Soybean and *Medicago truncatula* CYP93C genes have been isolated which encode a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavonone intermediate or an isoflavone. Plants can now be genetically engineered to produce isoflavones that provide potential human health benefits and increase disease resistance in plants. Isoflavones can now be produced in transgenic plants species in which isoflavones do not naturally occur, i.e., in species other than legumes. Alternatively, introducing infection-inducible isoflavonoid biosynthesis into non-legumes qualitatively complements these plants' phytoalexin defenses against microbial pathogens, whereas over-expression of the isoflavonoid pathway in legumes quantitatively increases this defense response. Finally, modifying the extent of production of isoflavonoids in legume roots positively impacts nodulation efficiency and therefore plant yield.
<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Albania</td>
</tr>
<tr>
<td>AM</td>
<td>Armenia</td>
</tr>
<tr>
<td>AT</td>
<td>Austria</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
</tr>
<tr>
<td>AZ</td>
<td>Azerbaijan</td>
</tr>
<tr>
<td>BA</td>
<td>Bosnia and Herzegovina</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
</tr>
<tr>
<td>BY</td>
<td>Belarus</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
</tr>
<tr>
<td>CI</td>
<td>Côte d'Ivoire</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
</tr>
<tr>
<td>CN</td>
<td>China</td>
</tr>
<tr>
<td>CU</td>
<td>Cuba</td>
</tr>
<tr>
<td>CZ</td>
<td>Czech Republic</td>
</tr>
<tr>
<td>DE</td>
<td>Germany</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
</tr>
<tr>
<td>EE</td>
<td>Estonia</td>
</tr>
<tr>
<td>ES</td>
<td>Spain</td>
</tr>
<tr>
<td>FI</td>
<td>Finland</td>
</tr>
<tr>
<td>FR</td>
<td>France</td>
</tr>
<tr>
<td>GA</td>
<td>Gabon</td>
</tr>
<tr>
<td>GB</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>GE</td>
<td>Georgia</td>
</tr>
<tr>
<td>GH</td>
<td>Ghana</td>
</tr>
<tr>
<td>GN</td>
<td>Guinea</td>
</tr>
<tr>
<td>GR</td>
<td>Greece</td>
</tr>
<tr>
<td>HU</td>
<td>Hungary</td>
</tr>
<tr>
<td>IE</td>
<td>Ireland</td>
</tr>
<tr>
<td>IL</td>
<td>Israel</td>
</tr>
<tr>
<td>IS</td>
<td>Iceland</td>
</tr>
<tr>
<td>IT</td>
<td>Italy</td>
</tr>
<tr>
<td>JP</td>
<td>Japan</td>
</tr>
<tr>
<td>KE</td>
<td>Kenya</td>
</tr>
<tr>
<td>KG</td>
<td>Kyrgyzstan</td>
</tr>
<tr>
<td>KP</td>
<td>Democratic People's</td>
</tr>
<tr>
<td>KR</td>
<td>Republic of Korea</td>
</tr>
<tr>
<td>KW</td>
<td>North Korea</td>
</tr>
<tr>
<td>KZ</td>
<td>Kazakhstan</td>
</tr>
<tr>
<td>LC</td>
<td>Saint Lucia</td>
</tr>
<tr>
<td>LI</td>
<td>Liechtenstein</td>
</tr>
<tr>
<td>LK</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>LR</td>
<td>Liberia</td>
</tr>
<tr>
<td>LS</td>
<td>Lesotho</td>
</tr>
<tr>
<td>LT</td>
<td>Lithuania</td>
</tr>
<tr>
<td>LU</td>
<td>Luxembourg</td>
</tr>
<tr>
<td>LV</td>
<td>Latvia</td>
</tr>
<tr>
<td>MC</td>
<td>Monaco</td>
</tr>
<tr>
<td>MD</td>
<td>Republic of Moldova</td>
</tr>
<tr>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td>MK</td>
<td>The former Yugoslav</td>
</tr>
<tr>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td>MN</td>
<td>Mongolia</td>
</tr>
<tr>
<td>MR</td>
<td>Mauritania</td>
</tr>
<tr>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td>MX</td>
<td>Mexico</td>
</tr>
<tr>
<td>NE</td>
<td>Niger</td>
</tr>
<tr>
<td>NL</td>
<td>Netherlands</td>
</tr>
<tr>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td>PL</td>
<td>Poland</td>
</tr>
<tr>
<td>PT</td>
<td>Portugal</td>
</tr>
<tr>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td>RU</td>
<td>Russian Federation</td>
</tr>
<tr>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td>SE</td>
<td>Sweden</td>
</tr>
<tr>
<td>SG</td>
<td>Singapore</td>
</tr>
<tr>
<td>SI</td>
<td>Slovenia</td>
</tr>
<tr>
<td>SK</td>
<td>Slovakia</td>
</tr>
<tr>
<td>SN</td>
<td>Senegal</td>
</tr>
<tr>
<td>SZ</td>
<td>Swaziland</td>
</tr>
<tr>
<td>TD</td>
<td>Chad</td>
</tr>
<tr>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>TJ</td>
<td>Tajikistan</td>
</tr>
<tr>
<td>TM</td>
<td>Turkmenistan</td>
</tr>
<tr>
<td>TR</td>
<td>Turkey</td>
</tr>
<tr>
<td>TT</td>
<td>Trinidad and Tobago</td>
</tr>
<tr>
<td>UA</td>
<td>Ukraine</td>
</tr>
<tr>
<td>UG</td>
<td>Uganda</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
</tr>
<tr>
<td>UZ</td>
<td>Uzbekistan</td>
</tr>
<tr>
<td>VN</td>
<td>Viet Nam</td>
</tr>
<tr>
<td>VU</td>
<td>Yugoslavia</td>
</tr>
<tr>
<td>ZW</td>
<td>Zimbabwe</td>
</tr>
</tbody>
</table>
GENETIC MANIPULATION OF ISOFLAVONOIDS

TECHNICAL FIELD OF THE INVENTION

The invention relates to gene manipulation in plants.

BACKGROUND OF THE INVENTION

The flavonoids are a major class of phenylpropanoid-derived plant natural products. Their fifteen carbon (C_6-C_3-C_6) backbone can be arranged as a 1,3-diphenylpropane skeleton (flavonoid nucleus) or as a 1,2-diphenylpropane skeleton (isoflavonoid nucleus). Although 1,3-diphenylpropane flavonoid derivatives are almost ubiquitous among terrestrial plants, the 1,2-diphenylpropane isoflavonoids are restricted primarily to the Leguminosae, although they occur rarely in other families such as the Apocynaceae, Pinaceae, Compositae, and Moraceae (Tahara, S. and R. K. Ibrahim, 1995, “Prenylated isoflavonoids - an update,” Phytochemistry 38: 1073-1094).

The limited taxonomic distribution of the isoflavonoids is directly related to the occurrence of the enzyme complex isoflavone synthase (IFS), which catalyzes the aryl migration reaction leading to the formation of an isoflavone from a flavanone. While flavanones are ubiquitous in higher plants, the IFS reaction, which is a two-step process specific for isoflavonoid biosynthesis (Kochs, G. and H. Grisebach, 1986, “Enzymic synthesis of isoflavones,” European J Biochem 155: 311-318), is limited to the Leguminosae and the other diverse taxa in which isoflavonoids are occasionally found.

The presence of isoflavonoids provides several advantages to plants. One such advantage is provided by the function of isoflavonoids as antimicrobial phytoalexins in plant-microbe interactions. For example, the simple isoflavones daidzein and genistein act as initial precursors in the biosynthesis of various antimicrobial isoflavonoid phytoalexins in a wide variety of legumes (Dixon, R. A. and N. L. Paiva, 1995, “Stress-induced phenylpropanoid metabolism,” Plant Cell 7: 1085-1097). Isoflavonoid compounds have been shown to accumulate in infected plant cells to
levels known to be antimicrobial in vitro. The temporal, spatial and quantitative aspects of accumulation are consistent with a role for these compounds in disease resistance (Rahe, J. E., 1973, “Occurrence and levels of the phytoalexin phaseollin in relation to delimitation at sites of infection of Phaseolus vulgaris by Colletotrichum lindemuthianum,” Canadian J Botany 51: 2423-2430; Hadwiger, L. A. and D. M. Webster, 1984, “Phytoalexin production in five cultivars of pea differentially resistant to three races of Pseudomonas syringae pv. pisi,” Phytopathology 74: 1312-1314; Long, et al., 1985, “Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to Pseudomonas syringae pv. glycinea,” Phytopathology 75: 235-239; Bhattacharyya, M. K. and E. W. B. Ward, 1987, “Biosynthesis and metabolism of glyceollin I in soybean hypocotyls following wounding or inoculation with Phytophthora megasperma f. sp. glycinea,” Physiol and Mol Plant Pathology 31: 387-405). Moreover, it has been reported that many plant pathogens are much more sensitive to phytoalexins of non-host species than they are to the phytoalexins of their natural hosts, because they can often detoxify the host’s phytoalexins. (VanEtten, et al., 1989, “Phytoalexin detoxification: importance for pathogenicity and practical implications,” An Rev Phytopathology 27: 143-164).

Isoflavonoids also function in plant-microbe interactions in the establishment of bacterial or fungal symbioses with plants. Isoflavonoids have been reported to regulate bacterial nodulation genes, acting as a major nod gene inducer (Kossnak, et al., 1987, “Induction of Bradyrhizobium japonicum common nod genes by isoflavones isolated from Glycine max,” Proc Natl Acad Sci USA 84: 7428-7432) and/or transcription activator (Dakora, et al., 1993, “Common bean root exudates contain elevated levels of daidzein and coumestrol in response to Rhizobium inoculation,” Mol Plant-Microbe Interact 6: 665-668). Isoflavonoids have also been shown to have a role on the establishment of the symbiotic vesicular arbuscular mycorrhizal (VAM) association of the fungus Glomus with legume roots. (Kape, et al., 1992, “Legume root metabolites and VA-mycorrhiza development,” J Plant Physiol 141: 54-60). Xie et al have reported that the isoflavonoids coumestrol, daidzein and genistein have small but significant stimulatory effects on the degree of mycorrhizal colonization of soybean, and that one effect of isoflavonoids on the
soybean mycorrhizal symbiosis could be via induction of nodulation factors from co-colonizing Rhizobia, since nod-factors have also been shown to stimulate fungal colonization (Xie, et al., 1995, "Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans," *Plant Physiology* 108: 1519-1525).


At present, the only dietary sources of isoflavonoids for humans are certain legumes such as soybean or chickpea. The development of methods to genetically manipulate isoflavonoids in plants, either to widen the source of dietary isoflavonoids for humans, or to exploit the biological activities of isoflavonoids for plant protection and improvement, is wholly dependent on the availability of cloned genes encoding the various enzymes of isoflavonoid biosynthesis. Of these, the isoflavone synthase (IFS) complex constitutes the first committed reactions, and as such represents the means to introduce isoflavonoids into plants that do not possess the pathway.

In 1984, Hagmann and Grisebach provided the first evidence for the enzymatic conversion of flavanone to isoflavone (the IFS reaction) in a cell free system (Hagmann, M. and H. Grisebach, 1984, “Enzymatic rearrangement of flavanone to isoflavone,” *FEBS Letters* 175: 199-202). They demonstrated that microsomes from elicitor-treated soybean cell suspension cultures could catalyze the conversion of 2(S)-naringenin to genistein, or of 2(S)-liquiritigenin to daidzein, in the presence of NADPH. The crude microsomal enzyme preparation, which was stable at -70°C but had a half-life of only 10 minutes at room temperature, was absolutely dependent on NADPH and molecular oxygen. It was subsequently shown that the reaction proceeded in two steps. The flavanone was converted in a cytochrome P450-catalyzed reaction requiring NADPH and O₂ to the corresponding 2-hydroxyisoflavanone. This
relatively unstable compound, which could, however, be identified by mass spectrometric analysis, then underwent dehydration to yield the isoflavone. The dehydration reaction appeared to be catalyzed by an enzyme present predominantly in the cytoplasmic supernatant, although it was not possible to remove all this activity from the microsomes. The corresponding 2-hydroxyisoflavanone spontaneously converted to genistein, for example, in methanol at room temperature. Kinetic analysis indicated that the 2-hydroxyisoflavanone was formed prior to genistein, consistent with its being an intermediate in isoflavone formation. (Kochs, G. and H. Grisebach, 1986, “Enzymic synthesis of isoflavones,” *European J Biochem* 155: 311-318).

