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Review

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Manipulating Isoflavone Levels in Plants

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Abstract

Metabolic engineering for production of isoflavones in non-legume plants could distribute the health benefits of these phytoestrogens in more widely-consumed grains. Series of investigation to check the ability of the heterologous isoflavone synthase enzyme to interact with the endogenous phenylpropanoid pathway have been conducted. Overall, results provide possibility of production of isoflavonoids in several plant tissue systems including soybean and non-legumes. In tissue that undergoes naturally enhanced synthesis of anthocyanins, genistein production was enhanced. In a monocot cell system, introduced expression of a transcription factor regulating genes of the anthocyanin pathway was effective in conferring the ability to produce genistein in the presence of the isoflavone synthase gene. However, in this case the intermediate accumulated to high levels indicating an inefficiency in its conversion. Introduction of a third gene, chalcone reductase, provided the ability to synthesize an additional substrate of isoflavone synthase resulting in production of the isoflavone daidzein. These research efforts provide insight into requirements for metabolic engineering for isoflavone production in non-legume dicot and monocot tissues.

Introduction

Isoflavonoids represent a class of secondary metabolites produced in legumes by a branch of the phenylpropanoid pathway and include such compounds as isoflavones, isoflavanones, isoflavans, pterocarpans, rotenoids, quinone derivatives, coumestans, 3-aryl-4-hydroxycoumarins, 3-arylcoumarins, isoflav-3-enes, alpha-methyldexoybenzoins, 2-arylbenzofuarns, isoflavonol, and coumaronochromone. In plants, these compounds are known to be involved in interactions with other organisms and to participate in the defense responses of legumes against phytopathogenic microorganisms (Fader et al. 2000). Isoflavonoid-derived compounds also are involved in symbiotic relationships between roots and rhizobial bacteria which eventually result in nodulation and nitrogen-fixation (Van Rhijn and Vanderleyden 1995; Pueppke 1996), and overall they have been shown to act as antibiotics, repellents, attractants, and signal compounds (Rivera-Vargas et al. 1993; Graham 1998).

Isoflavonoids have also been reported to have physiological activity in animal and human studies. For example, it has been reported that the isoflavones found in soybean seeds possess antihemolytic, antifungal, estrogenic, tumor-suppressing, hypolipidemic, and serum cholesterol-lowering effects (Fader et al. 2000). These epidemiological studies indicate that isoflavones in soybean protein products, when taken as a dietary supplement, may produce many significant health benefits. Recently, the U.S. Food and Drug Administration has recently approved a health claim suggesting that soy protein included in a diet low in saturated fat and cholesterol may reduce the risk of coronary heart disease (http://www.fda.gov/). As a result, many food manufacturers are striving to provide products containing soy and/or isoflavones to consumers.

Free isoflavones rarely accumulate to high levels in soybeans. Instead they are usually conjugated to carbohydrates or organic acids. Soybean seeds contain three types of isoflavones: The aglycones, daidzein, genistein and glycitein; The glucosides, diadzin, genistin and glycitin; The malonylglucosides, 6′′-O-malonyldaizin, 6′′-O-malonylgenistin and 6′′-O-malonylglycitin. During the processing acetylglucoside forms are produced: 6′-O-acetyldaidzin, 6′-O-acetylgenistin, 6′-O-acetylglycitin. The content of isoflavones in soybean seeds is quite variable and is affected by both genetics and environmental conditions such as growing location and temperature during seed fill (Dixon et al. 1995). In addition, isoflavone content in legumes can be stress-induced by pathogenic attack, wounding, high UV light

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exposure and pollution (Dixon and Paiva 1995). The genistein isoflavonoid forms make up the most abundant group soybean seed and most food products, while daidzein and glycitein forms are present in lower levels (Murphy 1999).

