

# Regulation of Isoflavonoid Biosynthesis in Soybean Seeds

Sangeeta Dhaubhadel

*Southern Crop Protection and Food Research Center,  
Agriculture and Agri-Food Canada, London, Ontario, N5V 4T3  
Canada*

## 1. Introduction

Isoflavonoids are biologically active plant natural products synthesized via general phenylpropanoid pathway. They accumulate predominantly in plant species belonging to family *Leguminosae*. Isoflavonoids play numerous roles in the interaction between plants and environment, where they act as inducers of nodulation genes during symbiosis between legumes and *Rhizobium* bacteria (Ferguson & Mathesius 2003, Phillip 1992) and also function as precursor molecules for the production of phytoalexins during plant-microbe or plant-insect interactions (Aoki et al. 2000, Dixon 1999, Dixon & Ferreria 2002). Many epidemiological and clinical trials have suggested a positive role for isoflavonoids in human health and nutrition (Aerenhouts et al. 2010, Cederroth & Nef 2009). The core isoflavones have structural similarity to beta-estradiol (Fig. 1) and possess affinity for oestrogen receptors (Molteni et al. 1995). Due to their structural resemblance with beta-estradiol, isoflavonoids have been associated with chemo-preventive activities against hormone dependent cancers such as breast cancer and post-menopausal ailments (Dixon 2004, Limer & Speirs 2004). Research data over the past decade suggests that the dietary intake of isoflavonoids may also be associated with many additional health benefits such as reduction of risk of cardiovascular diseases, osteoporosis, loss of bone mass intensity (reviewed in Messina 1999, Rochfort & Panozzo 2007, Zhang & Yu 2009). Due to these pharmaceutical and nutraceutical properties associated with isoflavonoids and their use in functional foods, there is a growing interest in these compounds and the plants that produce them.

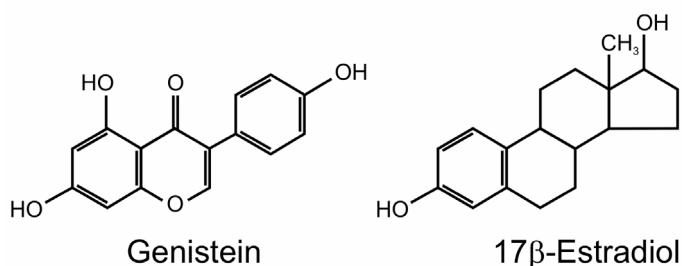


Fig. 1. Structure of isoflavone genistein and 17β-Estradiol.

Soybean (*Glycine max* [L.] Merr.) seeds contain large amount of isoflavonoids and are primary source of these compounds in human diet. Since isoflavonoids are phytoestrogens, the presence of these compounds in some foods such as soy-based formula for infants is controversial (Chen & Rogan 2004, Setchell 2001). Despite its continuous use for more than four decades, no clear correlation between soy isoflavones and negative effects in infant health has been reported. Nevertheless, seed isoflavonoid content has gained considerable attention in soybean breeding programmes for developing soybean cultivars with both high and low isoflavonoid levels that satisfies consumer's needs.

The amount of isoflavonoids present in soybean seed is a complex trait that is highly variable and is determined by multiple genetic and environmental factors that are largely unknown (Gutierrez-Gonzalez et al. 2010, Hoeck et al. 2000). This complexity of mechanism of isoflavonoid accumulation in soybean seeds poses a challenge to breeders for cultivar development. Thus understanding the mechanism of regulation of isoflavonoid synthesis and their accumulation in soybean, identifying the genetic factors involved in the regulation, dissecting the mechanism of how those factors interact with each other and with the environmental components are critical.

## 2. Biosynthesis of isoflavonoids in soybean

Isoflavonoids are synthesized by a legume specific branch of general phenylpropanoid pathway. Other branches of phenylpropanoid pathway are common to all plant species and produce lignin, proanthocyanidins, anthocyanin, phlobaphenes (Fig. 2). Chalcone is the first step in the production of flavonoids and isoflavonoids (Hahlbrock & Scheel 1989) which requires an enzymatic reaction catalyzed by the enzyme chalcone synthase (CHS), a plant specific polyketide synthase (Schröder 2000). Soybean contains nine *CHS* genes (*CHS1-CHS9*) among which *CHS7* and *CHS8* are critical for isoflavonoid biosynthesis (Dhaubhadel et al. 2007). Legume plants produce two kinds of chalcones, tetrahydroxy chalcones (naringenin chalcone) and trihydroxy chalcone (isoliquiritigenin chalcone) whereas non-legume plants only produce tetrahydroxy chalcones (naringenin chalcone). The production of isoliquiritigenin in legumes is a result of additional enzymatic reaction catalyzed by a legume specific enzyme chalcone reductase (CHR). Both naringenin and isoliquiritigenin chalcones then get converted into their corresponding flavanones by chalcone isomerase (CHI). Soybean *CHI* gene family consists of seven members that are grouped into four subfamilies: *CHI1*, *CHI2*, *CHI3*, and *CHI4* (Ralston et al. 2005). These genes are further categorized into different types based on their catalytic abilities where type I *CHIs* (such as *CHI2*) is common to all higher plants, convert naringenin chalcone to naringenin, and type II *CHIs* (such as *CHI1*) are almost entirely exclusive to legumes, possess additional capacity to catalyze the conversion of isoliquiritigenin to liquiritigenin (Ralston et al. 2005, Shimada et al. 2003).