Involvement of cytochrome P450 in the 2-hydroxyisoflavanone synthase reaction was confirmed by inhibition by CO, replacing O₂ with N₂, and examining the effects of a range of known P450 inhibitors of which anecymidol was the most effective. The enzyme co-migrated with the endoplasmic reticulum markers cinnamate 4-hydroxylase (another cytochrome P450) and cytochrome b₅ reductase on Percoll gradients. The enzyme is stereoselective, and (2R)-naringenin is not a substrate. (Kochs, G. and H. Grisebach, 1986, “Enzymic synthesis of isoflavones,” *European J Biochem* 155: 311-318).

The origin of the 2-hydroxyl group was determined from studies on the IFS present in microsomes from elicited cell cultures of *Pueraria lobata*. ¹⁸O from ¹⁸O₂ was incorporated into the 2-hydroxyl group, resulting in a 2-hydroxyisoflavanone with molecular ion shifted by two mass units, whereas there was no corresponding shift in the molecular ion of daidzein, consistent with the subsequent dehydration reaction (Hashim, et al., 1990, “Reaction mechanism of oxidative rearrangement of flavanone in isoflavone biosynthesis,” *FEBS Letters* 271: 219-222). The currently accepted model for the reaction pathway of IFS as illustrated in Fig. 1, therefore, involves P450-catalyzed hydroxylation coupled to aryl migration, a reaction with mechanistic similarities to the well described proton migration mechanism of some P450 reactions (Hakamatsuka, et al., 1991, “P-450-dependent oxidative rearrangement in isoflavone

Currently, there have been no reports on purification to homogeneity or molecular cloning of the cytochrome P450 of the IFS complex because of the extreme lability of the enzyme. The 2-hydroxyisoflavanone synthase cytochrome P450 from *Pueraria* has been solubilized with Triton X-100, and partially purified by DEAE-Sepharose chromatography; the enzymatic reaction could be reconstituted by addition of NADPH cytochrome P450 reductase that separated from the hydroxylase on the ion exchange column (Hakamatsu, et al., 1991, *Tetrahedron* 47: 5969-5978). A 2-hydroxyisoflavanone dehydratase has been purified from elicitor-treated *P. lobata* cells, and has been shown to be a soluble monomeric enzyme of subunit Mr 38,000 (Hakamatsu, et al., 1998, “Purification of 2-hydroxyisoflavanone dehydratase from the cell cultures of *Pueraria lobata*,” *Phytochemistry* 49: 497-505). It is not yet clear whether this enzyme physically associates with the P450 hydroxylase catalyzing the aryl migration, or even whether this activity is essential for isoflavone formation in *planta* in view of the spontaneous conversion of 2-hydroxyisoflavanone to isoflavone.

Flavanone is a potential substrate for more than one type of hydroxylation reaction at the 2-position. Thus, elicitor-treated cell cultures of alfalfa and *Glycyrrhiza echinata* have been shown to accumulate the dibenzoylmethane licodione (Kirikae, et al., 1993, “Biosynthesis of a dibenzoylmethane, licodione, in cultured alfalfa cells induced by yeast extract,” *Biosci Biotech Biochem* 57: 1353-1354). Licodione synthase is, by classical criteria, a cytochrome P450, the activity of which is induced by yeast elicitor in *Glycyrrhiza* cells (Otani, et al., 1994, “Licodione synthase, a cytochrome P450 monoxygenase catalyzing 2-hydroxylation of 5-deoxyflavanone, in cultured *Glycyrrhiza echinata* L. cells,” *Plant Physiol* 105: 1427-1432). The reaction it catalyzes involves 2-hydroxylation of flavanone followed by hemiacetal opening instead of aryl migration, and the reaction was thought to have mechanistic similarities to the flavone synthase II enzyme previously characterized from soybean (Kochs, G. and H. Grisebach, 1987, “Induction and characterization of a NADPH-dependent flavone synthase from cell cultures of soybean,” *Z. Naturforsch* 42C: 343-
348). A gene encoding the flavone synthase II/licodione synthase from *Glycyrrhiza*
has been cloned (Akashi, et al., 1998, “Identification of a cytochrome P450 cDNA
encoding (2S)-flavanone 2-hydroxylase of licorice (*Glycyrrhiza echinata* L.: Fabaceae) which represents licodione synthase and flavone synthase II,” *FEBS Letters*
431: 287-290), and a different cytochrome P450 gene encoding flavone synthase II has
recently been cloned from *Gerbera hybrida* (Martens, S. and G. Forkmann, “Cloning
and expression of flavone synthase II from Gerbera hybrids,” *Plant J* 20: 611-618).

Although the reactions catalyzed by IFS are critical for the formation of all
isoflavonoids in plants, there have been no previous reports of the isolation of genes
encoding components of isoflavone synthase, although genes encoding most of the
other enzymes of the isoflavonoid pathway, including downstream enzymes
converting simple isoflavones to antimicrobial phytoalexins, have been characterized
(Dixon, et al., 1995, “The isoflavonoid phytoalexin pathway: from enzymes to genes
to transcription factors,” *Physiologia Plantarum* 93: 385-392). Thus, the
unavailability of isoflavone synthase genes has made it heretofore impossible to utilize
the downstream genes for regulating isoflavonoid concentrations in legumes and other
plants that do have the isoflavonoid pathway, or for engineering antimicrobial and
pharmacologically active isoflavonoids in transgenic plants of species that do not have
the isoflavonoid pathway.

Genes encoding the enzyme catalyzing the first step of the isoflavone synthase
reaction have now been isolated and purified from soybean and *Medicago truncatula*
(barrel medic).

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 depicts the currently accepted model for the reaction pathway of IFS
wherein the flavanone is converted in a cytochrome P450-catalyzed reaction requiring
NADPH and O₂ to the corresponding 2-hydroxyisoflavanone which then undergoes
dehydration to yield the isoflavone.

Fig. 2 depicts the nucleotide sequence of soybean CYP93C1v2.
Fig. 3 depicts the amino acid sequence of soybean CYP93C1v2 compared to licorice CYP93B1.

Fig. 4 depicts the nucleotide sequence of *Medicago truncatula* mtIFSE3.

Fig. 5 depicts the amino acid sequence of *Medicago truncatula* mtIFSE3 compared to soybean CYP93C1v2.

Fig. 6A and Fig. 6B depict HPLC traces of extracts from pooled tissues (leaves, shoots, flowers) of *Arabidopsis thaliana* ecotype Columbia harboring an empty tDNA vector (Fig. 6A) and *Arabidopsis thaliana* ecotype Columbia harboring the soybean CYP93C1v2 cDNA sequence (Fig. 6B). The empty vector transformed line contains a number of flavonol glycosides and other phenolic compounds that are also present in the CYP93C1v2 transformed line. These compounds were identified as (a) rhamnose (Rha)-glucose (Glc)-quercetin (Q), (b) uncharacterized conjugate of Q, (c) Rha-Glc-Rha-Kaempferol (K), (d) Glu-Rha-Q, (e) Rha-Rha-Q, (f) Glc-Rha-K, (g) sinapic acid, (h) Rha-Rha-K. Three additional compounds were observed in the CYP93C1v2 transformed line (Fig. 6B), and labeled “1,” “2” and “3.” Fig. 6C depicts a total ion chromatogram of partially purified peaks 2 and 3, and the insets show the specific ions generated from these compounds. Peak 2 has a parental molecular mass ion of 579.5 consistent with genistein conjugated to a glucose-rhamnose disaccharide, and two further mass ions of 417.5 and 271.3, representing Rha-genistein and free genistein, respectively. Peak 3, which has a parental molecular ion of mass 417.5, is thereby identified as Rha-genistein.

Fig. 7A and Fig. 7B depict HPLC traces of the same extracts as shown in Fig. 6A (empty-vector transformed) and Fig. 6B, (CYP93C1v2 transformed), but following digestion with β-glucosidase. Peaks 2 and 3 remained at the same retention time as in Fig. 6A and 6B. However, Peak 1 disappeared, and was replaced with a new Peak 4 of much later retention time. Fig. 7C shows the total ion chromatograph of purified Peak 4, and the inset shows the parental molecular ion, with mass of 271.2, consistent with Peak 4 being free genistein. Fig. 7D shows a total ion chromatograph, and the parental molecular ion, of an authentic sample of genistein.
Fig. 8A, 8B, 8C and 8D are high performance liquid chromatography (HPLC) chromatograms depicting the presence of new peaks at RT 29.96 and 37.7 min representing the presence of the isoflavone daidzein formed from the flavanone liquiritigenin, or the isoflavone genistein formed from the flavanone naringenin, in insect cell microsomes expressing CYP93C1v2. Fig. 8A depicts the presence of NADPH during incubation with liquiritigenin. Fig. 8B depicts the absence of NADPH during incubation with liquiritigenin. Fig. 8C depicts the presence of NADPH during incubation with naringenin. Fig. 8D depicts the lack of a reaction when soybean CYP93E expressed in insect cells is incubated with liquiritigenin in the presence of NADPH.

Fig. 9A and Fig. 9B are mass spectra of BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) derivatives. Fig. 9A depicts the mass spectrum of the BSTFA derivative of the product of the reaction catalyzed by CYP93C1v2 in insect cells using liquiritigenin as substrate, and Fig. 9B shows the mass spectrum of the BSTFA derivative of an authentic sample of daidzein.

SUMMARY OF THE INVENTION

In one aspect, the invention is a method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising introducing a DNA segment encoding the enzyme into the plant to form a transgenic plant, wherein the transgenic plant expresses the DNA segment under the control of a suitable constitutive or inducible promoter when the transgenic plant is exposed to conditions which permit expression. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. Plants transformed by this method may also preferably express chalcone synthase,
chalcone reductase, and chalcone isomerase genes to cause in vivo formation of
daidzein or a daidzein derivative, and the chalcone synthase, chalcone reductase, and
chalcone isomerase genes may also be transgenes. Plants transformed by this method
may also preferably further comprise downstream genes, for example, isoflavone O-
methyltransferase, isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone
reductase, to metabolize a formed isoflavone to biologically active isoflavonoid
derivatives or conjugates. The plant can comprise isoflavone 4'-O-methyl-transferase
to cause formation of biochanin A or a biochanin A derivative from the isoflavonone
intermediate. An exemplary flavanone substrate for this transformation method is
liquiritigenin and/or naringenin.

In another aspect, the present invention is a method for increasing the level of
isoflavonoid compounds in naturally isoflavonoid-producing plants comprising
introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a
flavanone to yield an isoflavonoid to form a transgenic plant, wherein the transgenic
plant expresses the DNA segment under the control of a suitable constitutive or
inducible promoter when the transgenic plant is exposed to conditions which permit
expression. With this method, the resulting isoflavonoid can be an isoflavanone
intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.
The DNA segment can comprise isolated genomic DNA or recombinant DNA.

Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from
a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36
to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another
preferred DNA segment comprises a Medicago truncatula homolog of a CYP93C
gene, more preferably, the sequence from about nucleotide 92 to about nucleotide
1657 of the sequence depicted in SEQ ID NO:4. An exemplary flavanone substrate
for this transformation method is liquiritigenin and/or naringenin.

