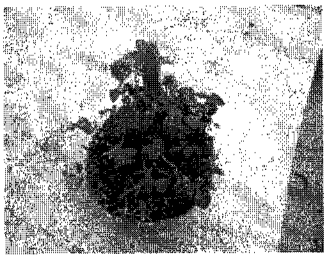

FIG.3I

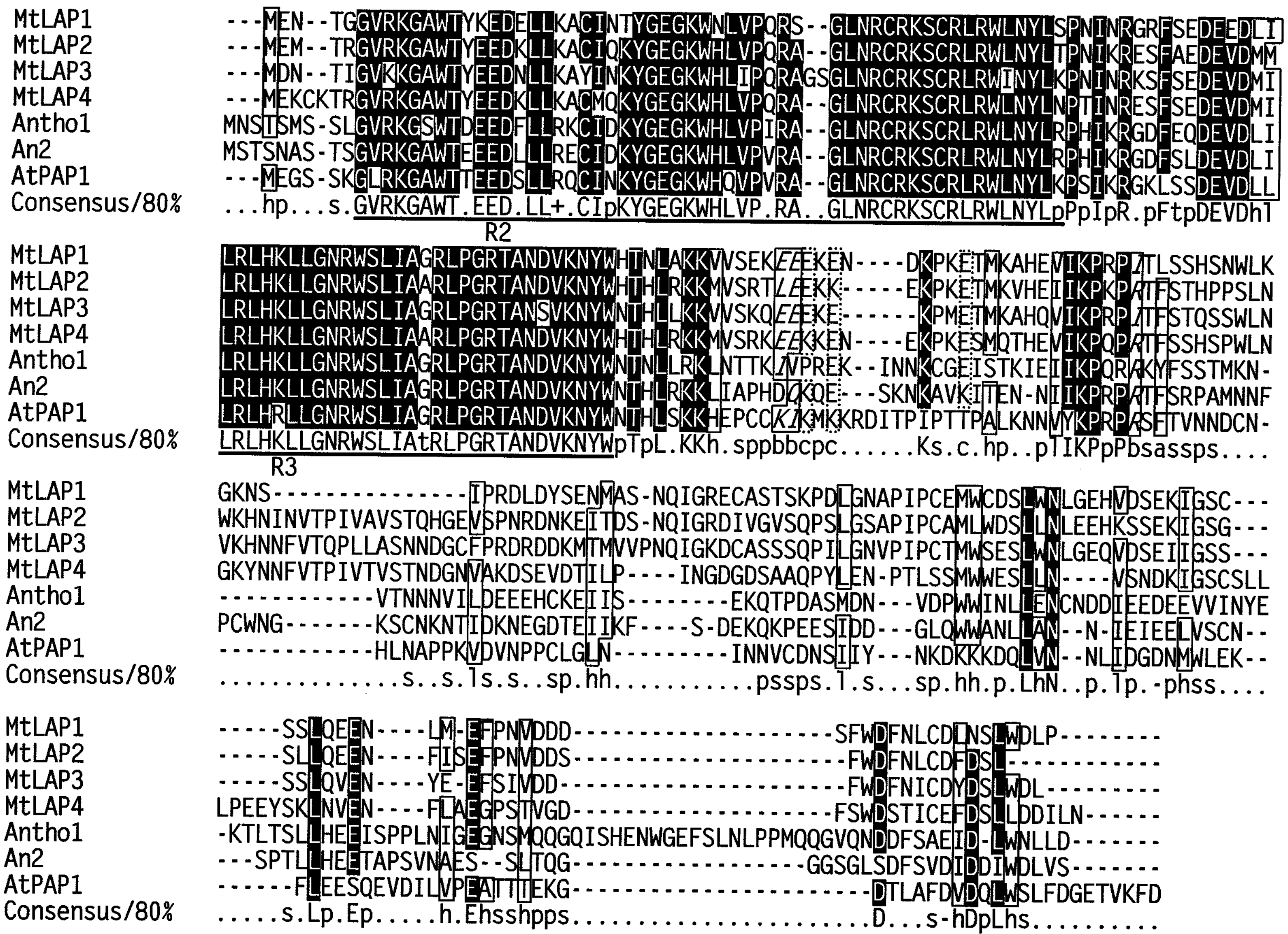
MtLAP1 ---MEN--TGGVRKGAWTYKEDELLKACINTYGEGKWNLVPQRS--GLNRCRKSCRLRWLNYLSPNINRGRFSEDEEDLI
MtLAP2 ---MEM--TRGVRKGAWTYEEDKLLKACIQKYGEGKWHLVPQRA--GLNRCRKSCRLRWLNYLTPNIKRESFAEDEVDMM
MtLAP3 ---MDN--TIGVKKGAWTYEEDNLLKAYINKYGEGKWHLIPQRAGSGLNRCRKSCRLRWINYLKPNINRKSFSEDEVDMI
MtLAP4 ---MEKCKTRGVRKGAWTYEEDKLLKACMQKYGEGKWHLVPQRA--GLNRCRKSCRLRWLNYLNPTINRESFSEDEVDMI
Antho1 MNSTSMS-SLGVRKGSWTDEEDFLLRKCIDKYGEGKWHLVPIRA--GLNRCRKSCRLRWLNYLRPHIKRGDFEQDEVDLI
An2 MSTSNAS-TSGVRKGAWTEEEDLLLRECIDKYGEGKWHLVPVRA--GLNRCRKSCRLRWLNYLRPHIKRGDFSLDEVDLI
AtPAP1 ---MEGS-SKGLRKGAWTTEEDSLLRQCINKYGEGKWHQVPVRA--GLNRCRKSCRLRWLNYLKPSIKRGKLSSDEVDLL
Consensus/80% ...hp...s.GVRKGAWT.EED.LL+.CIpKYGEGKWHLVP.RA..GLNRCRKSCRLRWLNYLpPpIpR.pFtpDEVDhl
R2
MtLAP1 LRLHKLLGNRWSLIAGRLPGRTANDVKNYWHTNLAKKVVSEKEEEKEN----DKPKETMKAHEVIKPRPITLSSHSNWLK
MtLAP2 LRLHKLLGNRWSLIAARLPGRTANDVKNYWHTHLRKKMVSRTLEEKK-----EKPKETMKVHEIIKPKPRTFSTHPPSLN
MtLAP3 LRLHKLLGNRWSLIAGRLPGRTANSVKNYWNTHLLKKVVSKQEEEKE------KPMETMKAHQVIKPRPITFSTQSSWLN
MtLAP4 LRLHKLLGNRWSLIAARLPGRTANDVKNYWHTHLRKKMVSRKEEKKEN----EKPKESMQTHEVIKPQPRTFSSHSPWLN
Antho1 LRLHKLLGNRWSLIAGRLPGRTANDVKNYWNTNLLRKLNTTKINVPREK--INNKCGEISTKIEIIKPQRAKYFSSTMKN-
An2 LRLHKLLGNRWSLIAGRLPGRTANDVKNYWNTHLRKKLIAPHDQKQE---SKNKAVKITEN-NIIKPRPRTFSRPAMNNF
AtPAP1 LRLHRLLGNRWSLIAGRLPGRTANDVKNYWNTHLSKKHEPCCKIKMKKRDITPIPTTPALKNNVYKPRPASFTVNNDCN-
Consensus/80% LRLHKLLGNRWSLIAtRLPGRTANDVKNYWpTpL.KKh.sppbbcpc......Ks.c.hp..pIKPpPbsassps....
R3
MtLAP1 GKNS---------------IPRDLDYSENMAS-NQIGRECASTSKPDLGNAPIPCEMWCDSLWNLGEHVDSEKIGSC---
MtLAP2 WKHNINVTPIVAVSTQHGEVSPNRDNKEITDS-NQIGRDIVGVSQPSLGSAPIPCAMLWDSLLNLEEHKSSEKIGSG---
MtLAP3 VKHNNFVTQPLLASNNDGCFPRDRDDKMTMVVPNQIGKDCASSSQPILGNVPIPCTMWSESLWNLGEQVDSEIIGSS---
MtLAP4 GKYNNFVTPIVTVSTNDGNVAKDSEVDTILP----INGDGDSAAQPYLEN-PTLSSMWWESLLN----VSNDKIGSCSLL
Antho1 -------------VTNNNVILDEEEHCKEIIS--------EKQTPDASMDN---VDPWWINLLENCNDDIEEDEEVVINYE
An2 PCWNG-------KSCNKNTIDKNEGDTEIIKF----S-DEKQKPEESIDD---GLQWWANLLAN--N-IEIEELVSCN--
AtPAP1 -------------HLNAPPKVDVNPPCLGLN---------INNVCDNSIIY---NKDKKKDQLVN--NLIDGDNMWLEK--
Consensus/80% ....s..s.ls.s..sp.hh...........pssps.l.s...sp.hh.p.LhN..p.lp.-phss....
MtLAP1 -----SSLQEEN----LM-EFPNVDDD------------------SFWDFNLCDLNSLWDLP--------
MtLAP2 -----SLLQEEN----FISEFPNVDDS-------------------FWDFNLCDFDSL-----------
MtLAP3 -----SSLQVEN----YE-EFSIVDD--------------------FWDFNICDYDSLWDL---------
MtLAP4 LPEEYSKLNVEN----FLAEGPSTVGD------------------FSWDSTICEFDSLLDDILN------
Antho1 -KTLTSLLHEEISPPLNIGEGNSMQQGQISHENWGEFSLNLPPMQQGVQNDDFSAEID-LWNLLD-------
An2 ---SPTLLHEETAPSVNAES--SLTQG----------------GGSGLSDFSVDIDDIWDLVS-------
AtPAP1 ------FLEESQEVDILVPEATTTEKG--------------------DTLAFDVDQLWSLFDGETVKFD
Consensus/80% .....s.Lp.Ep.....h.Ehsshpps...................D...s-hDpLhs..........