The biosynthetic pathway for isoflavonoids in soybean and their relationship with several other classes of phenylpropanoids is presented in Figure 1. Many of the enzymes involved in the synthesis of isoflavonoids in legumes have been identified and many of the genes in the pathway have been cloned. These include three P450-dependent monooxygenases, cinnamate 4-hydroxylase isoflavone 2′-hydroxylase and dihydroxypterocarpan 6a-hydroxylase. The isoflavone synthase (IFS), which mediates the first step in the phenylpropanoid branch that commits metabolic intermediates to the synthesis of isoflavonoids, has been cloned using a soybean EST database mining. In this central reaction carrying by IFS, 2S-flavanone is converted into an isoflavone such as genistein and diadzein. The enzymatic reaction for this oxidative aryl migration step was first reported by Hagmann and Grisebach (Kochs and Grisebach 1986). The reaction involves a P450 monoxygenase-mediated conversion of the 2S-flavanone to a 2-hydroxyisoflavanone, followed by conversion to the isoflavonoid. This last step is possibly mediated by a soluble dehydratase. However, the 2-hydroxyisoflavanone intermediate was described as unstable and could convert directly to genistein.

Cytochrome P450-dependant monooxygenases comprise a large group of heme-containing enzymes, most of which catalyze NADPH- and O_2-dependant hydroxylation reactions. Most of these enzymes do not use NADPH directly, but rely upon an interaction with a flavoprotein known as a P450 reductase that transfers electrons from the cofactor to the P450. Cloning of plant P450s by traditional protein purification strategies has been difficult, as these membrane-bound proteins are often very unstable and are typically present in low abundance. PCR-based cloning strategies using sequence homologies between P450s has increased dramatically the number of P450 genes cloned. However, the *in vivo* activity of many of these cloned genes remains unknown and they are classified simply as P450s, and are grouped into families based solely on sequence homology (Chapple 1998). Proteins that are greater than 55% identical are designated as members of the same subfamily, while P450s that are 97% identical, or greater, are assumed to be allelic variants of the same gene (Chapple 1998). Efforts to determine *in vivo* activities of existing P450 clones are increasing. Most efforts involve expressing genes or cDNAs for P450s in yeast or insect cell systems, and then screening for a particular activity. For example, isoflavone 2′-hydroxylase (Akashi et al. 1998) and dihydroxypterocarpan 6a-hydroxylase (Schopfer et al. 1998) were identified in this manner.

The physiological activities associated with isoflavonoids in both plants and humans makes the manipulation of their contents in crop plants highly desirable, although there are controversial issues regarding effects and working modes of phytoestrogens in human body (Table 1). For example, increasing levels of isoflavonoid in soybean seeds would increase the effi-

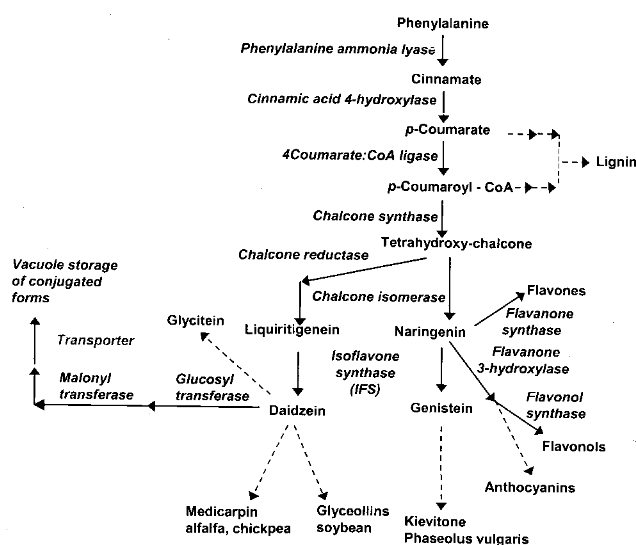

Figure 1. A partial diagram of phenylpropanoid pathway showing intermediate and enzymes involved in isoflavone synthesis as well as some branch pathways. Dotted arrows represent multiple steps. Enzymes are indicated in italics.

Table 1. A partial analysis of published issues regarding physiological aspects of isoflavones in human diet. Research publications related with two main subjects of the study, 'isoflavone' and 'breast cancer' were counted up to 44 in PubMed (www.ncbi.nlm.nih.gov) since 2000. Among them, the number of paper which showed positive (Pros), negative (Cons) or controversial effects of isoflavones on human health were counted. Some publications have no clear conclusions were discarded from this classification.