In another aspect, the invention is a method for synthesizing an isoflavanone
intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C
gene segment in a suitable bacterial, fungal, algal, or insect cell system. An
exemplary gene segment consists essentially of the sequence from about nucleotide 36
to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another
exemplary gene segment consists essentially of the sequence from about nucleotide 92
to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a method of reducing the levels of
isoflavonoid compounds in a naturally isoflavonoid-producing plant comprising
introducing and expressing an antisense or gene silencing construct that contains an
intact CYP93C gene or segments thereof into the plant. An exemplary gene consists
essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the
sequence depicted in SEQ ID NO:1. Another exemplary gene consists essentially of
the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence
depicted in SEQ ID NO:4.

In another aspect, the invention is a naturally non-isoflavonoid-producing plant
cell transformed by introducing a DNA segment encoding the enzyme catalyzing the
aryl migration of a flavanone to form an isoflavanone intermediate or an isoflavone,
wherein the transformed plant cell expresses the DNA segment under the control of a
suitable constitutive or inducible promoter when exposed to conditions which permit
expression. The DNA segment can comprise isolated genomic DNA or recombinant
DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA
segment from a soybean CYP93C gene consists essentially of the sequence from about
nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.
Another preferred DNA segment comprises a *Medicago truncatula* homolog of a
CYP93C gene, more preferably, the sequence from about nucleotide 92 to about
nucleotide 1657 of the sequence depicted in SEQ ID NO:4. Plants transformed by this
method may also preferably express chalcone synthase, chalcone reductase, and
chalcone isomerase genes to cause in vivo formation of daidzein or a daidzein
derivative, and the chalcone synthase, chalcone reductase, and chalcone isomerase
genes may also be transgenes. Plants transformed by this method may also preferably
further comprise downstream genes, for example, isoflavone O-methyltransferase,
isoflavone 2'-hydroxylase, isoflavone reductase, and vestitone reductase, to
metabolize a formed isoflavanone intermediate to biologically active isoflavonoid
derivatives or conjugates. The plant can comprise isoflavone 4’-O-methyl-transferase to cause formation of biochanin A or a biochanin A derivative from the isoflavonone intermediate.

In another aspect, the invention is a naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein the transformed plant cell expresses the DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression. With this method, the resulting isoflavonoid can be an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate. The DNA segment can comprise isolated genomic DNA or recombinant DNA. Preferably, the DNA segment is a CYP93C gene. An exemplary DNA segment from a soybean CYP93C gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another preferred DNA segment comprises a *Medicago truncatula* homolog of a CYP93C gene, more preferably, the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is a transgenic plant cell having reduced levels of isoflavonoid compounds, the plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into the plant cell. An exemplary gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. Another exemplary gene consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

In another aspect, the invention is an isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1. An exemplary gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin. Another
exemplary gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

In another aspect, the invention is a protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

In another aspect, the invention is an isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion is a *Medicago truncatula* homolog of a CYP93C gene. An exemplary gene or DNA segment consists essentially of about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4. An exemplary gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin. Another exemplary gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

In another aspect, the invention is a protein encoded by a portion of an isolated gene or a DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the portion is a *Medicago truncatula* homolog of a CYP93C gene.

In yet another aspect, the invention is a food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein the transgenic plant has been transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a food comprising at least one isoflavonoid, wherein the isoflavonoid is isolated from a transgenic plant transformed
with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a composition comprising at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, and wherein the composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

In yet another aspect, the invention is a composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, wherein the isoflavonoid is isolated from at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing the nutritional value of a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.
In yet another aspect, the invention is a method of using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, to provide a nutraceutical benefit to a human or animal administered the isoflavonoid. The isoflavonoid can be administered by ingestion of at least a portion of the plant. The isoflavonoid can also be administered by ingestion of a composition comprising an isoflavonoid isolated from the plant.

In yet another aspect, the invention is a method of using an isoflavonoid isolated from a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, to provide a pharmaceutical benefit to a patient administered the isoflavonoid.

In yet another aspect, the invention is a method of increasing disease resistance in a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing nodulation efficiency of a leguminous plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid released from the roots.
when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic leguminous plant exhibiting increased nodulation efficiency transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid released from the roots when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a method of increasing bacterial or fungal symbiosis in a plant by transforming the plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic plant exhibiting increased bacterial or fungal symbiosis transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.

In yet another aspect, the invention is a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.
In yet another aspect, the invention is seed from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is progeny from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is progeny from seed of a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the recombinant DNA sequence.

In yet another aspect, the invention is use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, for the preparation of a nutraceutical preparation for achieving a nutritional effect.

In yet another aspect, the invention is use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein the transgenic plant exhibits an increased level of an isoflavonoid
when compared to the level of the isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment, for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.

**DETAILED DESCRIPTION**

One aspect of the present invention is an isolated gene which encodes the first step of the isoflavone synthase reaction: a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavone. Genes and corresponding cDNA of the soybean or *Medicago truncatula* CYP93 family have been isolated. The enzymes encoded by the genes of the present invention are isoflavone synthases (IFS) and can catalyze the aryl migration of a flavanone to yield an isoflavone either directly or through the intermediacy of a 2-hydroxyisoflanone. One isolated soybean gene is classified as *CYP93Clv2*.

Cytochrome P450 enzymes belong to a large superfamily of enzymes that are abundant in every living organism. The P450 nomenclature committee has determined that each P450 should carry a “CYP” designation and arbitrarily divided the superfamily into families (alphabetical designation), subfamilies (numerical designation) and allelic variants (“v” plus numerical designation) based on amino acid identity of >40%, >55%, and >97%, respectively (Nelson, et al. 1993. “The P450 superfamily update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature,” *DNA Cell Biol* 12:1). Thus, CYP93Clv2 is a variant of the first described P450 belonging to the third subfamily (C) of the ninety-third P450 family.

Utilizing the procedures presented herein, any plant known to produce isoflavonoids may also serve as sources of suitable DNA, or coding sequences may be synthesized in vitro based on the sequences for the IFS genes of the present invention. CYP93 family members can also be obtained from other plant species by polymerase chain reaction amplification methods known to those skilled in the art, using primer sequences corresponding to regions of nucleotide conservation between CYP93 family members. Furthermore, the genes of the present invention are defined by their
catalytic activity: the aryl migration of a flavanone to yield an isoflavone. The gene sequences presented as SEQ ID NO:1 and SEQ ID NO:4 are exemplary, and it is understood that modifications to these genes which do not alter the catalytic activity of its encoded protein fall within the scope of the present invention. While a preferred IFS gene contains the entire open reading frame, portions of or the entire 5' and 3' untranslated regions as well as portions of the vector sequence can also be present. With the isolation and functional identification of these isoflavone synthase (IFS) genes that encode the first key step in isoflavone formation, the aryl migration reaction, it is now possible to introduce the isoflavonoid pathway into all plant species, including those that do not naturally possess this pathway.

Another aspect of the present invention is a genetically modified plant which has been transformed with a gene of the present invention. For example, when the CYP93C1v2 gene is transferred into the model plant Arabidopsis thaliana, which does not naturally produce isoflavonoids, the isoflavone genistein accumulates as a series of glycoconjugates (Example 1). This demonstrates that the genes of the present invention can be genetically engineered into plants which do not naturally contain the isoflavonoid pathway, and the transgenic plants can then produce isoflavonoids, resulting in plants with improved disease resistance and/or value added health benefits for humans. In the present invention, unless otherwise stated, as used herein, the term “plant” or “progeny” includes plant parts, plant tissue, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, explants, plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, capsules, stems, leaves, seeds, roots, root tips, and the like. Furthermore, the present invention includes the IFS genes expressed in various parts of the plant, e.g., in aerial portions of the plant useful for increasing disease resistance or production of health promoting isoflavonoid nutraceuticals, in seeds useful for increasing levels of isoflavones and their conjugates, or in roots useful for increasing disease resistance or production of nodulation gene inducing isoflavones.
In another aspect, the present invention is a method of improving disease resistance and a transgenic plant with increased disease resistance. By transforming a plant which does not naturally make isoflavones with an IFS gene of the present invention, disease resistance can be genetically engineered into the plant by providing the necessary enzyme to convert its natural flavanones into isoflavonoids. The introduction and subsequent expression of an IFS gene of the present invention into a crop species which naturally possesses the isoflavonoid pathway results in increased levels of the isoflavonoid defense compounds.

In another aspect, the present invention is a method of increasing levels of isoflavonoids that might be beneficial to the establishment of bacterial or fungal symbioses with plants and a transgenic plant with an increased capacity for symbiotic association with bacteria or fungi. Bacterial nodulation can be stimulated in transgenic leguminous plants by expression of an IFS gene of the present invention and decreased by expression of antisense constructs or constructs designed to promote gene silencing that contain an intact IFS gene or segments thereof. Mycorrhizal colonization of leguminous plants can also be increased through the introduction and expression of an IFS gene of the present invention.

In yet another aspect, the present invention is a method of producing isoflavonoid compounds in plants or any other organism to be used in nutraceuticals or pharmaceuticals to confer human or animal health benefits. Edible transgenic plants high in isoflavonoids can be utilized as food for humans and animals. Edible compositions high in isoflavonoids can also be made by incorporation of the transgenic plants or plant materials, or by incorporation of isoflavonoids isolated from the transgenic plants. Compositions useful for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical can be made by incorporation of the transgenic plants or plant materials, or by incorporation of isoflavonoids isolated from the transgenic plants. The nutritional value of a plant can be increased by transforming the plant with an IFS gene of the present invention and, as a result, accumulating high amounts of isoflavonoids in the plant.
The soybean IFS gene of the present invention was isolated and purified according to the detailed procedures outlined in Example 2. The DNA sequence is shown in SEQ ID NO:1 and Fig. 2, and the encoded protein sequence of the isolated soybean CYP93C clone is shown in SEQ ID NO:2 and Fig. 3. For comparison, Fig. 3 also shows the protein sequence alignment between the isolated CYP93C clone (SEQ ID NO:2) and CYP93B1 (SEQ ID NO:3), the licorice licodione synthase.

The DNA and protein sequences of the soybean CYP93C1 open reading frame were deposited in the Genbank data base under accession # AF022462. The deposition was made by Siminszky, Dewey and Corbin, and the sequence described as representing a gene induced in soybean in response to herbicide safeners. However, the function of the gene was not known and there was no understanding that it could be involved in isoflavonoid biosynthesis at the time the deposit was made (Siminszky, B., Corbin, F.T., Ward, E.R., Fleischmann, T.J. and Dewey, R.E., 1999, “Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides.” Proc. Natl. Acad. Sci. USA 96: 1750-1755). The sequence of the clone characterized herein differs from CYP93C1 in three nucleotide substitutions in the open reading frame that change proline 140 to leucine, threonine 156 to isoleucine, and glutamate 295 to lysine. Thus, the soybean gene identified herein has been classified as CYP93C1v2.