FIG.4A

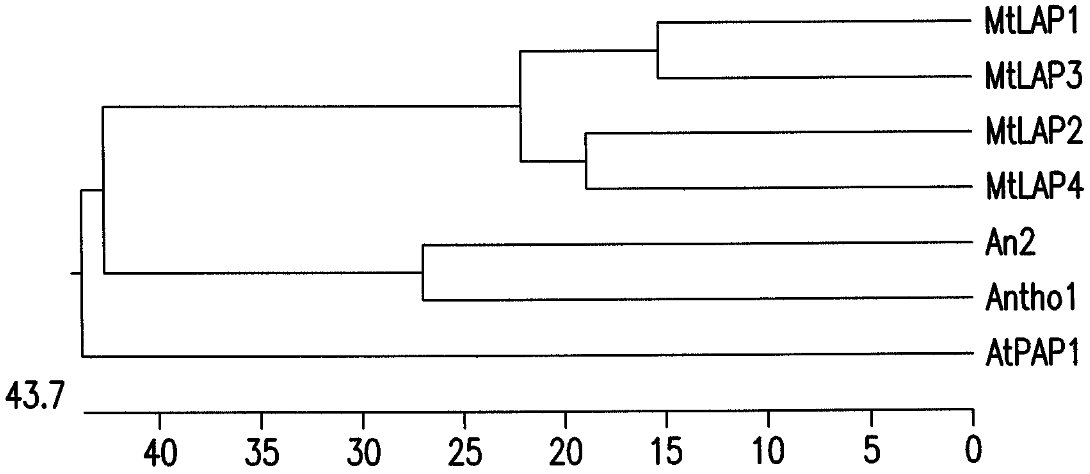
MtLAP1
MtLAP3
MtLAP2
MtLAP4
An2
Antho1
AtPAP1
43.7
40
35
30
25
20
15
10
5
0

FIG.4B

FIG.5

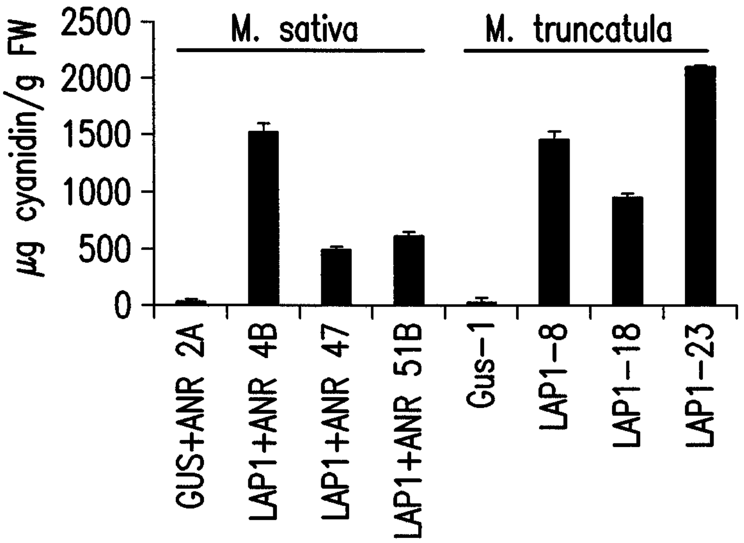
μg cyanidin/g FW
2500
2000
1500
1000
500
0
M. sativa
M. truncatula
GUS+ANR 2A
LAP1+ANR 4B
LAP1+ANR 47
LAP1+ANR 51B
Gus-1
LAP1-8
LAP1-18
LAP1-23

FIG.6A

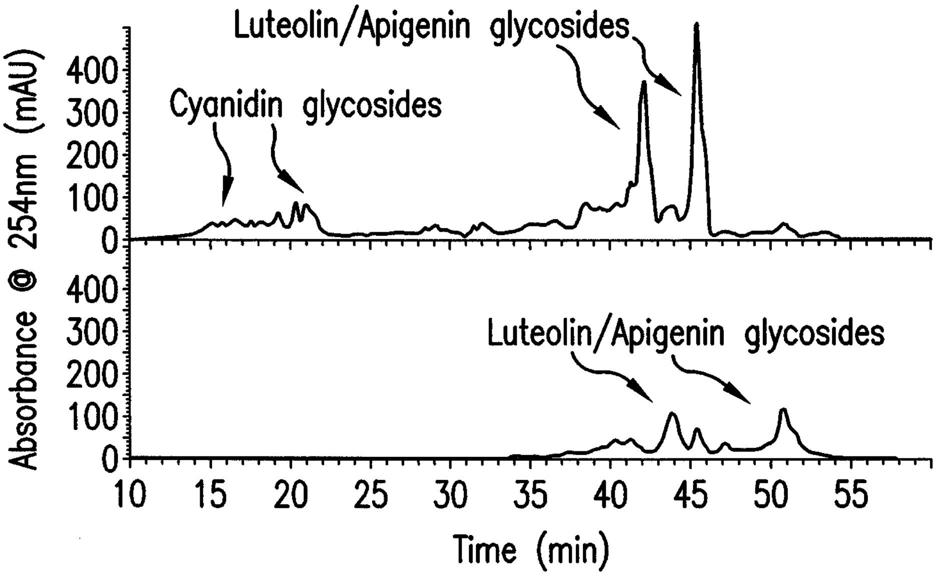
Absorbance @ 254nm (mAU)
400
300
200
100
0
Luteolin/Apigenin glycosides
Cyanidin glycosides
400
300
200
100
0
Luteolin/Apigenin glycosides
10
15
20
25
30
35
40
45
50
55
Time (min)

FIG.6B

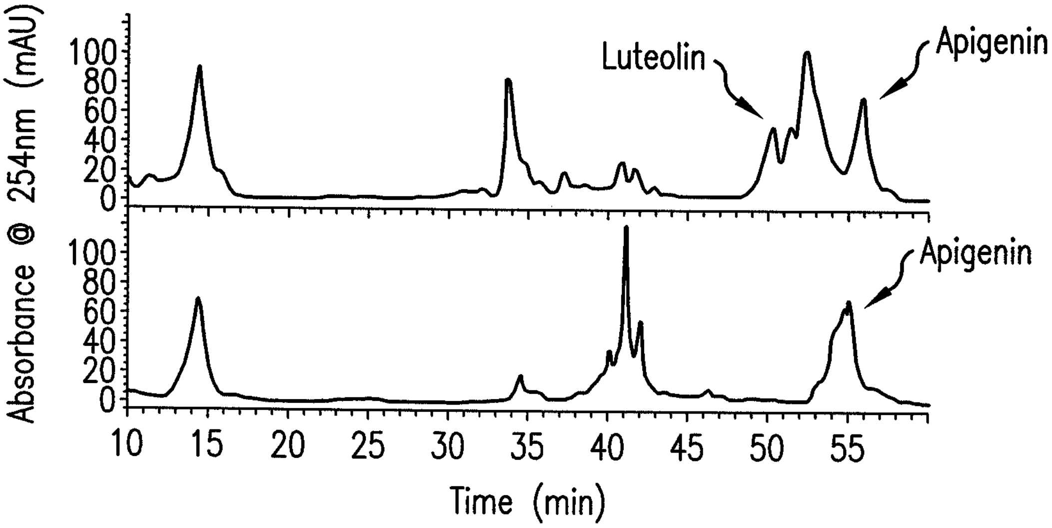
Absorbance @ 254nm (mAU)
100
80
60
40
20
0
Luteolin
Apigenin
100
80
60
40
20
0
Apigenin
10
15
20
25
30
35
40
45
50
55
Time (min)