	Pros	Cons	Unclear or Controversial
Effect of isoflavone on breast cancer or cancer cell line	14	3	6
Effect of isoflavone on relieving postmanoposal symptoms	3	-	1
Result of isoflavone consumption of (estrogen dependent) breast cance patient while TAM therapy	-	1	-
Effect of isoflavone on mammary tumorigenesis	2	-	-
Role of isoflavone and isoflavone metabolites against breast and prostate cancers	1	1	-
Role of isoflavone in soy-based infant formula	-	2	2

ciency of extraction and lower the cost of isoflavone-related products sold today for use in either reduction of serum cholesterol or in estrogen replacement therapy. Decreasing levels of isoflavonoid in soybean seeds would be beneficial for production of soy-based infant formulas where the estrogenic effects of isoflavonoid are undesirable. Raising levels of isoflavonoid phytoalexins in vegetative plant tissue could increase plant defenses to pathogen attack, thereby improving plant disease resistance and lowering pesticide use rates. Manipulation of isoflavonoid levels in roots could lead to improved nodulation and increased efficiencies of nitrogen fixation. To date, however, it has proven difficult to develop soybean or other plant lines with consistently high levels of isoflavonoid. Because isoflavone synthase is the central reaction in pathways producing isoflavonoids, identification of this functional gene is extremely important, and its manipulation via molecular techniques is expected to allow production of soybeans and other plants with high, stable levels of isoflavonoid. Introduction of the isoflavone synthase gene in non-legume crop species including, but not limited to, corn, wheat, rice, sunflower, and canola could lead to synthesis of isoflavonoids. The expression of isoflavonoids would confer to these species disease resistance and/or properties which produce human/livestock health benefits.

Substrates for isoflavone synthase may be limiting for synthesizing very high levels of isoflavonoids in soybean, or for synthesizing isoflavonoids in non-legumes. It is desirable to increase the flux of metabolites through the phenylpropanoid pathway to provide additional amounts of substrate to those occurring naturally. Different stress conditions such as UV irradiation, phosphate starvation, and prolonged exposure to cold can cause activation of the phenylpropanoid pathway. While these treatments may produce the desired substrate availability, it is more desirable to have a genetic means of activating the phenylpropanoid pathway. It is known that expression of genes encoding certain transcription factors can regulate the expression of various genes that encode enzymes of the phenylpropanoid pathway. These include, but are not limited to, the C1 myb-type transcription factor of maize and the AmMyb305 of Antirrhinum majus. The C1 myb-type transcription factor of maize, in conjunction with the myc-type transcription factor R, activates chalcone synthase and chalcone isomerase genes (Grotewold et al. 1998). The Antirrhinum majus AmMyb305 activates the phenylalanine ammonia lyase promoter (Sablowski et al. 1814). Transcription factors such as these may be expressed in host plant cells to activate expression of genes in the phenylpropanoid pathway thereby increasing the encoded enzyme activities and the flux of compounds through the pathway. Increases in the precursors to substrates of isoflavone synthase would enhance the production of isoflavonoids.

Alteration of isoflavone production in soybean maturing seed model system

The ability to alter the isoflavonoid levels in transgenic soybean plants expressing the gene from soybean IFS clone was tested by transforming somatic embryo cultures with a vector containing the gene, allowing the plant to regenerate, and measuring the levels of isoflavonoids produced.

Construction of Vectors for Transformation of Glycine max

A vector containing a chimeric isoflavone synthase gene was constructed as follows. The 1.6 Kb isoflavone synthase coding region was amplified using a standard PCR reaction in a GeneAmp PCR System using Pfu polymerase (Stratagene). Consequently, the 3.2 Kb fragment containing the beta-conglycinin/P-IFS-phaseolin 3′ chimeric gene was ligated with a vector which has 35S-HPT gene. The resulting plasmid was named pWSJ001, has also a T7promoter/HPT/T7terminator cassette for expression of the HPT enzyme in certain strains of *E.coli* that are lysogenic for lambda DE3. The lambda DE3 carries the T7 RNA Polymerase gene under *lac*V5 control and is found in commercially available *E.coli* strains such as NovaBlue (DE3) (from Novagen). Plasmid pWSJ001 contains the 35S/HPT/*NOS* 3′cassette for constitutive expression of the HPT enzyme in plants. These two expression systems allow selection for growth in the presence of hygromycin to be used as a means of identifying cells that contain plasmid DNA sequences in both bacterial and plant systems.

Soybean transformation and analysis of transgenic soybean somatic embryos[1)]

Soybean embryogenic suspension cultures obtained from soybean cultivar Jack were transformed with pWSJ001 by the method of particle gun bombardment. The media and culture system used for the soybean somatic embryo cultures described in previous reports (Fader et al. 2000).