The cDNA insert from CYP93C1v2 was used to probe 240,000 phage plaques from a *Medicago truncatula* root cDNA library (van Buuren, M.L., I.E. Maldonado-Mendoza, A.T. Trieu, L.A. Blaylock, and M.J. Harrison, 1999, “Novel genes induced during an arbuscular mycorrhizal (AM) symbiosis formed between *Medicago truncatula* and *Glomus versiforme,*” Mol. Plant-Microbe Interact. 12, 171-181). Five positive plaques were purified, in vivo excised, and sequenced. A full length clone designated mtIFSE3 was completely sequenced on both strands, and shown to encode the *Medicago truncatula* homolog of soybean CYP93C1. The nucleotide sequence of mtIFSE3 is shown in SEQ ID NO:4 and Fig. 4, and the protein sequence, in SEQ ID NO:5. An alignment between the protein sequences of mtIFSE3 and CYP93C1v2 is shown in Fig. 5.
An IFS gene of the soybean or *Medicago truncatula* CYP93C subfamily or corresponding cDNA sequence, the open reading frame of which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavone, either directly or through the intermediacy of a 2-hydroxyisoflavonone, can be used to introduce the isoflavonoid pathway into any plant species that does not naturally possess this pathway. Soybean CYP93C1v2 acts on the flavanones liquiritigenin to yield daidzein, and naringenin to yield genistein. Liquiritigenin is only formed in plants that possess the enzyme chalcone reductase (CHR) (Welle, R. and Grisebach, H., 1989, “Phytoalexin synthesis in soybean cells: elicitor induction of reductase involved in biosynthesis of 6'-deoxychalcone.” *Arch Biochem Biophys* 272: 97-102), and a form of chalcone isomerase that is active against 2',4,4'-trihydroxychalcone, the product of the co-action of chalcone synthase (CHS) with CHR (Dixon, R.A., Blyden, E.R., Robbins, M.P., van Tunen, A.J. and Mol, J.N.M., 1988, “Comparative biochemistry of chalcone isomerases.” *Phytochemistry* 27: 2801-2808). Such genes are common in legumes, but not in most other plant families. Thus, to form daidzein in transgenic plants that do not possess the isoflavonoid pathway, it would be necessary to introduce three new genes, namely CHR, to co-act with CHS to form 2',4,4'-trihydroxychalcone, a suitable CHI to convert 2',4,4'-trihydroxychalcone to liquiritigenin, and IFS, assuming that the 2'-hydroxyisoflavanone intermediate can spontaneously dehydrate in planta, a phenomenon that is demonstrated below. Without CHR present, no liquiritigenin would be formed, and IFS would only be able to act on naringenin to yield, assuming spontaneous dehydration of the 2'-hydroxyisoflavanone, genistein.

The IFS genes of the present invention can be introduced into non-leguminous plants such as by standard *Agrobacterium* based or biolistic transformation procedures (Horsch, et al., 1985, “A simple and general method for transferring genes into plants,” *Science* 227:1229-1231; and Klein, et al., 1988, “Stable genetic transformation of intact *Nicotiana* cells by the particle bombardment process,” *Proc Natl Acad Sci USA* 85:8502-8505). Both procedures require the construction of a plasmid vector containing a desirable transcriptional promoter driving expression of the gene of interest (in this case IFS), followed by a transcriptional terminator and a
selectable marker gene for resistance, such as to an antibiotic or a herbicide. The biolistic procedure coats metal particles with plasmid DNA containing the gene of interest and places them on a micro carrier disk. Using the biolistic apparatus, the particles are physically propelled into plant tissue. The plant tissue is then put under selection (e.g., antibiotic or herbicide) followed by regeneration. The two

*Agrobacterium*-based procedures are “in planta” and “ex-planta”, respectively. Both procedures require the above gene construct to be placed into a T-DNA vector, which is then transferred into *Agrobacterium tumefaciens*. The in planta procedure places the transformed *Agrobacterium* in the presence of plant material (flower or meristem) and the plants are allowed to seed followed by selection (e.g., antibiotic or herbicide) during germination. The ex-planta procedure also places *Agrobacterium* in the presence of plant material (callus, cell culture, leaf disk, hypocotyl) which is placed directly under selection (e.g., antibiotic or herbicide) followed by regeneration.

Thus, the isoflavonoid pathway can be introduced into any plant species that does not possess the enzyme catalyzing the IFS reaction by expressing the IFS gene in transgenic plants under the control of a suitable constitutive or inducible promoter.

**Example 1: Transformation of Arabidopsis thaliana with Soybean CYP93C1V2**

Soybean CYP93C1v2 cDNA was placed in the binary plant transformation vector pCHF3, in which it is under control of the cauliflower mosaic virus 35S promoter, using standard recombinant DNA methods (Sambrook, et al. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed, Cold Spring Harbor Laboratory Press, New York). The gene was then transformed into plants of the crucifer, *Arabidopsis thaliana* ecotype Columbia, using *Agrobacterium tumefaciens* and a standard floral infiltration procedure (Clough, S.J. and Bent, A.F., 1998, “Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana”. *Plant J* 16: 735-743). Transgenic plants were selected by germinating the seedlings on kanamycin, and those surviving selection were allowed to set seed. T<sub>2</sub> seedlings expressing CYP93C1v2 were identified by standard DNA and RNA gel blot analysis (Sambrook, et al. 1989. *Molecular cloning: A Laboratory Manual*, 2nd Ed,
Cold Spring Harbor Laboratory Press, New York), and analyzed for accumulation of genistein in leaves by HPLC analysis, according to a method developed to profile the flavonoid components of \textit{Arabidopsis} leaves (Graham, T.L., 1998, “Flavonoid and flavonol glycoside metabolism in \textit{Arabidopsis}”. \textit{Plant Physiol Biochem} 36: 135-144).

Figure 6A shows a typical HPLC trace of a leaf extract from an untransformed plant. The major components are glycosides (containing glucose and rhamnose) of the flavonols kaempferol and quercetin. Plants harboring the soybean CYP93C1v2 gene showed an additional three peaks on HPLC analysis (Fig. 6B), indicated by the arrows labeled as “1,” “2” and “3.” No free genistein, free 2-hydroxyisoflavanone or 2-hydroxyisoflavanone conjugates were observed. However, following treatment of extracts with almond \(\beta\)-glucosidase (Fig 7B), one of the new peaks disappeared, and free genistein was now observed, consistent with the peak being a glucoside of genistein. LC-MS analysis confirmed the identities of the new compounds as a glucoside of genistein, glucose-rhamnose-genistein, and rhamnose-genistein (Figs. 6C and 7C and 7D, insets). Therefore, expression of CYP93C1v2 in transgenic \textit{Arabidopsis} leads to formation of genistein with no requirement for an enzyme to catalyze the dehydration of the presumed 2-hydroxyisoflavanone intermediate. \textit{Arabidopsis} plants then modify the genistein by exactly the same chemistry they use to conjugate their endogenous flavonols, namely by conjugation to glucose and rhamnose. Transgenic production of conjugates of genistein are suitable for nutraceutical applications, because genistein is also glycosylated in soybean, its natural dietary source (Graham, T.L., 1991, “Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates.” \textit{Plant Physiol} 95: 594-603).

In addition to introducing the isoflavonoid pathway into plants that do not possess this pathway, the level of isoflavonoid compounds can be controlled in plants that do possess the pathway by manipulating the level of expression of the IFS gene. Increasing the levels of isoflavonoid compounds in leguminous plants by expression of the IFS gene of the present invention in transgenic plants under the control of a suitable constitutive or inducible promoter can be accomplished by standard methods.
such as *Agrobacterium*-based or biolistic transformation methods known in the art. Alternatively, the level of isoflavonoid compounds in plants can be reduced by expression of antisense constructs or constructs designed to promote gene silencing that contain an intact IFS gene, or segments thereof, in transgenic plants using methods known in the art. (Bourque, J.E., 1995, “Antisense strategies for genetic manipulations in plants,” *Plant Science* 105:125-149; and Angell, S. M. and D. C. Baulcombe, 1997, “Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA,” *EMBO J* 16:3675-3684). Antisense constructs for gene silencing are constructed by placing the whole or part of the cDNA in a three prime to five prime orientation behind a desirable transcriptional promoter and ahead of a transcriptional terminator in a plasmid vector. The vector may be used for biolistic transformation or the new antisense gene may be transferred to a T-DNA vector for *Agrobacterium*-based transformation. The actual mechanism of silencing by antisense constructs is unknown. Homology-dependent gene silencing or co-suppression requires the over-expression of a homologous gene; therefore, to achieve co-suppression a construct is made using a strong promoter, the gene of interest (in this case IFS) and a transcriptional terminator. The gene should be transferred to plants as described above. Gene silencing is an epigenetic phenomenon that may or may not occur with a particular gene construct. When it does occur, the inhibition of gene expression can be greater than with the antisense approach.

Isoflavones can be synthesized from flavanones, utilizing recombinant IFS expressed in any suitable bacterial, fungal, algal, or insect cell system. For example, naringenin is extracted in large amounts from grapefruits. A CYP93C1 enzyme can be used convert naringenin to 2,5,7,4'-tetrahydroxyisoflavanone, which spontaneously converts to the valuable nutraceutical genistein under weak acid conditions. Furthermore, daidzin can be synthesized from liquiritigenin utilizing recombinant CYP93C1 and an isoflavone glucosyltransferase (Köster, J. and W. Barz, 1981, “UDP-Glucose: isoflavone 7-O-glucosyltransferase from roots of chick pea (*Cicer arietinum* L.).” *Arch Biochem Biophys* 212: 98-104).
Example 2: Methodology Used to Isolate and Identify IFS cDNA Clones

In an attempt to obtain cDNA clones encoding IFS, a functional genomics approach was followed. IFS activity is present in soybean seeds, which accumulate daidzein and genistein. Furthermore, IFS activity can be induced in soybean tissues in response to infection with Phytophthora infestans, associated with the accumulation of the isoflavonoid phytoalexin glyceollin (Bhattacharyya, M. K. and E. W. B. Ward, 1987, "Biosynthesis and metabolism of glyceollin I in soybean hypocotyls following wounding or inoculation with Phytophthora megasperma f. sp. glycinea," Physiol Mol Plant Path 31: 387-405). It was also known that an enzyme catalyzing a similar reaction to IFS, namely the 2-hydroxylation of flavanone but without aryl migration, belongs to the CYP93B1 subclass of cytochrome P450s (Akashi, et al, 1998, "Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of licorice (Glycyrrhiza echinata L.: Fabaceae) which represents licodione synthase and flavone synthase II," FEBS Letters 431: 287-290). We therefore searched an expressed sequence tag (EST) database of partial soybean sequences obtained by mass sequencing of two cDNA libraries: a Phytophthora-infected hypocotyl cDNA library (48 hours after infection) and a mid to late developmental stage seed library. Nine candidate P450 sequences were identified, of which three belonged to the CYP93 family. DNA probes were made from the EST clones of the three CYP93 candidates and were used to probe an RNA blot of transcripts from alfalfa suspension cells at various times after exposure to yeast elicitor, a treatment known to induce IFS activity at the onset of isoflavonoid phytoalexin accumulation (Kessmann, et al., 1990, "Stress responses in alfalfa (Medicago sativa L.) III. Induction of medicarpin and cytochrome P450 enzyme activities in elicitor-treated cell suspension cultures and protoplasts," Plant Cell Reports 9: 38-41). One P450 probe cross-hybridized and detected alfalfa transcripts that were strongly induced by elicitation. This probe was derived from a clone with high homology to soybean CYP93C1 as described below, and the insert in the EST clone was full length. The insert was excised and then cloned into the baculovirus expression system for functional identification by heterologous expression in insect cells (Pauli, H. H. and T. M. Kuchan, 1998, "Molecular cloning and functional heterologous expression of two alleles encoding (S)-N-methylcoclaurine 3'-

The carbon monoxide difference spectrum of microsomes isolated from insect cells expressing the soybean CYP93C clone indicated the presence of expressed cytochrome P450, as seen from an absorption peak at 450 nm that was not present in similar spectra from insect microsomes originating from cells transformed with a control vector. Unlabeled liquiritigenin was then fed to the microsomes in the presence of NADPH. The substrate remained unconverted in microsomes from cells harboring the control vector. However, in microsomes expressing the CYP93C clone, a new peak of RT 29.96 min was observed by high performance liquid chromatography (Fig 8A). The amount of this peak was reduced 10-fold if NADPH was omitted from the incubations (Fig. 8B). The UV spectrum of the product, obtained by diode array detection, was identical to that of authentic daidzein ($\lambda_{\text{max}}$ 248 nm, sh 302 nm, $\lambda_{\text{min}}$ 222 nm). The product was collected, derivatized, and analyzed by GC-MS. The mass spectrum of the BSTFA derivative was identical to that of an authentic sample of daidzein (Fig. 9). Microsomes containing the CYP93C clone also metabolized naringenin to yield genistein, although somewhat less efficiently than the reaction with liquiritigenin (Fig. 8C). Insect cell microsomes expressing a different soybean cytochrome P450 cDNA, CYP93E, did not convert liquiritigenin to daidzein when incubated in the presence of NADPH (Fig. 8D). These results indicate that the soybean CYP93C encodes IFS.