FIG.6C

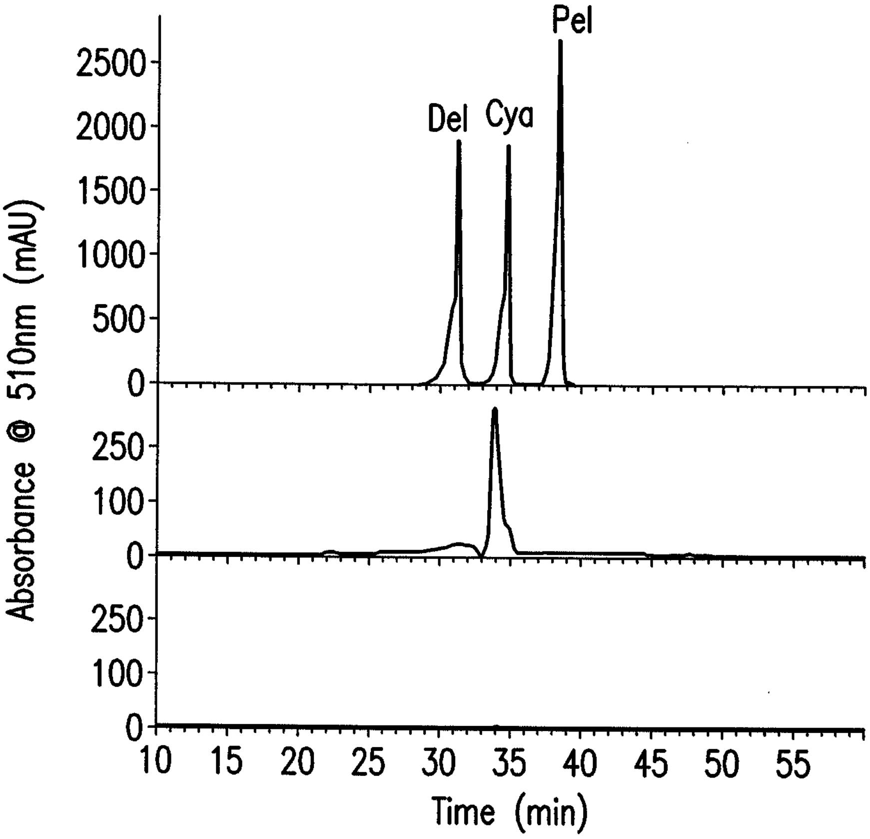
Absorbance @ 510nm (mAU)
2500
2000
1500
1000
500
0
Del
Cya
Pel
250
100
0
250
100
0
10
15
20
25
30
35
40
45
50
55
Time (min)

FIG.6D

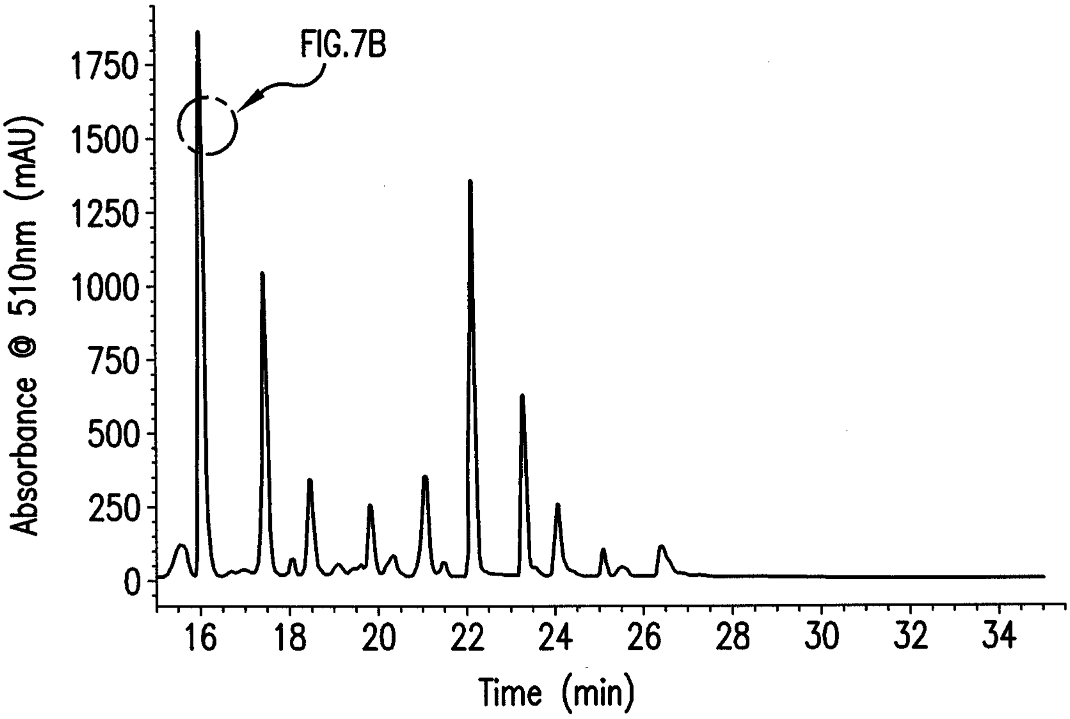
FIG.7B
Absorbance @ 510nm (mAU)
1750
1500
1250
1000
750
500
250
0
16
18
20
22
24
26
28
30
32
34
Time (min)

FIG.7A

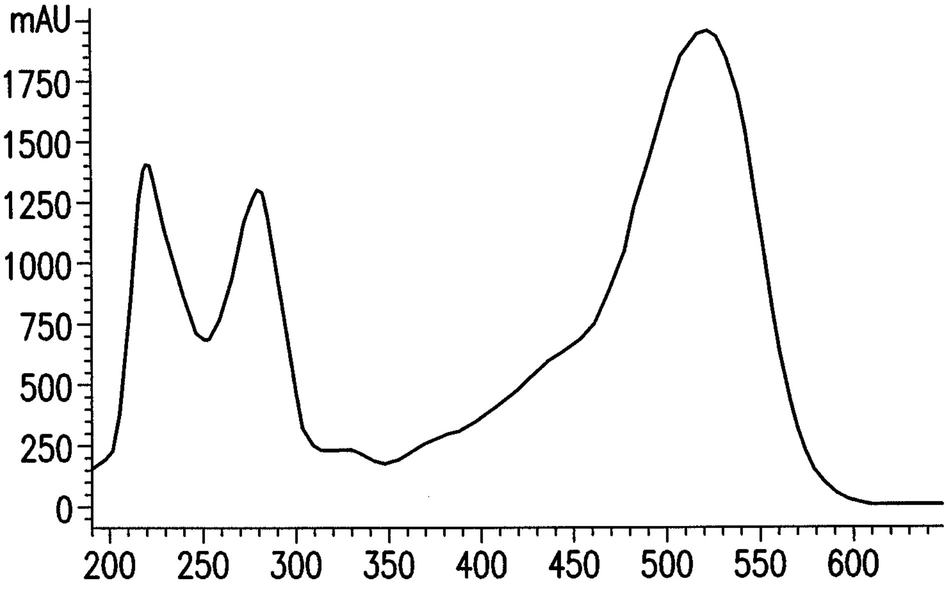
mAU
1750
1500
1250
1000
750
500
250
0
200
250
300
350
400
450
500
550
600