Seed from IFS-transformed soybean plants are analyzed for quantification of isoflavonoid levels. Extracts are prepared and analyzed by HPLC as described in previous reports.

The result of individual somatic embryo analysis of 14 transgenic soybean somatic embryo line was presented in Figure 2. Over the 3 times difference in total content of isoflavonoids observed between the transgenic soybean somatic embryo

[1)]Published in Fader et al. (2000) WIPO Publication WO0044909

lines showed lowest and highest level of isoflavones. This expand in terms of the ability to produce isoflavonoids in transgenic somatic embryos which is a model system of maturing seed could represent the potential availabilities in the use of IFS gene. This result presented a possibility of manipulation of isoflavonoids production in soybean seeds by the single gene (IFS) transformation.

Expression of Soy IFS in transgenic *Arabidopsis*[2)]

Neither Southern blot analysis nor the analysis of the *Arabidopsis* ESTs and genomic sequence database reveals a gene with high homology to IFS. The most closely related gene is cyp93d1, which has neither reported expression nor activity as noted above and has less then 40% sequence identity to IFS. Though isoflavones are not synthesized in *Arabidopsis*, the naringenin substrate of IFS is an intermediate in the anthocyanin biosynthetic pathway that is present in *Arabidopsis* (Figure 1). Naringenin may be accessible to an introduced foreign IFS enzyme, creating the possibility of synthesizing the isoflavone genistein in *Arabidopsis*.

To test the function of the soybean IFS gene in a non-legume plant, a chimeric gene including the 35S promoter of CaMV, the soybean IFS1 coding region and the *Nos* 3′ was introduced into *Arabidopsis*. Extracts of kanamycin-selected transformants were assayed by HPLC to look for the presence of genistein. A peak corresponding to the position of the genistein standard was detected in samples from five independent primary transformants, while no corresponding peak was detected in samples from control plants (Figure 3). The identity of the peak in the transformant extracts was verified by GC/MS to be the isoflavone genistein (data not shown). These results indicate that the soybean IFS gene expresses an enzyme that is active in *Arabidopsis*, and that the naringenin intermediate of the anthocyanin pathway is available as a substrate for the introduced foreign enzyme. Naringenin was not detected in the control plants or in IFS transformants (data not shown), suggesting that IFS is competing with other enzymes of the phenylpropanoid pathway for a limited or transient amount of this intermediate. The amount of genistein produced is approximately two ng per mg of fresh weight, as determined by comparison with a quantitated genistein standard.

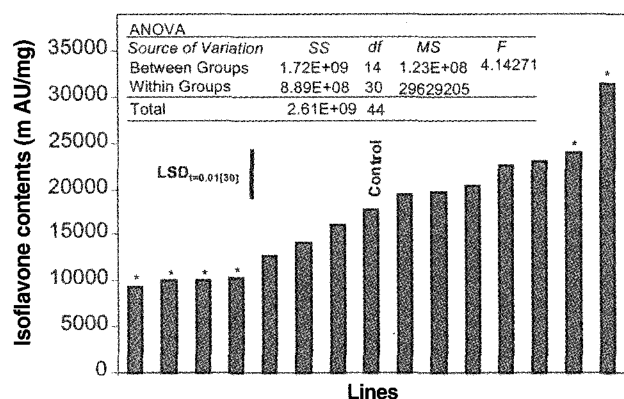

Figure 2. Fifteen soybean somatic embryo lines were analyzed by HPLC for levels of isoflavones. The graph shows the distribution of total isoflavone levels (areas of peaks representing all different forms of the isoflavones are summed) in 14 transgenic lines and a control lines and presented on a dry weight basis. The bars represent the mean of 3 samples for each line. The asterisks mark the lines with mean isoflavone concentrations that are significantly greater or less than the control based on the LSD test at P<0.01. The highest level of isoflavones is about 1.7 fold higher than the controls. Lines accumulating lower level of isoflavones are presumably co-suppression events.