**Example 3: Method of Increasing Dietary Isoflavonoid Intake**

Transgenic tomato plants are produced by the introduction of CYP93C1v2 via standard *Agrobacterium*-based procedures. In a preferred embodiment, the CYP93C1v2 coding sequence is under control of a gene promoter giving specific expression in the fruit. Progeny containing the coding region of the CYP93C1v2 gene are selected at the seedling stage by standard polymerase chain reaction and/or DNA blot analysis known to those skilled in the art. Plants scoring positive for possession of the transgene are grown to fruiting, and fruit analyzed for the presence of
isoflavones by the HPLC methods shown in Fig. 7 and Fig. 8 of the present invention. Fruit harvested from the transgenic tomato plants are ingested to increase the dietary intake of isoflavonoids.

It is to be understood that the above description is of preferred exemplary embodiments of the invention and is intended to be illustrative of the invention, but is not to be construed to limit the scope of the invention in any way. Modifications may be made in the structural features of the invention without departing from the scope of the invention.

In summary, isoflavones can now be genetically engineered to provide potential human health benefits of dietary isoflavones and to increase disease resistance in plants. Isoflavones can now be produced in transgenic plants species in which isoflavones do not naturally occur, i.e., in species other than legumes. For example, engineering constitutive production of daidzein and/or genistein or their conjugates into tomato, potato, corn, or other popular components of the human diet, leads to human health benefits, such as reduced cancer risk, reduced incidence of osteoporosis, and treatment for alcoholism. Alternatively, introducing infection-inducible isoflavonoid biosynthesis into non-legumes qualitatively complements these plants' phytoalexin defenses against microbial pathogens, whereas over-expression of the isoflavonoid pathway in legumes quantitatively increases this defense response.

Finally, modifying the extent of production of isoflavonoids in legume roots positively impacts nodulation efficiency and therefore plant yield.
WE CLAIM:

1. A method for introducing into a naturally non-isoflavonoid-producing plant species the enzyme catalyzing the aryI migration of a flavanone to form an isoflavanone intermediate or an isoflavone, comprising:
   introducing a DNA segment encoding said enzyme into said plant to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

2. The method of Claim 1, wherein said DNA segment comprises isolated genomic DNA.

3. The method of Claim 1, wherein said DNA segment comprises recombinant DNA.

4. The method of Claim 1, 2 or 3, wherein said DNA segment comprises a CYP93C gene.

5. The method of Claim 4, wherein said DNA segment consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

6. The method of Claim 1, 2 or 3, wherein said DNA segment comprises a Medicago truncatula homolog of a CYP93C gene.

7. The method of Claim 6, wherein said DNA segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

8. The method of Claim 1, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.
9. The method of Claim 2, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.

10. The method of Claim 3, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant to cause in vivo formation of daidzein or a daidzein derivative.

11. The method of Claim 8, 9 or 10, wherein said plant is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

12. The method of Claim 1, 2, 3, 8, 9 or 10, wherein said plant further comprises downstream genes to metabolize said formed isoflavonone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

13. The method of Claim 12, wherein said downstream gene is selected from the group consisting of isoflavone O-methyltransferase, isoflavone 2’-hydroxylase, isoflavone reductase, and vestitone reductase.

14. The method of Claim 13, wherein said plant comprises downstream gene 4’-O-methyltransferase to form biochanin A or a biochanin A derivative.

15. The method of Claim 1, 2, 3, 5, 7, 8, 9, 10, 13 or 14, wherein said flavanone is liquiritigenin.

16. The method of Claim 4, wherein said flavanone is liquiritigenin.

17. The method of Claim 6, wherein said flavanone is liquiritigenin.

18. The method of Claim 11, wherein said flavanone is liquiritigenin.

19. The method of Claim 12, wherein said flavanone is liquiritigenin.

20. The method of Claim 1, 2, 3, 5, 7, 8, 9, 10, 13 or 14, wherein said flavanone is naringenin.
21. The method of Claim 4, wherein said flavanone is naringenin.

22. The method of Claim 6, wherein said flavanone is naringenin.

23. The method of Claim 11, wherein said flavanone is naringenin.

24. The method of Claim 12, wherein said flavanone is naringenin.

25. A method for increasing the level of isoflavonoid compounds in naturally isoflavonoid-producing plants comprising:
   introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transgenic plant, wherein said transgenic plant expresses said DNA segment under the control of a suitable constitutive or inducible promoter when said transgenic plant is exposed to conditions which permit expression.

26. The method of Claim 25, wherein said isoflavonoid is selected from the group consisting of an isofavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

27. The method of Claim 25, wherein said DNA segment comprises isolated genomic DNA.

28. The method of Claim 26, wherein said DNA segment comprises isolated genomic DNA.

29. The method of Claim 25, wherein said DNA segment comprises recombinant DNA.

30. The method of Claim 26, wherein said DNA segment comprises recombinant DNA.

31. The method of Claim 25, 26, 27, 28, 29 or 30, wherein said DNA segment comprises a CYP93C gene.
32. The method of Claim 31, wherein said DNA segment consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

33. The method of Claim 25, 26, 27, 28, 29 or 30, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

34. The method of Claim 33, wherein said DNA segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

35. The method of Claim 25, 26, 27, 28, 29, 30, 32 or 34, wherein said flavanone is liquiritigenin.

36. The method of Claim 31, wherein said flavanone is liquiritigenin.

37. The method of Claim 33, wherein said flavanone is liquiritigenin.

38. The method of Claim 25, 26, 27, 28, 29, 30, 32 or 34, wherein said flavanone is naringenin.

39. The method of Claim 31, wherein said flavanone is naringenin.

40. The method of Claim 33, wherein said flavanone is naringenin.

41. A method for synthesizing an isoflavonone intermediate or an isoflavone from a flavanone by expressing a recombinant CYP93C gene segment in a suitable bacterial, fungal, algal, or insect cell system.

42. The method of Claim 41, wherein said gene segment consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

43. The method of Claim 41, wherein said gene segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.
44. A method of reducing the levels of isoflavonoid compounds in a naturally
isoavonoid-producing plant comprising introducing and expressing an antisense or
gene silencing construct that contains an intact CYP93C gene or segments thereof into
said plant.

45. The method of Claim 44, wherein said gene consists essentially of the
sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted
in SEQ ID NO:1.

46. The method of Claim 44, wherein said gene consists essentially of the
sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted
in SEQ ID NO:4.

47. A naturally non-isoavonoid-producing plant cell transformed by
introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a
flavanone to form an isoavonone intermediate or an isoavone, wherein said
transgenic plant cell expresses said DNA segment under the control of a suitable
constitutive or inducible promoter when exposed to conditions which permit
expression.

48. The plant cell of Claim 47, wherein said DNA segment comprises isolated
genomic DNA.

49. The plant cell of Claim 47, wherein said DNA segment comprises
recombinant DNA.

50. The plant cell of Claim 47, 48 or 49, wherein said DNA segment
comprises a CYP93C gene.

51. The plant cell of Claim 50, wherein said DNA segment consists essentially
of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence
depicted in SEQ ID NO:1.

52. The plant cell of Claim 47, 48 or 49, wherein said DNA segment
comprises a *Medicago truncatula* homolog of a CYP93C gene.
53. The plant cell of Claim 52, wherein said DNA segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

54. The plant cell of Claim 47, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant cell to cause in vivo formation of daidzein or a daidzein derivative.

55. The plant cell of Claim 48, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant cell to cause in vivo formation of daidzein or a daidzein derivative.

56. The plant cell of Claim 49, wherein chalcone synthase, chalcone reductase, and chalcone isomerase genes are also expressed in said plant cell to cause in vivo formation of daidzein or a daidzein derivative.

57. The plant cell of Claim 54, 55 or 56, wherein said plant cell is further transformed to comprise said chalcone synthase, chalcone reductase, and chalcone isomerase genes.

58. The plant cell of Claim 47, 48, 49, 54, 55 or 56, wherein said plant cell further comprises downstream genes to metabolize said formed isoflavanone intermediate or isoflavone to biologically active isoflavonoid derivatives or conjugates.

59. The plant cell of Claim 58, wherein said downstream gene is selected from the group consisting of isoflavone O-methyltransferase, isoflavone 2’-hydroxylase, isoflavone reductase, and vestitone reductase.

60. The plant cell of Claim 59, wherein said plant cell comprises downstream gene 4’-O-methyltransferase to cause in vivo formation of biochanin A or a biochanin A derivative.
61. A naturally isoflavonoid-producing plant cell transformed by introducing a DNA segment encoding the enzyme catalyzing the aryl migration of a flavanone to yield an isoflavonoid to form a transformed plant cell, wherein said transformed plant cell expresses said DNA segment under the control of a suitable constitutive or inducible promoter when exposed to conditions which permit expression.

62. The plant cell of Claim 61, wherein said isoflavonoid is selected from the group consisting of an isoflavanone intermediate, an isoflavone, an isoflavone derivative, and an isoflavone conjugate.

63. The plant cell of Claim 61, wherein said DNA segment comprises isolated genomic DNA.

64. The plant cell of Claim 62, wherein said DNA segment comprises isolated genomic DNA.

65. The plant cell of Claim 61, wherein said DNA segment comprises recombinant DNA.

66. The plant cell of Claim 62, wherein said DNA segment comprises recombinant DNA.

67. The plant cell of Claim 61, 62, 63, 64, 65 or 66, wherein said DNA segment comprises a CYP93C gene.

68. The plant cell of Claim 67, wherein said DNA segment consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

69. The plant cell of Claim 61, 62, 63, 64, 65 or 66, wherein said DNA segment is a *Medicago truncatula* homolog of a CYP93C gene.

70. The plant cell of Claim 69, wherein said DNA segment consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.
71. A transgenic plant cell having reduced levels of isoflavonoid compounds, said plant cell transformed by introducing an antisense or gene silencing construct that contains an intact CYP93C gene or segments thereof into said plant cell.

72. The plant cell of Claim 71, wherein said gene consists essentially of the sequence from about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

73. The plant cell of Claim 71, wherein said gene consists essentially of the sequence from about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO:4.

74. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 of the CYP93 family that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

75. The gene or DNA segment of Claim 74, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

76. The gene or DNA segment of Claim 74, wherein said gene is the soybean gene encoding the enzyme catalyzing the aryl migration of naringenin.

77. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion consists essentially of about nucleotide 36 to about nucleotide 1598 of the sequence depicted in SEQ ID NO:1.

78. An isolated gene or DNA segment comprising a portion which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.
79. The gene or DNA segment of Claim 78 consisting essentially of about nucleotide 92 to about nucleotide 1657 of the sequence depicted in SEQ ID NO: 4

80. The gene or DNA segment of Claim 78, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

81. The gene or DNA segment of Claim 79, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of liquiritigenin.

82. The gene or DNA segment of Claim 78, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

83. The gene or DNA segment of Claim 79, wherein said gene is the *Medicago truncatula* gene encoding the enzyme catalyzing the aryl migration of naringenin.

84. A protein encoded by a portion of an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said portion is a *Medicago truncatula* homolog of a CYP93C gene.

85. A food comprising edible transgenic plant material capable of being ingested for its nutritional value, said transgenic plant is transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

86. A food comprising at least one isoflavonoid, wherein said isoflavonoid is isolated from a transgenic plant transformed with an isolated gene or DNA segment
which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

87. A composition comprising at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment, said composition suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical.