FIG.7B

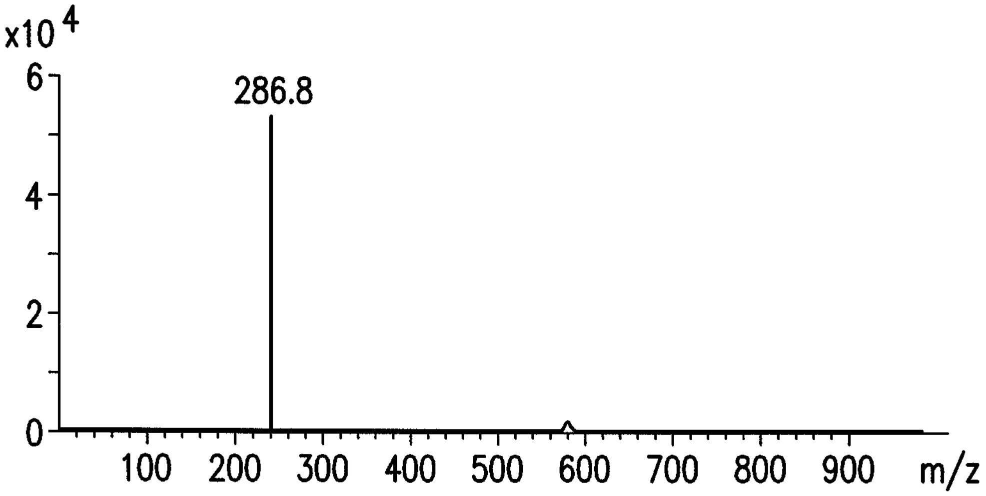

FIG.7C

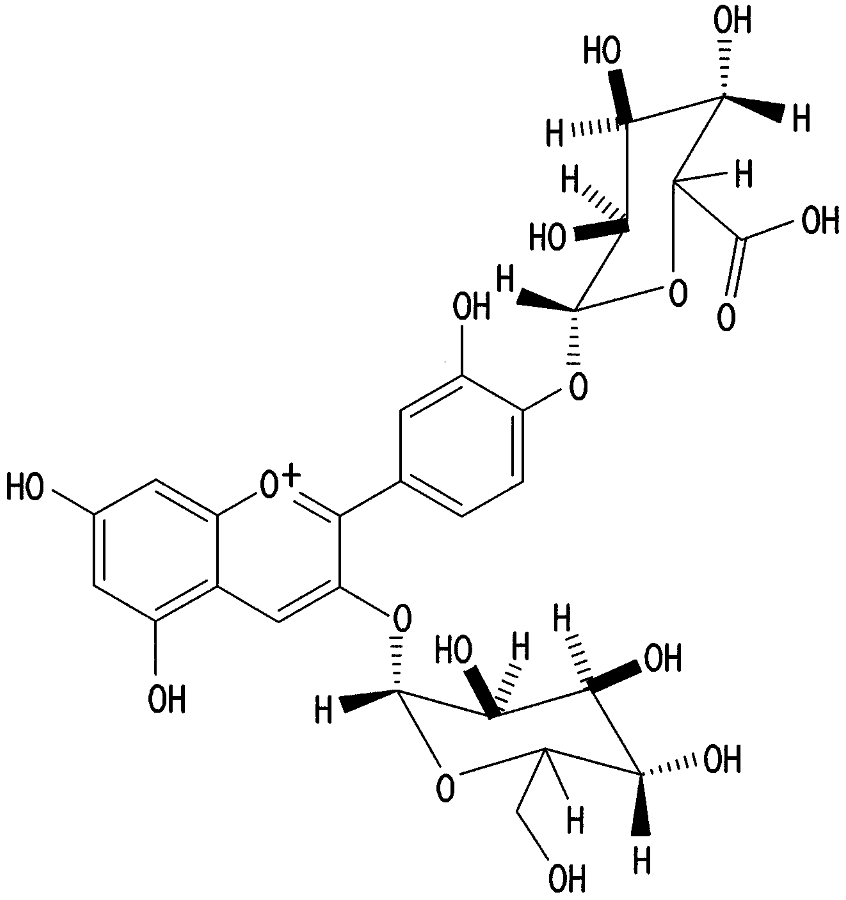

FIG.7D

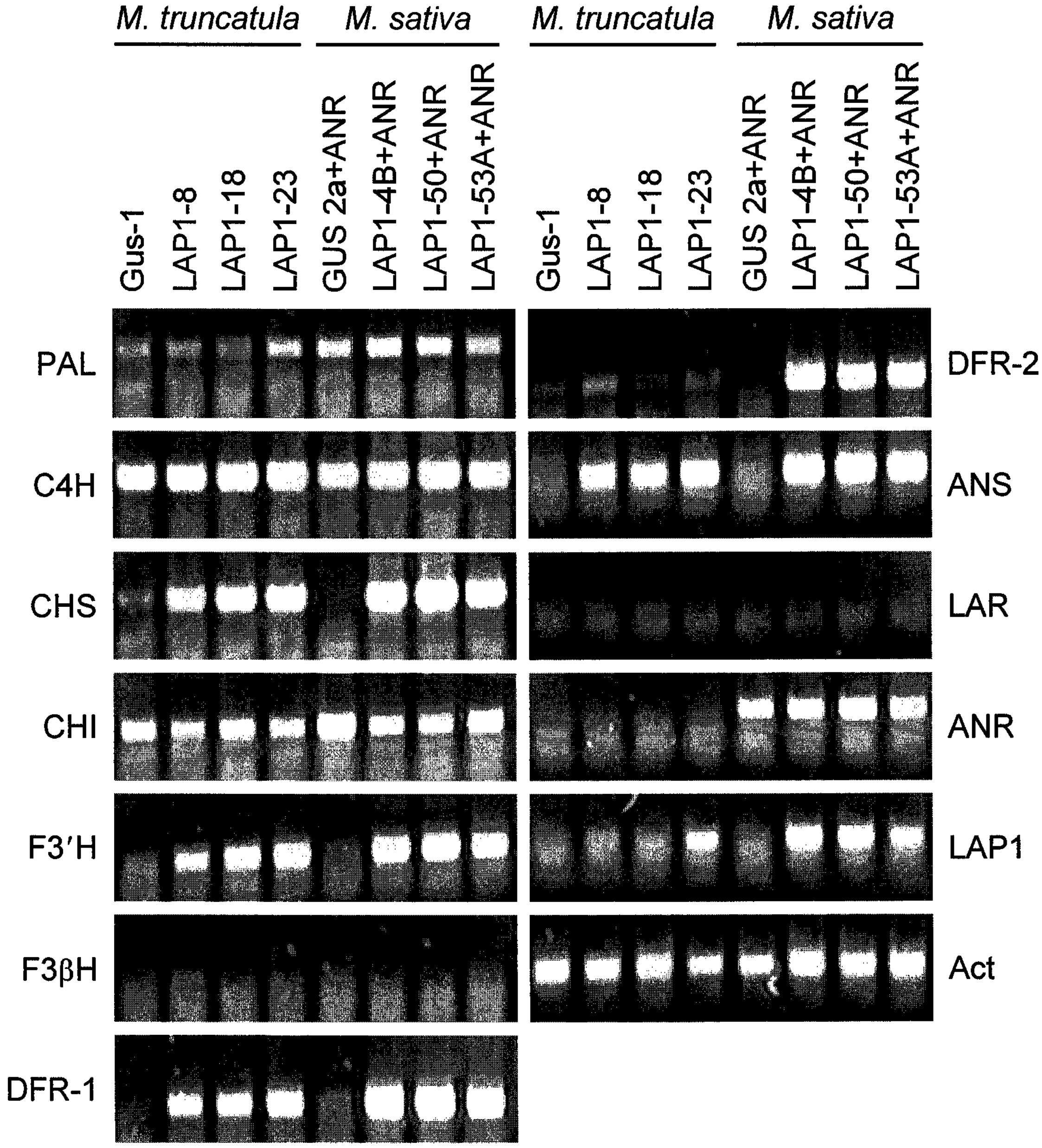
M. truncatula
M. sativa
M. truncatula
M. sativa
Gus-1
LAP1-8
LAP1-18
LAP1-23
GUS 2a+ANR
LAP1-4B+ANR
LAP1-50+ANR
LAP1-53A+ANR
Gus-1
LAP1-8
LAP1-18
LAP1-23
GUS 2a+ANR
LAP1-4B+ANR
LAP1-50+ANR
LAP1-53A+ANR
PAL
C4H
CHS
CHI
F3′H
F3βH
DFR-1
DFR-2
ANS
LAR
ANR
LAP1
Act