Production of genistein in monocot BMS cells requires induction of the phenylpropanoid pathway by C1 and R transcription factors[3)]

We used the maize BMS cell system to determine whether

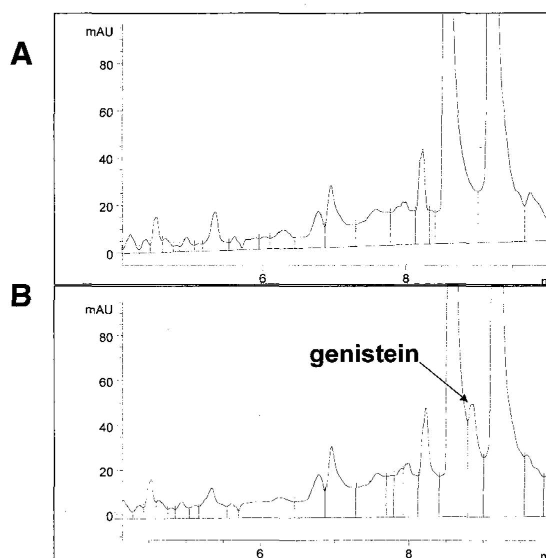

Figure 3. Synthesis of isoflavone genistein in non-legume dicot plant. Transgenic *Arabidopsis* plant which have 35S::IFS2 produce genistein.

[2)]Published in Jung et al. (2000) Nature Biotech 18: 208-212
[3)]Published in Yu et al. (2000) Plant Physiol 124: 781-7

isoflavones could be synthesized and accumulated in monocot cells. To enhance expression, the 35S/P-IFS gene construction prepared for monocot transformation additionally includes the intron 6 from the maize Adh1 gene (Mascarenhas et al. 1990), and the entire chimeric gene is bounded by SARs derived from the chicken lysozyme locus (Stief et al. 1989). Bombardment of BMS suspension culture cells with this construction, and an additional plasmid containing a 35S/P-bar gene, produced cell lines resistance to bialaphos. The presence of the IFS transgene was confirmed in 25 independent lines by PCR, and expression was confirmed in six randomly selected lines by RT-PCR (data not shown). All twenty-five independent lines were assayed by HPLC for production of genistein. In none of them could a peak with the properties of genistein be identified (Table 2).

BMS culture cells are white, and it is known that genes encoding enzymes required for the synthesis of anthocyanin are not active in these cells (Grotewold et al. 1998). A chimeric transcription factorcontaining maize C1 and R coding regions, called CRC, is able to activate expression of genes in BMS cells leading to the synthesis and accumulation of anthocyanin (Bruce et al. 2000), which can be visually detected by a reddish color. Since the naringenin intermediate for anthocyanin synthesis is the substrate for IFS, we tested the effect of CRC expression on making this substrate available for genistein synthesis.

Transformation of BMS cells with a Nos/P-CRC gene alone or in combination with the IFS gene described above produced stably transformed lines of both reddish and white color, with about a 1:1 ratio, most likely due to variation among the lines in CRC gene expression level. Transformation with a 35S/P-CRC gene, which was introduced in combination with IFS, produced red lines at a rate of 95%. All lines selected for further analysis containedeither the CRC gene alone, or the CRC and IFS genes that were introduced in combination, as determined by PCR analysis. Extracts were prepared from individual BMS cell lines and analyzed by HPLC to detect genistein.

Lines with CRC alone that were reddish in color, indicating accumulation of anthocyanin, did not produce genistein (Table 2). Lines that received both IFS and CRC, but were white, also produced no genistein. In all 23 lines tested that received both IFS and CRC and showed anthocyanin accumulation, genistein was produced as shown by the characteristic peak present in the HPLC profile (Figure 4B). The peak was confirmed as genistein by the identifying 414 and 399 m/z GC-MS profile. Thus the synthesis of genistein in BMS lines is strictly correlated with the red color that is induced by CRC expression, and the presence of the IFS gene.

We attribute the production of genistein in every red CRC line that harbors the IFS gene to effects of the A element SAR on expression of the IFS gene in BMS cells. We previously found that including this SAR greatly enhanced both the number of expressing lines, and the level of expression (Odell and Luckring, unpublished). This experiment shows that the soybean IFS coding region is expressed and produces a functional enzyme that is active in monocot cells, producing genistein when its substrate is present. The substrate is not efficiently used by IFS, however, since a large peak in the HPLC profile corresponding to naringenin is present in the red CRC lines, whether or not IFS is expressed (Figure 4A, B).

Table 2. Summary of BMS line genotypes with corresponding phenotypes. Each line is derived from an independent transformation event. Presence of genistein and naringenin were determined by HPLC analysis.