88. A composition comprising an isoflavonoid suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, said isoflavonoid isolated from at least a portion of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

89. A method of increasing the nutritional value of a plant by transforming said plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits increased levels of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.
90. A method of using a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment, to provide a nutraceutical benefit to a human or animal administered said isoflavonoid.

91. The method of claim 90, wherein said isoflavonoid is administered by ingestion of at least a portion of said plant.

92. The method of claim 90, wherein said isoflavonoid is administered by ingestion of a composition comprising an isoflavonoid isolated from said plant.

93. A method of using an isoflavonoid isolated from a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment, to provide a pharmaceutical benefit to a patient administered said isoflavonoid.

94. A method of increasing disease resistance in a plant by transforming said plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.
95. A method of increasing nodulation efficiency of a leguminous plant by transforming said plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid released from the roots of said transgenic plant when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise said isolated gene or DNA segment.

96. A leguminous transgenic plant exhibiting increased nodulation efficiency, wherein said transgenic plant is transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein said transgenic plant exhibits an increased level of an isoflavonoid released from the roots of said transgenic plant when compared to the level of the isoflavonoid released from the roots of plants of the same species which do not comprise said isolated gene or DNA segment.

97. A method of increasing bacterial or fungal symbiosis in a plant by transforming said plant with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment.

98. A transgenic plant exhibiting an increased level of bacterial or fungal symbiosis, wherein said transgenic plant is transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, and wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise the isolated gene or DNA segment.
99. A transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

100. Seed from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

101. Progeny from a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

102. Progeny from seed of a transgenic plant comprising at least one recombinant DNA sequence encoding a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said recombinant DNA sequence.

103. Use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said
isolated gene or DNA segment, for the preparation of a nutraceutical preparation for achieving a nutritional effect.

104. Use of a transgenic plant transformed with an isolated gene or DNA segment which encodes a cytochrome P450 that can catalyze the aryl migration of a flavanone to yield an isoflavanone intermediate or an isoflavone, wherein said transgenic plant exhibits an increased level of an isoflavonoid when compared to the level of said isoflavonoid in plants of the same species which do not comprise said isolated gene or DNA segment, for the preparation of a pharmaceutical preparation for achieving a therapeutic effect.
Fig. 1
Fig. 4
<table>
<thead>
<tr>
<th>mtIFS</th>
<th>MLVELAVTLILALFLHLRTPTATSKALRHLPNPPSPKPRLPFGHGLHL 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP93C1v2</td>
<td>MLIELALGLVLALFLHLRTPTATSKALRHLPNPPSPKPRLPFGHGLHL 50</td>
</tr>
<tr>
<td></td>
<td>LPDKHILLYAALIDLSKKGPGFSLYFSGMPTVAVSTPELFLKGLQTHEATS 100</td>
</tr>
<tr>
<td></td>
<td>LKTQQIRKLVIRVMAQGAEQKPDLEELKWTNISTSMMLGAEAEVRD 200</td>
</tr>
<tr>
<td></td>
<td>LQDEIYKIFGEYSVNNIFWPLNKFVGNYDKTICEIFNMDPITIKVIIK 250</td>
</tr>
<tr>
<td></td>
<td>QSEIRNKSQNGEVVEQCVNQLDLCLELIEDEIYKIKGLVLOD 300</td>
</tr>
<tr>
<td></td>
<td>RREIVRRKMKNENVEVGEVSQVFLDLTCLELIEDEIYKIKGLVLOD 300</td>
</tr>
<tr>
<td></td>
<td>FFSAFTSTAVTSTELINNFRVLKKEIKQSLOVDVQ 350</td>
</tr>
<tr>
<td></td>
<td>FFSAFTSTAVTSTELINNFRVKKEIKQSLOVQ 350</td>
</tr>
<tr>
<td></td>
<td>NLPYIKAIVKEARLHPVLVVKCRCTQCEIDGYVIVPNALILFNVWAV 400</td>
</tr>
<tr>
<td></td>
<td>NLPYIIRAIVKTFRMHPLVPVVRKCTECEEGNYVPEALIGALFNVWQV 400</td>
</tr>
<tr>
<td></td>
<td>GROKVKWPLFRPFIENYVGEASAIDLQHFTLLPFGSGRRCNP 450</td>
</tr>
<tr>
<td></td>
<td>GROKVKWDRPSEFRFPFLTGAEGAPLDLQHGQFOLLPPGSGRRCNP 450</td>
</tr>
<tr>
<td></td>
<td>GVNLATAGMNTMTATISLIQCFDLQVPGQHQGEILNMDYAKVSMEERPGLTVP 500</td>
</tr>
<tr>
<td></td>
<td>GVNLATGMSMTALLASLIQCFDLQVLPQOGQILKGGDIAKVSMEERAGLTVP 500</td>
</tr>
<tr>
<td></td>
<td>RAHNLISVPLARAGVAKLSS 522</td>
</tr>
<tr>
<td></td>
<td>RAHSVLGCVPLARIGVASKLSS 521</td>
</tr>
</tbody>
</table>

**Fig. 5**
SEQUENCE LISTING

THE SAMUEL ROBERTS NOBLE FOUNDATION, INC.
STEEL, Christopher L.
DIXON, Richard A.

GENETIC MANIPULATION OF ISOFLAVONOIDS

11137/05002

60/123,267
1999-03-08

5

PatentIn Ver. 2.1

1
1717
DNA
Glycine max

CDS
(36) .. (1598)

1

53
Met Leu Leu Glu Leu Ala

1
5

101
Leu Gly Leu Leu Val Leu Ala Leu Phe Leu His Leu Arg Pro Thr Pro

15
20

149
Thr Ala Lys Ser Lys Ala Leu Arg His Leu Pro Asn Pro Pro Ser Pro

25
30
35

197
Lys Pro Arg Leu Pro Phe Ile Gly His Leu His Leu Lys Asp Lys

40
45
50

245
Leu Leu His Tyr Ala Leu Ile Asp Leu Ser Lys Lys His Gly Pro Leu

55
60
65
70

293
Phe Ser Leu Tyr Phe Gly Ser Met Pro Thr Val Val Ala Ser Thr Pro

75
80
85

341
Glu Leu Phe Lys Leu Phe Leu Gln Thr His Glu Ala Thr Ser Phe Asn

90
95
100

1
aca agg ttc caa acc tca gcc ata aga cgc ctc acc tat gat agc tca
Thr Arg Phe Glu Thr Ser Ala Ile Arg Arg Leu Thr Tyr Asp Ser Ser
 105  110  115

gtg gcc atg gtt ccc ttc gga cct tac tgg aag ttc gtt agg aag ctc
Val Ala Met Val Pro Phe Gly Pro Tyr Trp Lys Phe Val Arg Lys Leu
 120  125  130

atc atg aac gac ctt ctc aac gcc acc act gta aac aag ttg agg aag cct
Ile Met Asn Asp Leu Leu Asn Ala Thr Thr Val Asn Lys Leu Arg Pro
 135  140  145  150

ttg agg acc caa cag atc cgc aag ttc ctt agg gtt atg gcc caa ggc
Leu Arg Thr Glu Glu Ile Arg Lys Phe Leu Arg Val Met Ala Glu Gly
 155  160  165

gca gag gca cag aag ccc ctt gac ttg acc gag gag ctt ctc aag tgg
Ala Glu Ala Glu Pro Leu Asp Leu Thr Glu Glu Leu Leu Lys Trp
 170  175  180

acc aac agc acc atc tcc atg atg atg ctc ggc gag gct gag gag atc
Thr Asn Ser Thr Ile Ser Met Met Met Leu Gly Glu Ala Glu Glu Ile
 185  190  195

aga gac atc gct cgc gag gtt ctt aag atc ttt ggc gaa tac agc ctc
Arg Asp Ile Ala Glu Val Leu Lys Ile Phe Gly Glu Tyr Ser Leu
 200  205  210

act gac ttc atc tgg cca ttg aag cat ctc aag gtt gga aag tat gag
Thr Asp Phe Ile Trp Pro Leu Lys His Leu Lys Val Gly Lys Tyr Glu
 215  220  225  230

aag agg atc gac gac atc ttg aac aag ttc gac cct gtc gtt gaa aag
Lys Arg Ile Asp Asp Ile Leu Asn Lys Phe Asp Pro Val Val Glu Arg
 235  240  245

gtc atc aag aag cgc cgt gag atc gtg aag agg aag aag aac gga gag
Val Ile Lys Arg Arg Glu Ile Val Arg Arg Lys Asn Gly Glu
 250  255  260

gtt gtt gag ggt gag gtc agc ggg gtt ttc ctt gac act tgt ctt gaa
Val Val Glu Gly Glu Ser Gly Val Asp Thr Leu Leu Glu
 265  270  275

ttc gct gag gat gag acc atg gag gac aag aat tac aa aat acc aag gac cct atc
Phe Ala Glu Asp Glu Thr Met Glu Ile Thr Lys Thr Asp His Ile
 280  285  290

aag gtt ctt gtt gtc gac ttc cgg gca gaa aca gac tcc aca ggg
Lys Gly Leu Val Val Asp Phe Phe Ser Ala Gly Thr Asp Ser Thr Ala
 295  300  305  310

gtg gca aca gag tgg gca ttg gca gaa ctc atc aac aat cct aag gtt
Val Ala Thr Glu Trp Ala Leu Ala Glu Leu Ile Asn Asn Pro Lys Val
 315  320  325

ttg gaa aag gct cgt gag gac gtc tac aag tgt ggt gaa aag gag aca
Leu Glu Lys Ala Arg Glu Glu Val Tyr Ser Val Val Gly Lys Asp Arg
 330  335  340
ctt gtg gac gaa gtt gac act caa aac ctt cct tac att aga gca atc
Leu Val Asp Glu Val Asp Thr Gln Asn Leu Pro Tyr Ile Arg Ala Ile
345 350 355

gtg aag gag aca ttc cgc atg cac ccg cca ctc cca gtc gtc aaa aga
Val Lys Glu Thr Phe Arg Met His Pro Pro Leu Pro Val Val Lys Arg
360 365 370

aag tgc aca gaa gag tgt gag attaat gga tat gtc atc cca gag gga
Lys Cys Thr Glu Glu Cys Glu Ile Asn Gly Tyr Val Ile Pro Glu Gly
375 380 385 390

gca tgg att ctc ttc aat gta tgg caa gta gga aga gac ccc aaa tac
Ala Leu Ile Leu Phe Asn Val Trp Gln Val Gly Arg Asp Pro Lys Tyr
395 400 405

ctg gag cag cca ctt gct gat tgc aag gtc cta gag cag cag
Trp Asp Arg Pro Ser Phe Arg Pro Glu Arg Phe Leu Glu Thr Gly
410 415 420

gct gaa ggg gaa gca ggg cct ctt gat ctt agg gga caa cat ttt caa
Ala Glu Gly Glu Ala Gly Pro Leu Asp Leu Arg Gly Gln His Phe Gln
425 430 435

ctt ctc cca ttt ggg tct ggg agg aga atg tgc ctt gga gtc aat ctc
Leu Leu Pro Phe Gly Ser Gly Arg Met Cys Pro Gly Val Asn Leu
440 445 450

gct act tgc gga atg gca aca ctt ctc cta ctt att cag tgc ttc
Ala Thr Ser Gly Met Ala Thr Leu Ala Ser Leu Ile Gln Cys Phe
455 460 465 470

gac ttt ctt gtt ctt gta ggt cca cta gaa cag ata tgg gga gag gtt ggt
gac
Asp Leu Gln Val Leu Gly Pro Glu Gly Gly Gly Ile Leu Lys Gly Gly Asp
475 480 485

gcc aag gtt atc gaa gag aga gcc ggc ctc act gtt cca aag gca
Ala Lys Val Ser Met Glu Glu Arg Ala Gly Leu Thr Val Pro Arg Ala
490 495 500