FIG.8

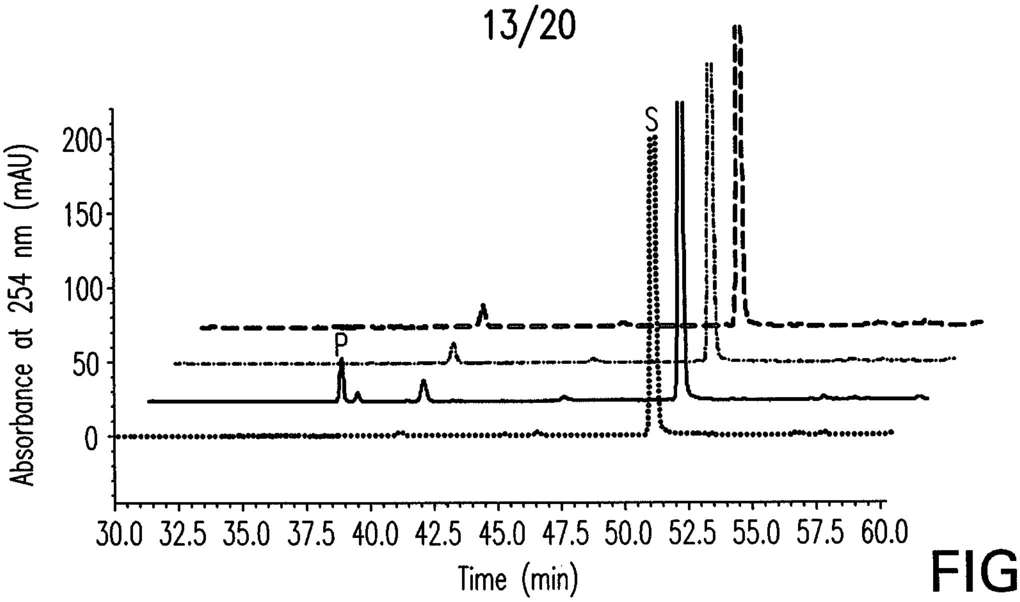

FIG.9A

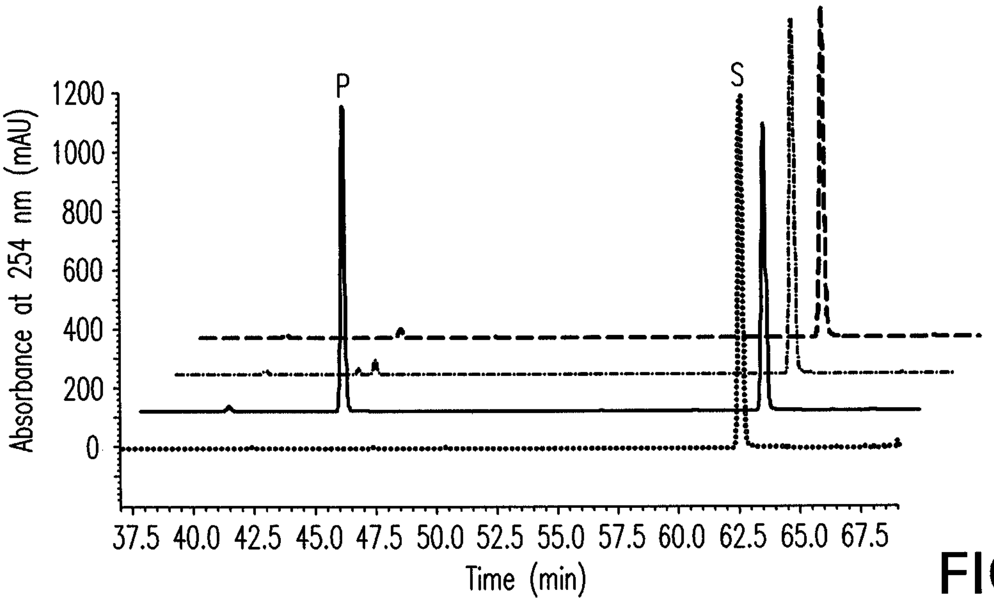

FIG.9B

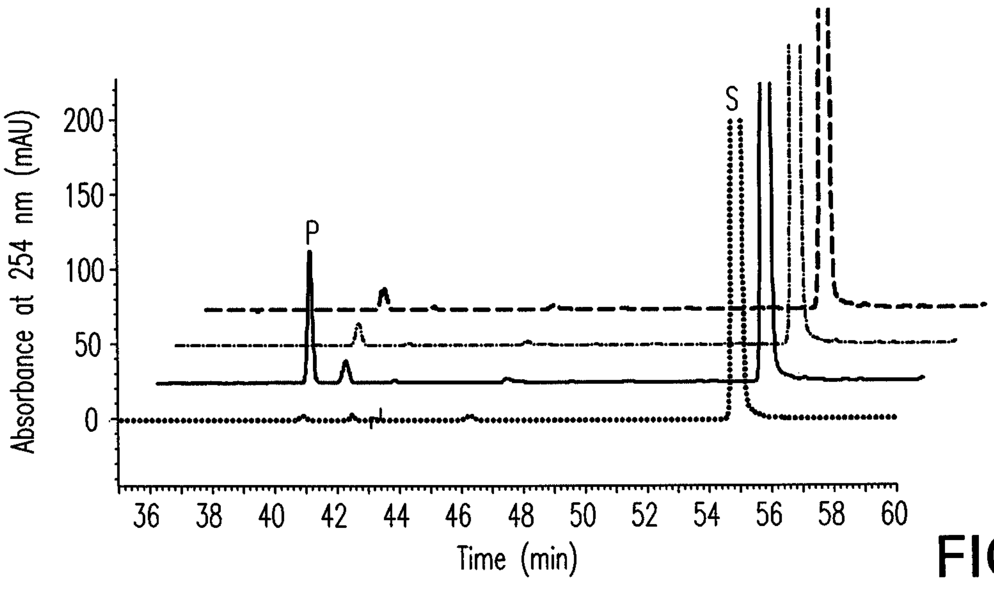

FIG.9C

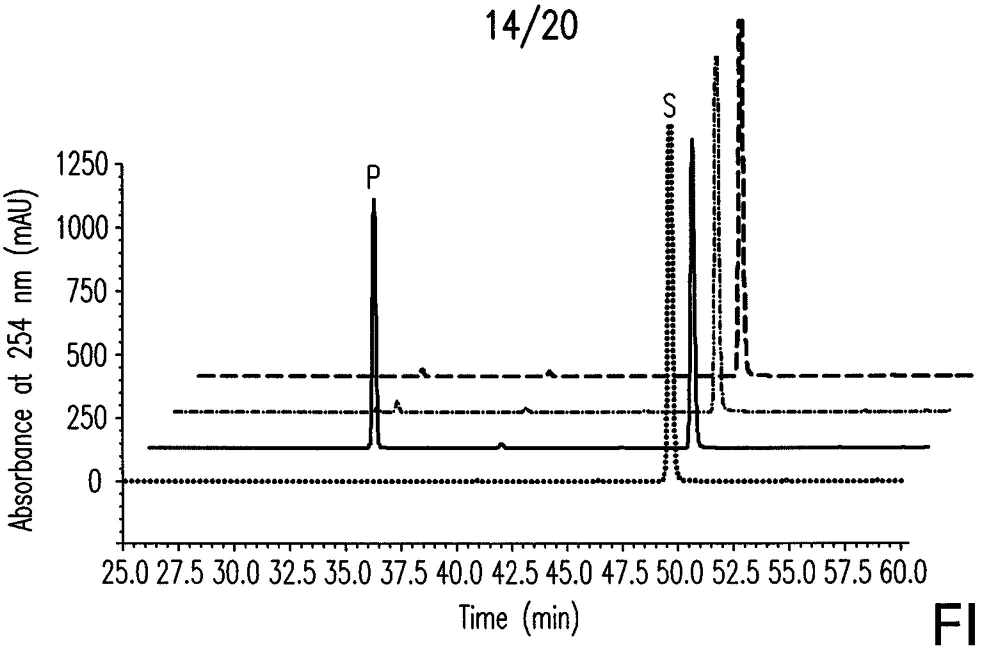

FIG.9D