The branch point enzyme that introduces isoflavonoid specific branch in the phenylpropanoid pathway is 2-hydroxyisoflavanone synthase (isoflavone synthase, IFS). IFS is a microsomal cytochrome P450 monooxygenase enzyme that catalyzes a 2, 3 aryl ring migration of flavanones to their corresponding flavones and subsequent hydroxylation of the resulting C-2 radical. This reaction also requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen molecule and yields 2-hydroxyisoflavanone. The reaction product of IFS, 2-hydroxyisoflavanone, is extremely unstable and undergoes dehydration by forming a double bond between C-2 and C-3 by a dehydratase to form

genestein or daidzein (Hakamatsuka et al. 1998). Two *IFS* genes, *IFS1* and *IFS2*, have been identified in soybean (Jung et al. 2000, Steele et al. 1999) about a decade ago by two research groups independently that brought a major break through in the attempts to metabolically engineer isoflavonoids in nonlegumes. These two IFSs differ from one another by 14 amino acid residues; however, both *IFS1* and *IFS2* convert the naringenin and liquiritigenin flavanones to their corresponding isoflavones (Dhaubhadel et al. 2003, Jung et al. 2000).

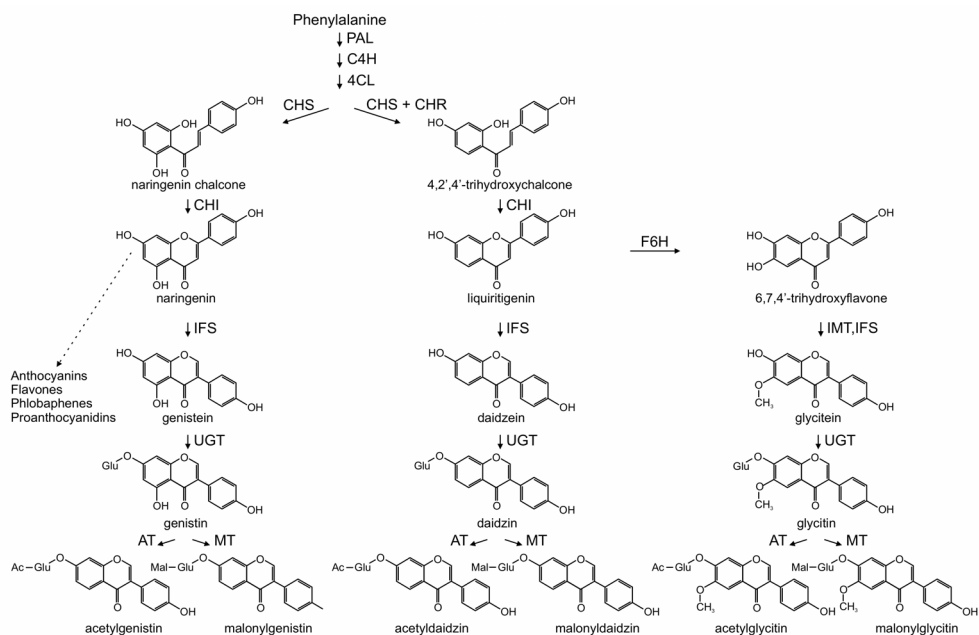


Fig. 2. Soybean seed isoflavonoid biosynthesis pathway. Dotted arrow indicates multiple steps involved in the synthesis of other phenylpropanoids. PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate-CoA-ligase; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; F6H, flavonone-6-hydroxylase; UGT, glycosyl-transferase; IFS, 2-hydroxyisoflavanone synthase; IMT, isoflavone methyl-transferase; MT, malonyl-transferase; AT, acetyl-transferase; (modified from Dhaubhadel *et al.*, 2003).