cat gaa ctt tct tct gtt ctt gca agg atc ggc gtt gca tct aag
His Ser Leu Val Cys Val Pro Leu Ala Arg Ile Gly Val Ala Ser Lys
505 510 515

cgc ctc aag ttc ccg cct gaa acg ccc cag gct
Leu Leu Ser
520

cgcttgggtggttgataaactcactcattttcaccattttcactttttaattatattta
1638

tatataaggcccttccatgc
1717

<210> 2
<211> 521
<212> PRT
<213> Glycine max

3
| Met Leu Leu Glu Leu Ala Leu Gly Leu Leu Val Leu Ala Leu Phe Leu |
| 1      5   10  15  |
| His Leu Arg Pro Thr Pro Thr Ala Lys Ser Lys Ala Leu Arg His Leu |
| 20     25  30   |
| Pro Asn Pro Pro Ser Pro Lys Pro Arg Leu Pro Phe Ile Gly His Leu |
| 35     40  45   |
| His Leu Leu Lys Asp Lys Leu Leu His Tyr Ala Leu Ile Asp Leu Ser |
| 50     55  60   |
| Lys Lys His Gly Pro Leu Phe Ser Leu Tyr Phe Gly Ser Met Pro Thr |
| 65     70  75  80 |
| Val Val Ala Ser Thr Pro Glu Leu Phe Lys Leu Phe Leu Gln Thr His |
| 85     90   |
| Glu Ala Thr Ser Phe Asn Thr Arg Phe Gln Thr Ser Ala Ile Arg Arg |
| 100     105  110 |
| Leu Thr Tyr Asp Ser Ser Val Ala Met Val Pro Phe Gly Pro Tyr Trp |
| 115    120  125 |
| Lys Phe Val Arg Lys Leu Ile Met Asn Asp Leu Leu Asn Ala Thr Thr |
| 130    135  140 |
| Val Asn Lys Leu Arg Pro Leu Arg Thr Gln Gln Ile Arg Lys Phe Leu |
| 145    150  155  160 |
| Arg Val Met Ala Gln Gly Ala Glu Ala Gln Lys Pro Leu Asp Leu Thr |
| 165    170  175 |
| Glu Glu Leu Leu Lys Trp Thr Asn Ser Thr Ile Ser Met Met Met Leu |
| 180    185  190 |
| Gly Glu Ala Glu Glu Ile Arg Asp Ile Ala Arg Glu Val Leu Lys Ile |
| 195    200  205 |
| Phe Gly Glu Tyr Ser Leu Thr Asp Phe Ile Trp Pro Leu Lys His Leu |
| 210    215  220 |
| Lys Val Gly Lys Tyr Glu Lys Arg Ile Asp Asp Ile Leu Asn Lys Phe |
| 225    230  235  240 |
| Asp Pro Val Val Glu Arg Val Ile Lys Lys Arg Arg Glu Ile Val Arg |
| 245    250  255 |
| Arg Arg Lys Asn Gly Glu Val Val Glu Gly Glu Val Ser Gly Val Phe |
| 260    265  270 |
| Leu Asp Thr Leu Leu Glu Phe Ala Glu Asp Glu Thr Met Glu Ile Lys |
| 275    280  285 |
| Ile Thr Lys Asp His Ile Lys Gly Leu Val Val Asp Phe Phe Ser Ala |
| 290    295  300 |
| Gly Thr Asp Ser Thr Ala Val Ala Thr Glu Trp Ala Leu Ala Glu Leu |
| 305    310  315  320 |
Ile Asn Asn Pro Lys Val Leu Glu Lys Ala Arg Glu Glu Val Tyr Ser 325 330 335
Val Val Gly Lys Asp Arg Leu Val Asp Glu Val Asp Thr Gln Asn Leu 340 345 350
Pro Tyr Ile Arg Ala Ile Val Lys Glu Thr Phe Arg Met His Pro Pro 355 360 365
Leu Pro Val Val Lys Arg Lys Cys Thr Glu Glu Cys Glu Ile Asn Gly 370 375 380
Tyr Val Ile Pro Glu Gly Ala Leu Ile Leu Phe Asn Val Trp Gln Val 385 390 395 400
Gly Arg Asp Pro Lys Tyr Trp Asp Arg Pro Ser Glu Phe Arg Pro Glu 405 410 415
Arg Phe Leu Glu Thr Gly Ala Glu Gly Glu Ala Gly Pro Leu Asp Leu 420 425 430
Arg Gly Gln His Phe Gln Leu Leu Pro Phe Gly Ser Gly Arg Arg Met 435 440 445
Cys Pro Gly Val Asn Leu Ala Thr Ser Gly Met Ala Thr Leu Leu Ala 450 455 460
Ser Leu Ile Gln Cys Phe Asp Leu Gln Val Leu Gly Pro Gln Gly Gln 465 470 475 480
Ile Leu Lys Gly Gly Asp Ala Lys Val Ser Met Glu Glu Arg Ala Gly 485 490 495
Leu Thr Val Pro Arg Ala His Ser Leu Val Cys Val Pro Leu Ala Arg 500 505 510
Ile Gly Val Ala Ser Lys Leu Leu Ser 515 520

<210> 3
<211> 523
<212> PRT
<213> Glycyrrhiza echinata

<400> 3
Met Glu Pro Gln Leu Val Ala Val Ser Val Leu Val Ser Ala Leu Ile 1 5 10 15
Cys Tyr Phe Phe Arg Pro Tyr Phe His Arg Tyr Gly Lys Asn Leu 20 25 30
Pro Pro Ser Pro Phe Phe Arg Leu Pro Ile Ile Gly His Met His Met 35 40 45
Leu Gly Pro Leu Leu His Gln Ser Phe His Asn Leu Ser His Arg Tyr 50 55 60
Gly Pro Leu Phe Ser Leu Asn Phe Gly Ser Val Leu Cys Val Val Ala
65  70  75  80
Ser Thr Pro His Phe Ala Lys Gln Leu Leu Gln Thr Asn Glu Leu Ala
85  90  95
Phe Asn Cys Arg Ile Glu Ser Thr Ala Val Lys Leu Thr Tyr Glu
100 105 110
Ser Ser Leu Ala Phe Ala Pro Tyr Gly Asp Tyr Trp Arg Phe Ile Lys
115 120 125
Lys Leu Ser Met Asn Glu Leu Leu Gly Ser Arg Ser Ile Asn Asn Phe
130 135 140
Gln His Leu Arg Ala Gln Glu Thr His Gln Leu Leu Arg Leu Ser
145 150 155 160
Asn Arg Ala Arg Ala Phe Glu Ala Val Asn Ile Thr Glu Glu Leu Leu
165 170 175
Lys Leu Thr Asn Val Ile Ser Ile Met Met Val Gly Glu Ala Glu
180 185 190
Glu Ala Arg Asp Val Val Arg Asp Val Thr Glu Ile Phe Gly Glu Phe
195 200 205
Asn Val Ser Asp Phe Ile Trp Leu Phe Lys Met Asp Leu Gln Gly
210 215 220
Phe Gly Lys Arg Ile Glu Asp Leu Phe Gln Arg Phe Asp Thr Leu Val
225 230 235 240
Glu Arg Ile Ile Ser Lys Arg Glu Gln Thr Arg Lys Asp Arg Arg Arg
245 250 255
Asn Gly Lys Lys Gly Glu Gln Gly Ser Gly Asp Gly Ile Arg Asp Phe
260 265 270
Leu Asp Ile Leu Leu Asp Cys Thr Glu Asp Glu Asn Ser Glu Ile Lys
275 280 285
Ile Gln Arg Val His Ile Lys Ala Leu Ile Met Asp Phe Phe Thr Ala
290 295 300
Gly Thr Asp Thr Thr Ala Ile Ser Thr Glu Trp Ala Leu Val Glu Leu
305 310 315 320
Val Lys Lys Pro Ser Val Leu Gln Lys Val Arg Glu Glu Ile Asp Asn
325 330 335
Val Val Gly Lys Asp Arg Leu Val Glu Glu Ser Asp Cys Pro Asn Leu
340 345 350
Pro Tyr Leu Gln Ala Ile Leu Lys Glu Thr Phe Arg Leu His Pro Pro
355 360 365
Val Pro Met Val Thr Arg Arg Cys Val Ala Glu Cys Thr Val Glu Asn
370 375 380

6
Tyr Val Ile Pro Glu Asp Ser Leu Leu Phe Val Asn Val Trp Ser Ile
385 390 395 400
Gly Arg Asn Pro Lys Phe Trp Asp Asn Pro Leu Glu Phe Arg Pro Glu
405 410 415
Arg Phe Leu Lys Leu Glu Gly Asp Ser Ser Gly Val Val Asp Val Arg
420 425 430
Gly Ser His Phe Gln Leu Leu Pro Phe Gly Ser Gly Arg Arg Met Cys
435 440 445
Pro Gly Val Ser Leu Ala Met Gln Glu Val Pro Ala Leu Leu Gly Ala
450 455 460
Ile Ile Gln Cys Phe Asp Phe His Val Val Gly Pro Lys Gly Glu Ile
465 470 475 480
Leu Lys Gly Asp Ile Val Ile Asn Val Asp Glu Arg Pro Gly Leu
485 490 495
Thr Ala Pro Arg Ala His Asn Leu Val Cys Val Pro Val Asp Arg Thr
500 505 510
Ser Gly Gly Gly Pro Leu Lys Ile Ile Glu Cys
515 520

<210> 4
<211> 1811
<212> DNA
<213> Medicago truncatula

<220>
<221> CDS
<222> (92)...(1657)

<400> 4
caacacctaa gagtaactaa taagaacttt ctttctactt cttagtatag ttaacaactt 60
aagtaaatat actacaagga agctatacac c atg ttg gtt gaa ctt gca gtt
Met Leu Val Glu Leu Ala Val
1 5

act cta ttg ctc att gct ctc ttc tta cac ttg cgt cca aca cct act
Thr Leu Leu Leu Ile Ala Leu Phe Leu His Leu Arg Pro Thr Pro Thr
10 15 20

cca aaa tca aag gct ctt cgc cac ctt cca aat cca cca agc cct aaa
Ala Lys Ser Lys Ala Leu Arg His Leu Pro Asn Pro Pro Ser Pro Lys
25 30 35

cca cgt ctt cca ttc ata ggt cat ctt ctc ctt ttg gat aac cca ctt
Pro Arg Leu Pro Phe Ile Gly His Leu His Leu Leu Asp Asn Pro Leu
40 45 50 55

cct cac cac act ctt atc aag tta gga aag cgt tat ggc cct ttg tac
Leu His His Thr Leu Ile Lys Leu Gly Lys Arg Tyr Gly Pro Leu Tyr
60 65 70
act ctt tac ttt ggt tcc atg cct acc gtt gtt gca tcc act cct gac
Thr Leu Tyr Phe Gly Ser Met Pro Thr Val Val Ala Ser Thr Pro Asp
 75   80   85

ttg ttt aaa ctt ttc ctt cca acc cat gaa gct act tcc ttt aac aca
Leu Phe Lys Leu Phe Leu Gln Thr His Glu Ala Thr Ser Phe Asn Thr
 90  95 100

aga ttc cca acc acc tct gct att agt cgt ctt acc tat gac aac tct gtt
Arg Phe Gln Thr Ser Ala Ile Ser Arg Leu Thr Tyr Asp Asn Ser Val
105 110 115

gct atg gtt cca ttt gca cct tat tgg aag ttt att aga aag ctt atc
Ala Met Val Pro Phe Ala Pro Tyr Trp Lys Phe Ile Arg Lys Leu Ile
120 125 130 135