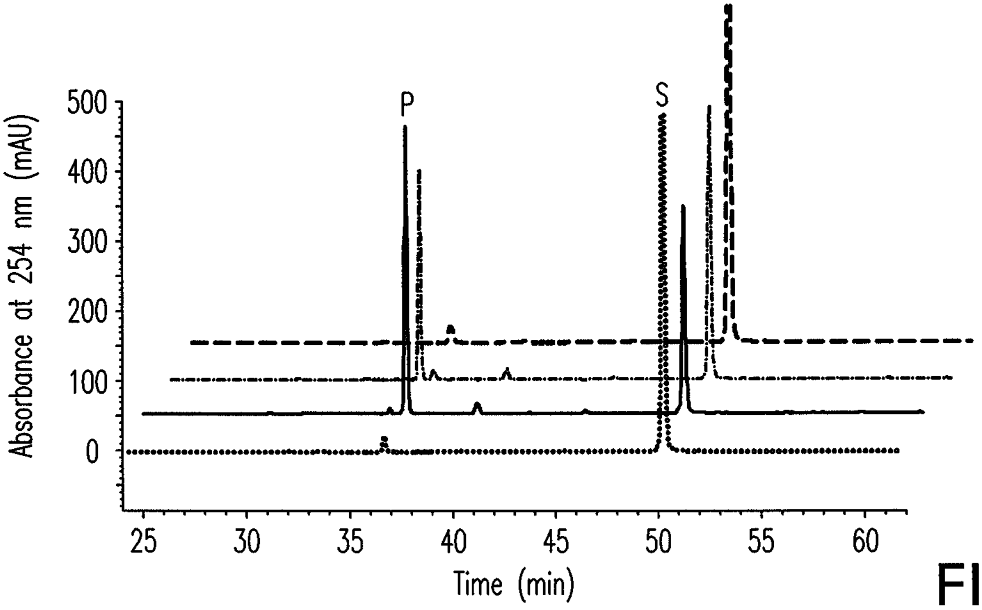

FIG.9E

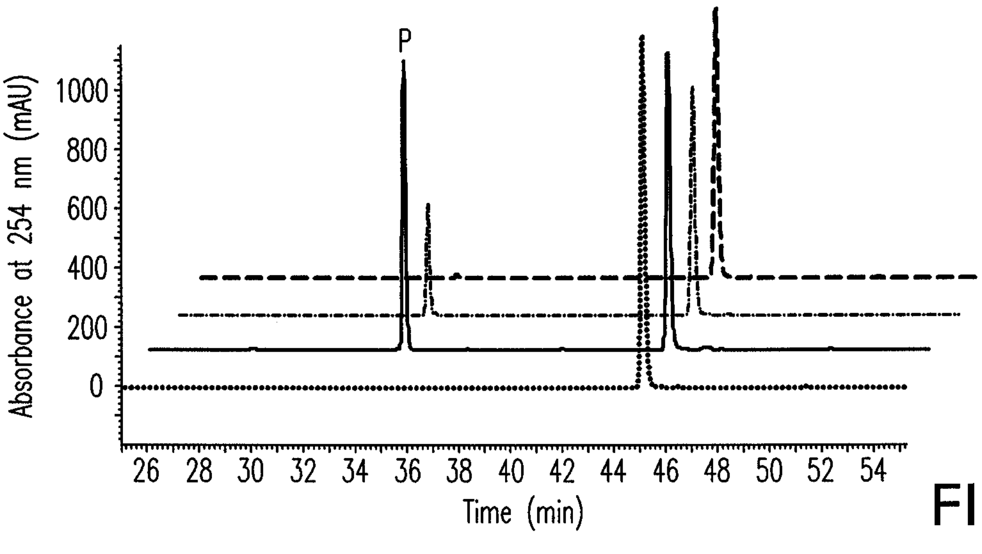

FIG.9F

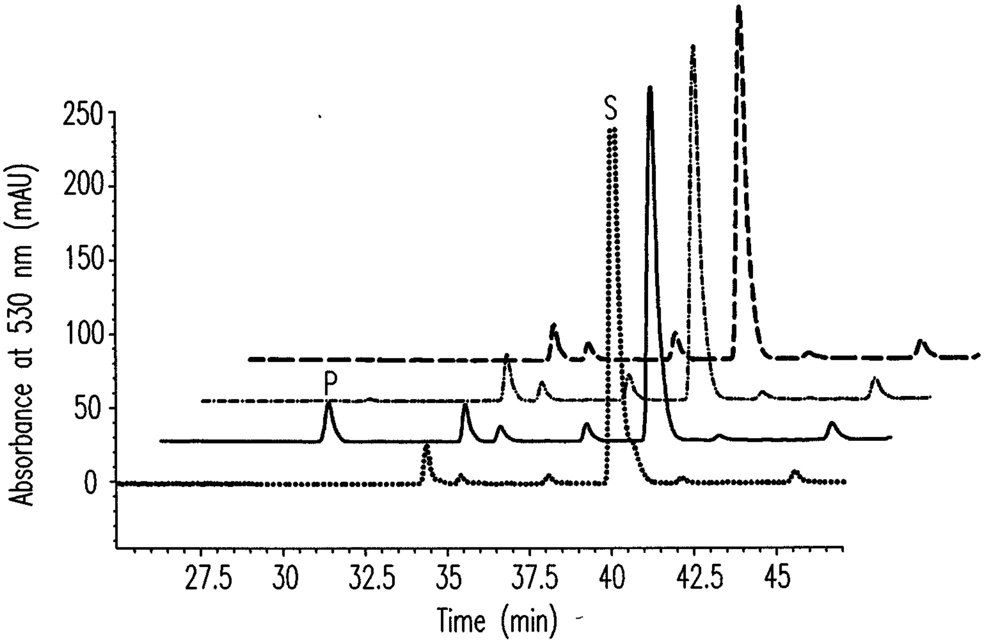
Absorbance at 530 nm (mAU)
250
200
150
100
50
0
S
P
27.5
30
32.5
35
37.5
40
42.5
45
Time (min)