No. of lines	Genotype	Tissue Color	Genistein	Naringenin
5	control	White	NO	NO
25	IFS	White	NO	NO
6	IFS+*Nos*/P-CRC	White	NO	NO
6	*Nos*/P-CRC	Red	NO	YES
7	IFS+*Nos*/P-CRC	Red	YES	YES
16	IFS+35S/P-CRC	Red	YES	YES

Production of daidzein in BMS cells by co-transformation of CRC, IFS and CHR[4)]

A second substrate of IFS, liquiritigenin, is produced through

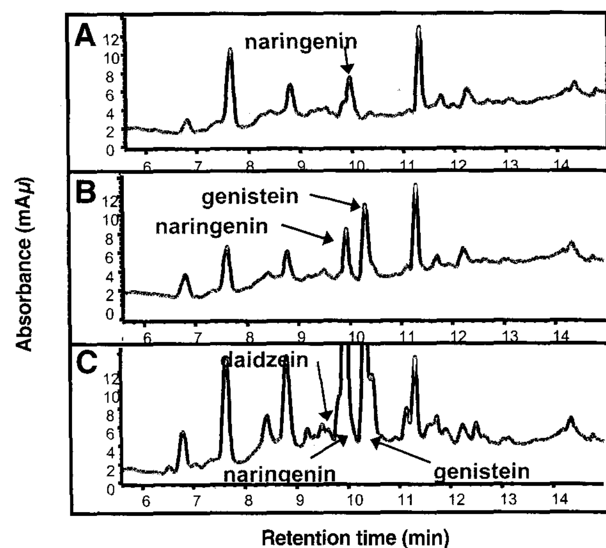

Figure 4. Production of isoflavone genistein and daidzein in transformed BMS corn cell lines. HPLC chromatograms at 260 nm of extracts from BMS cell lines transformed with A, CRC; B, CRC and IFS; C, CRC, IFS, and CHR. Peaks corresponding to retention times and spectra of naringenin, genistein, and daidzein are labeled.

[4)]Published in Yu et al. (2000) Plant Physiol 124: 781-7

the activity of chalcone reductase, thereby allowing the synthesis of the isoflavone daidzein (Figure 1). Chalcone reductase activity is present in legumes, but has not been reported in most non-legume plants including *Arabidopsis*, tobacco, and corn. We tested whether expression of CHR in cells engineered to produce genistein would allow the synthesis of daidzein, in addition to genistein. A soybean CHR cDNA clone was identified from the Dupont EST sequencing program by homology to known chalcone reductase sequences of soybean and alfalfa (Welle et al. 1991; Ballance and Dixon 1995). The identified cDNA encodes a protein with 99.7% amino acid identity to the soybean p10 CHR (Swissprot accession number P26690; Welle et al. 1991). The coding region was amplified using PCR and used to construct a 35S/P-CHR-*Nos* 3′chimeric gene. This CHR gene was co-bombarded into BMS cells with the IFS, CRC, and selection marker genes and bialaphos resistant lines selected. Thirty-two transformed lines were screened by PCR for the presence of all three trait genes. From the six lines shown to contain the CHR, IFS and CRC genes and a control line carrying only the IFS and CRC genes, extracts were prepared, hydrolyzed, and analyzed by HPLC and GC-MS. In one line the HPLC profile showed a peak with the retention time of the daidzein standard, that was not present in the control (Figure 4C). The GC-MS assay showed the diagnostic ions of derivatized daidzein: m/z: 398, 383, 218, 97, confirming that daidzein was produced in this cell line (data not shown).

Prospects for isoflavone metabolic engineering

We observed that through introducing the isoflavone synthase enzyme activity, both monocot and dicot plant tissues that do not naturally produce isoflavones can acquire the potential to synthesize this compound. However, since the availability of the substrate for isoflavone synthase relies on the activity level of a branch of the phenylpropanoid pathway (Figure 1), isoflavones are produced only in specific tissues or under certain environmental conditions where this branch pathway is active. Induced expression of the relevant pathway using transcription factors that activate a set of pathway genes was shown to be a viable strategy for providing the required substrate for isoflavone synthesis. Further investigation for the regulation mechanism of isoflavonoids synthesis will provide new possibilities to develop diverse soybean lines in term of their ability to produce isoflavones and this allows more chance to choose to soy based manufacturers.

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