atg aac gac ttc ctc aac gcc acc act gtt aac aac aag ttg agg cca ttg
Met Asn Asp Leu Asn Ala Thr Thr Thr Val Asn Lys Leu Arg Pro Leu
140 145 150

agg aqc cga gaa atc ctt aag gtt ctt aag gtc atg gct aat agt gct
Arg Ser Arg Glu Ile Leu Lys Val Leu Lys Val Met Ala Asn Ser Ala
155 160 165

gaa act cca cag cca ctt gat gtc act gag gag ctt ctc aag tgg aca
Glu Thr Gln Gln Pro Leu Asp Val Thr Glu Leu Leu Lys Trp Thr
170 175 180

aac aqc aca atc tct acc atg atg ttg gtt gag gcc gaa gag gtt aga
Asn Ser Thr Ile Ser Thr Met Met Leu Gly Glu Ala Glu Glu Val Arg
185 190 195


gat atg gct gtt gtt ctt aag atc ttt gga gaa tat agt gtt aca
Asp Ile Ala Arg Asp Val Leu Lys Ile Phe Gly Glu Tyr Ser Val Thr
200 205 210 215

aac ttt att tgg cct tgg aac aag ttt aag aac tat gat aag
Asn Phe Ile Trp Pro Leu Asn Lys Phe Phe Gly Asn Tyr Asp Lys
220 225 230

aga act gag gag att ttc aat aag tat gat cct atc att gaa aag gtt
Arg Thr Glu Glu Ile Arg Lys Tyr Asp Pro Ile Ile Glu Lys Val
235 240 245

atc aag aaa cga cca gag att gtt aac aag aaa aat gga gaa atc
Ile Lys Arg Arg Glu Gly Asp Val Lys Arg Lys Arg Gly Glu Ile
250 255 260


gta gaa ggc gag aat gtt ttt ctt gac act ttg ctt gaa ttt
Val Glu Gly Glu Glu Asp Val Val Phe Leu Asp Thr Leu Leu Glu Phe
265 270 275

gca cca gat gag acc atg gag atc aaa att aca aag gaa cca atc aag
Ala Gln Asp Glu Thr Met Glu Ile Lys Ile Thr Lys Glu Gln Ile Lys
280 285 290 295


gtt ctt gtt gat ttt ttc tct gca gga aca gac tcc acc gcc gtt
Gly Leu Val Val Asp Phe Phe Ser Ala Gly Thr Asp Asp Thr Ala Val
300 305 310

8
tct aca gaa tgg act tta tca gag ctc atc aat aat cct aga gtt ttg
Ser Thr Glu Trp Thr Leu Ser Glu Leu Ile Asn Asn Pro Arg Val Leu
315 320 325

aag aaa gct cga gag gag att gac tct gtt gtg gga aaa gat aga ctc
Lys Lys Ala Arg Glu Glu Ile Asp Ser Val Val Gly Lys Asp Arg Leu
330 335 340

gtt gat gaa tca gat gtt cag aat ctt cct tac att aaa gcc atc gta
Val Asp Glu Ser Asp Val Gln Asn Leu Pro Tyr Ile Lys Ala Ile Val
345 350 355

aaa gaa gca ttt cgc ttg cac cca cca cta cct gta gtc aaa aga aaa
Lys Glu Ala Phe Arg Leu His Pro Pro Leu Pro Val Val Lys Arg Lys
360 365 370 375

tgt aca caa gaa tgt gag atc gag ggg tat gtt cca gaa gga gca
Cys Thr Glu Glu Cys Glu Ile Asp Gly Tyr Val Val Pro Glu Gly Ala
380 385 390

ctt ata ctt ttc aat gtc tgg gca gtt gga aga gcc cca aaa tat tgg
Leu Ile Leu Phe Asn Val Trp Ala Val Gly Arg Asp Pro Lys Tyr Trp
395 400 405

gta aag cca ttg gaa ttt cgt cca gag agg ttc ata gaa aat gtt ggt
Val Lys Pro Leu Glu Phe Arg Pro Glu Arg Phe Ile Glu Asn Val Gly
410 415 420

gaa ggt gaa gca gct tca att gat ctt agg ggt cca cat ttc aca ctt
Glu Gly Glu Ala Ala Ser Ile Asp Leu Arg Gly Gln His Phe Thr Leu
425 430 435

cct cca ttt ggg tct gga aga agg atg tgt cct gga gtc aat ttg gct
Leu Pro Phe Gly Ser Gly Arg Arg Met Cys Pro Gly Val Asn Leu Ala
440 445 450 455

aag gaa atg cca gcc tgg aat ggg aga gag gga gat tat gtt
Lys Val Ser Met Glu Glu Arg Pro Gly Thr Val Pro Arg Ala His
490 495 500

aag atg ctc atg tgt gtt cct ctt gaa aga gct gtt ctc cca gaa gca cat
Lys Val Ser Met Glu Glu Arg Pro Gly Thr Val Pro Arg Ala His
490 495 500

ctt tcc tcc taaaatattc tgagaggag aatcaccacac atatatagcctc
Leu Ser Ser
520

tctcttgtac tacaaatta tgtgttaatt ttctttatatttt ttctgctaca aaggaagtgt
1757

tgtaactttgt aatgtcata aaaaatata aatatttata aatatttata tcctattcat tatt
1811
<210> 5
<211> 522
<212> PRT
<213> Medicago truncatula

Met Leu Val Glu Leu Ala Val Thr Leu Leu Leu Ile Ala Leu Phe Leu
1  5  10  15
His Leu Arg Pro Thr Pro Thr Ala Lys Ser Lys Ala Leu Arg His Leu
20  25  30
Pro Asn Pro Ser Pro Lys Pro Arg Leu Pro Phe Ile Gly His Leu
35  40  45
His Leu Leu Asp Asn Pro Leu Leu His His Thr Leu Ile Lys Leu Gly
50  55  60
Lys Arg Tyr Gly Pro Leu Tyr Thr Leu Tyr Phe Gly Ser Met Pro Thr
65  70  75  80
Val Val Ala Ser Thr Pro Asp Leu Phe Lys Leu Phe Leu Gln Thr His
85  90  95
Glu Ala Thr Ser Phe Asn Thr Arg Phe Gln Thr Ser Ala Ile Ser Arg
100 105 110
Leu Thr Tyr Asp Asn Ser Val Ala Met Val Pro Phe Ala Pro Tyr Trp
115 120 125
Lys Phe Ile Arg Lys Leu Ile Met Asn Asp Leu Leu Asn Ala Thr Thr
130 135 140
Val Asn Lys Leu Arg Pro Leu Arg Ser Arg Glu Ile Leu Lys Val Leu
145 150 155 160
Lys Val Met Ala Asn Ser Ala Glu Thr Gln Gln Pro Leu Asp Val Thr
165 170 175
Glu Glu Leu Leu Lys Trp Thr Asn Ser Thr Ile Ser Thr Met Met Leu
180 185 190
Gly Gly Ala Glu Glu Val Arg Asp Ile Ala Arg Asp Val Leu Lys Ile
195 200 205
Phe Gly Glu Tyr Ser Val Thr Asn Phe Ile Trp Pro Leu Asn Lys Phe
210 215 220
Lys Phe Gly Asn Tyr Asp Lys Arg Thr Glu Ile Phe Asn Lys Tyr
225 230 235 240
Asp Pro Ile Ile Glu Val Ile Lys Lys Arg Gln Glu Ile Val Asn
245 250 255
Lys Arg Lys Asn Gly Glu Ile Val Glu Gly Glu Gln Asn Val Val Phe
260 265 270
Leu Asp Thr Leu Leu Glu Phe Ala Gln Asp Glu Thr Met Glu Ile Lys
275 280 285
Ile Thr Lys Glu Gln Ile Lys Gly Leu Val Val Asp Phe Phe Ser Ala
290 295 300
Gly Thr Asp Ser Thr Ala Val Ser Thr Glu Trp Thr Leu Ser Glu Leu
305 310 315 320
Ile Asn Asn Pro Arg Val Leu Lys Ala Arg Glu Glu Ile Asp Ser
325 330 335
Val Val Gly Lys Asp Arg Leu Val Asp Glu Ser Asp Val Gln Asn Leu
340 345 350
Pro Tyr Ile Lys Ala Ile Val Lys Glu Ala Phe Arg Leu His Pro Pro
355 360 365
Leu Pro Val Val Lys Arg Lys Cys Thr Gln Glu Cys Glu Ile Asp Gly
370 375 380
Tyr Val Val Pro Glu Gly Ala Leu Ile Leu Phe Asn Val Trp Ala Val
385 390 395 400
Gly Arg Asp Pro Lys Tyr Trp Val Lys Pro Leu Glu Phe Arg Pro Glu
405 410 415
Arg Phe Ile Glu Asn Val Gly Glu Gly Glu Ala Ala Ser Ile Asp Leu
420 425 430
Arg Gly Gln His Phe Thr Leu Leu Pro Phe Gly Ser Gly Arg Arg Met
435 440 445
Cys Pro Gly Val Asn Leu Ala Thr Ala Gly Met Ala Thr Met Ile Ala
450 455 460
Ser Ile Ile Gln Cys Phe Asp Leu Gln Val Pro Gly Gln His Gly Glu
465 470 475 480
Ile Leu Asn Gly Asp Tyr Ala Lys Val Ser Met Glu Glu Arg Pro Gly
485 490 495
Leu Thr Val Pro Arg Ala His Asn Leu Met Cys Val Pro Leu Ala Arg
500 505 510
Ala Gly Val Ala Asp Lys Leu Leu Ser Ser
515 520
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/53 C12N15/82 C12N15/11 C12N9/02 C12N5/10
A01H5/00 A01H5/10 A23L1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

Further documents are listed in the continuation of box C.

X Patent family members are listed in annex.

* Special categories of cited documents:

  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier document but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, usa, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search 27 July 2000

Date of mailing of the international search report 10/08/2000

Name and mailing address of the ISA
European Patent Office, P. B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk, Tel. (+31-70) 340-2040, Tx. 31 651 apoo nl, Fax: (+31-70) 340-3016

Authorized officer Kania, T

Form PCT/ISA/210 (second sheet) (July 1999)
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PAIVA N L ET AL: &quot;REGULATION OF ISOFLAVONOID METABOLISM IN ALFALFA&quot; PLANT CELL, TISSUE AND ORGAN CULTURE, XX, KLUWER ACADEMIC PUBLISHERS, vol. 38, no. 2/03, 1994, pages 213-220, XPO00571330 ISSN: 0167-6857 the whole document</td>
<td>1-104</td>
</tr>
<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim No.</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>P,X</td>
<td>WO 99 19493 A (UNIV NORTH CAROLINA ;DEWEY RALPH E (US); CORBIN FREDERICK T (US)); 22 April 1999 (1999-04-22) see esp. example 2; SEQ ID NO:9</td>
<td>74-77</td>
</tr>
<tr>
<td>P,X</td>
<td>STEELE CHRISTOPHER L ET AL: &quot;Molecular characterization of the enzyme catalyzing the aryl migration reaction of isoflavonoid biosynthesis in soybean.&quot; ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 367, no. 1, 1 July 1999 (1999-07-01), pages 146-150, XP000925681 ISSN: 0003-9861 the whole document</td>
<td>74-77</td>
</tr>
</tbody>
</table>
## INTERNATIONAL SEARCH REPORT

**Information on patent family members**

<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AU 699496 B</td>
<td>03-12-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AU 3972795 A</td>
<td>06-06-1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA 2204131 A</td>
<td>23-05-1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0792356 A</td>
<td>03-09-1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 10508750 T</td>
<td>02-09-1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NZ 296033 A</td>
<td>25-02-1999</td>
</tr>
<tr>
<td>WO 9919493 A</td>
<td>22-04-1999</td>
<td>AU 9680698 A</td>
<td>03-05-1999</td>
</tr>
</tbody>
</table>

Form PCT/ISA/210 (patent family annex) (July 1990)