FIG.9G

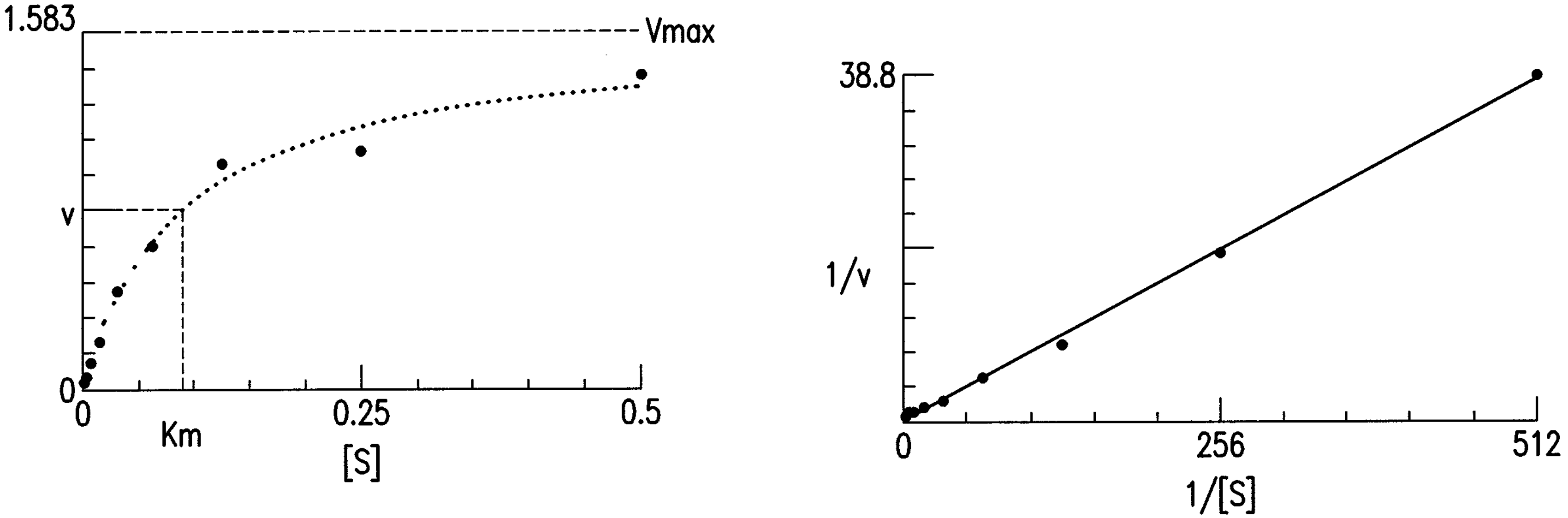

FIG.10

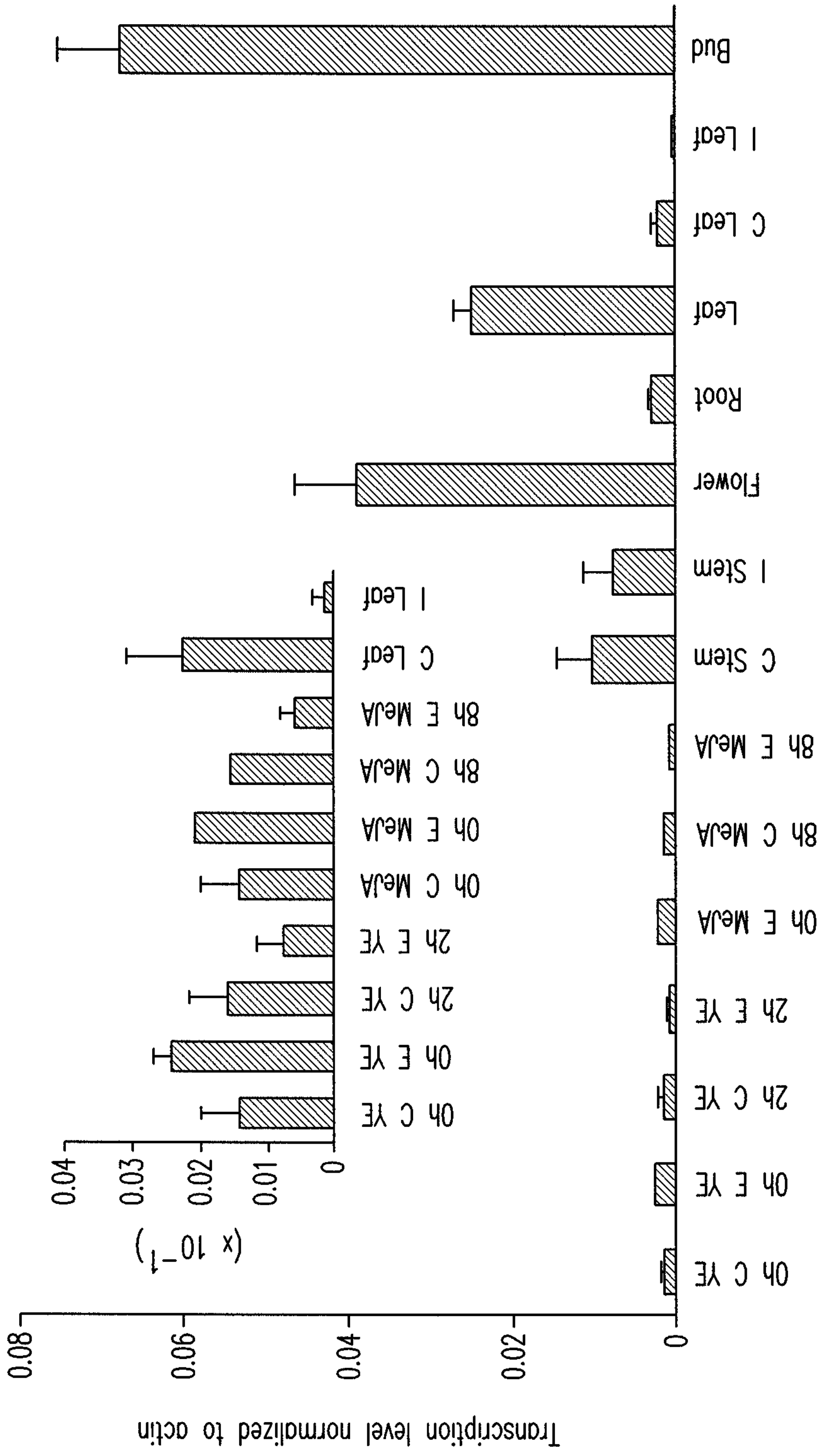
Transcription level normalized to actin
0
0.02
0.04
0.06
0.08
0h C YE
0h E YE
2h C YE
2h E YE
0h E MeJA
8h C MeJA
8h E MeJA
C Stem
I Stem
Flower
Root
Leaf
C Leaf
I Leaf
Bud
(x 10^{-1})
0
0.01
0.02
0.03
0.04
0h C YE
0h E YE
2h C YE
2h E YE
0h C MeJA
0h E MeJA
8h C MeJA
8h E MeJA
C Leaf
I Leaf

FIG.11

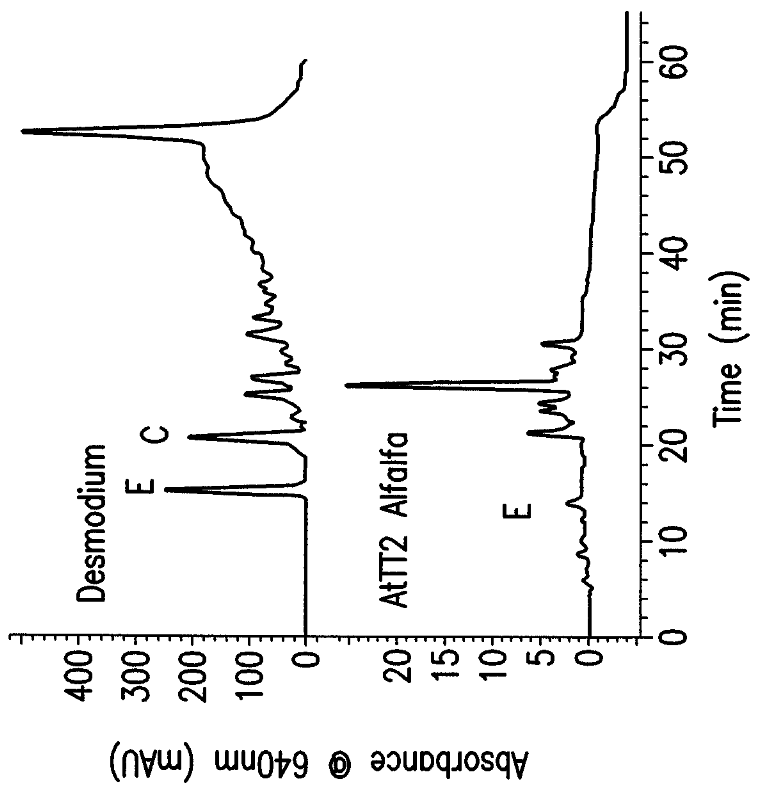

FIG.12A

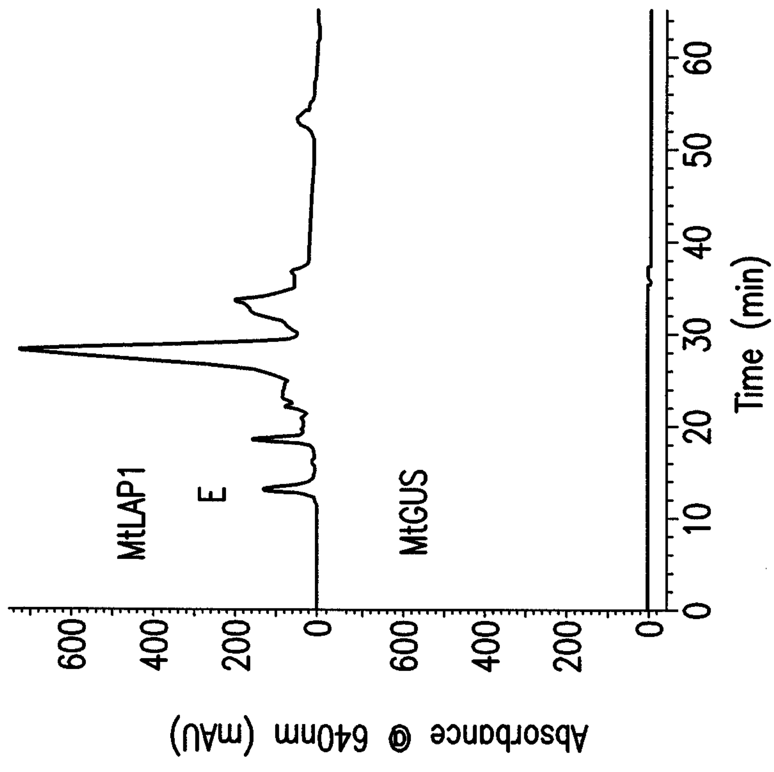

FIG.12B

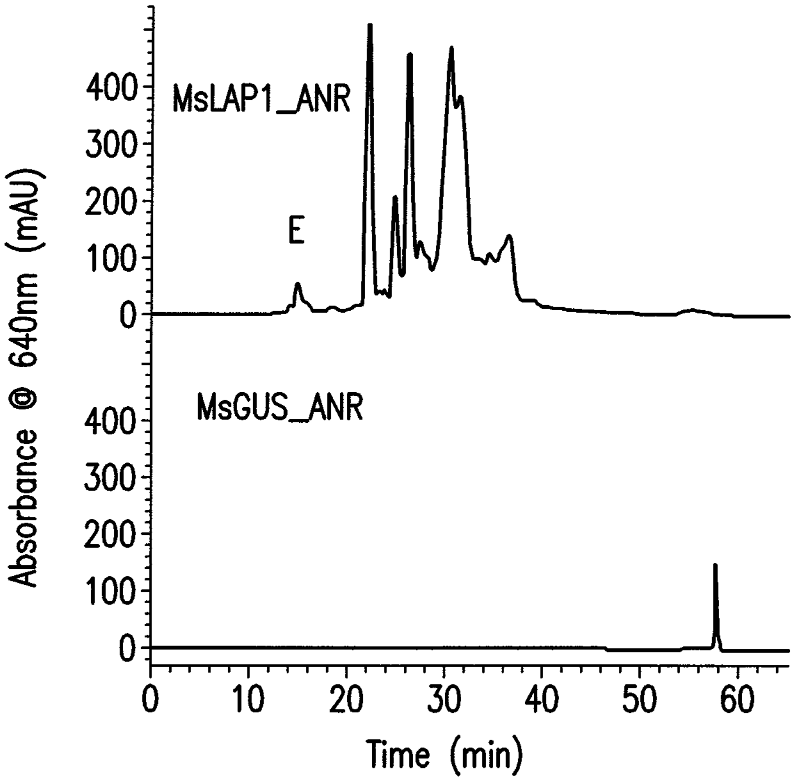

FIG.12C

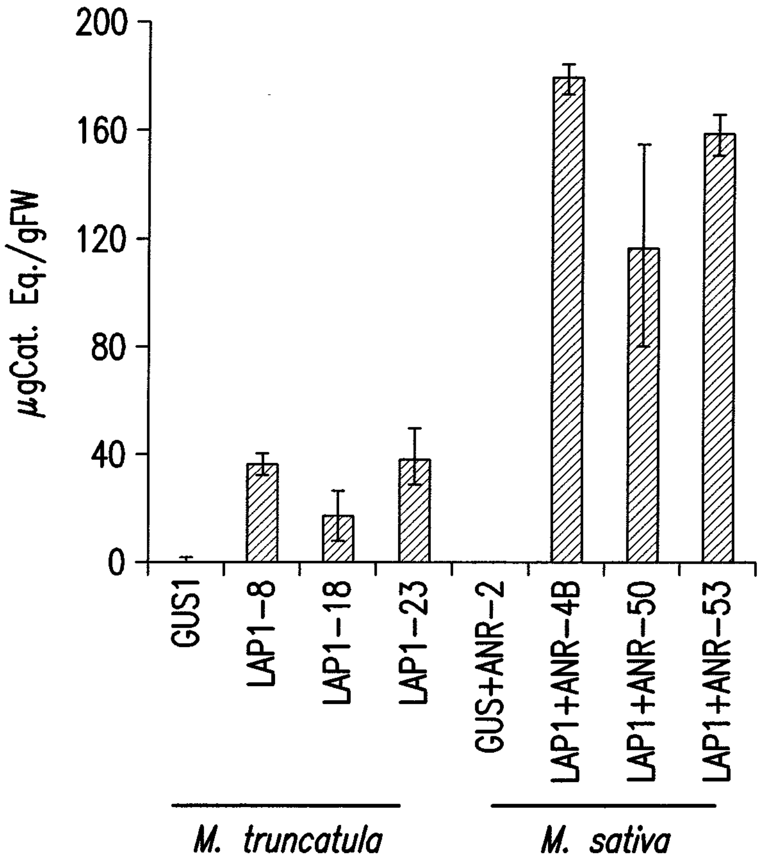

FIG.12D

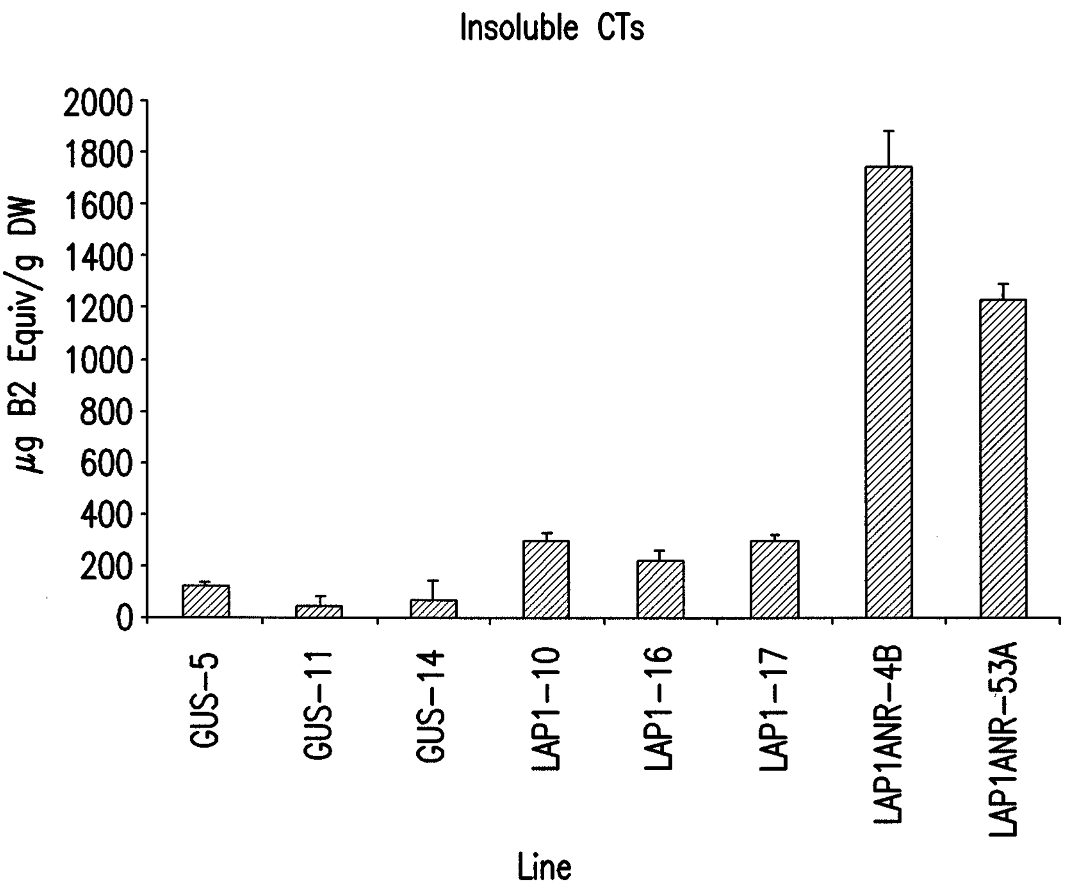
Insoluble CTs
μg B2 Equiv/g DW
2000
1800
1600
1400
1200
1000
800
600
400
200
0
GUS-5
GUS-11
GUS-14
LAP1-10
LAP1-16
LAP1-17
LAP1ANR-4B
LAP1ANR-53A
Line

FIG.13