Soybean seeds contain nine different isoflavonoids with three core isoflavone aglycones (daidzein, genistein and glycitein). The aglycones are the most bioactive forms of isoflavonoids. Each of these aglycones may also be present as their corresponding 7-O-glycosides (daidzin, genistin and glycitin) and malonyl glycosides (6''-O-malonyldaidzin, 6''-O-malonylgenistin and 6''-O-malonylglycitin) (Kudou et al. 1991) (Fig. 2). The malonyl glycoside form of isoflavonoids are thermally unstable and get converted into acetylglycosides (6''-O-acetyldaidzin, 6''-O-acetylgenistin and 6''-O-acetylglycitin) forms during processing of soybeans and soy foods (Griffith & Collison 2001). The conjugation of glycosyl- and malonyl-groups to the isoflavone aglycones is catalyzed by uridine diphosphate glycosyltransferase (UGT) and malonyltransferase (MT), respectively. The

addition of glycosyl- and malonyl groups to the aglycones confers the metabolite with the increased water solubility and reduced chemical reactivity. The conjugation process also increases the *in vivo* stability of the metabolite and alters their biological activity (Jones & Vogt 2001). In soybean, the UGT73F2 and GmMT7/GmIF7MaT are isoflavonoid specific UGT and MT that are involved in glycosylation and malonylation of soybean isoflavones, respectively (Dhaubhadel et al. 2008, Suzuki et al. 2007) providing stability and solubility to aglycone molecules and possibly helping in their compartmentalization to central vacuole of the cell or transport to the site of accumulation. Even though it has been found that malonyl derivatives of isoflavonoids are highly unstable, the majority of soybean seed isoflavonoids accumulate in the form of malonyl- conjugates followed by glycosyl- conjugates while the amount of daidzin, genistin and glycitein is negligible (Fig. 3). It is not known what stabilizes the malonyl derivatives of isoflavonoids *in planta*.

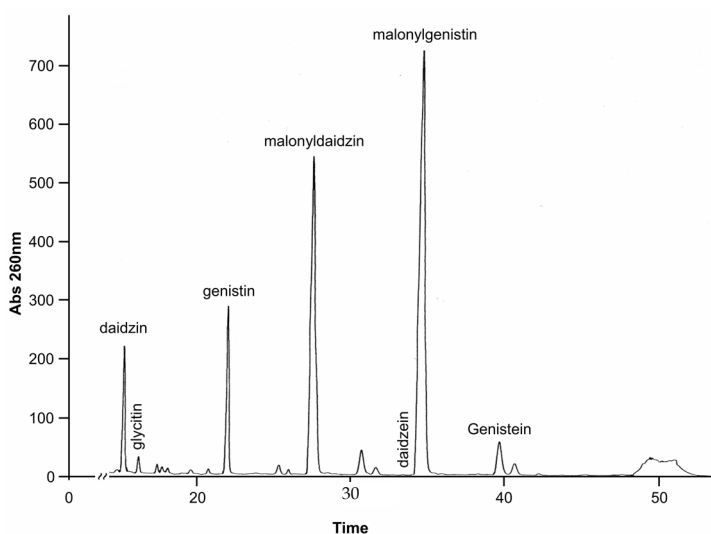


Fig. 3. Chromatogram showing seed isoflavonoid accumulation profiles analysed by HPLC. Isoflavonoids were extracted from mature soybean seeds in 50% acetonitrile and separated using HPLC.

### 3. Metabolic engineering of isoflavonoids

Due to health related benefits associated with the dietary intake of isoflavonoids such as prevention against various hormone-dependent cancers, (Dixon & Ferreria 2002), there is considerable interest in engineering non-legume plants such as tomato, broccoli or lettuce to introduce isoflavonoid phytoestrogen or to generate soybean plants with altered isoflavonoid content. Metabolic engineering of isoflavones in non-legume plants possessing active flavonoid branch of phenylpropanoid pathway has been conducted successfully (Yu et al. 2000). However, attempts in these lines have produced very low concentration of isoflavonoids (Jung et al. 2000, Yu et al. 2000). Since IFS is the entry point enzyme into isoflavonoid biosynthesis and is not present in nonlegume plants, these earlier efforts for metabolic engineering of isoflavonoids were focussed on introducing soybean IFS into corn,

tobacco and *Arabidopsis thaliana* (Jung et al. 2000, Liu et al. 2002, Yu et al. 2000). Introduction of soybean *IFS* gene into *Arabidopsis* could yield low levels of genistein glycosides (Liu et al. 2002). However, this constitutive production of isoflavonoids in *Arabidopsis* resulted into competition for flavanone between endogenous flavonol synthesis and IFS, affecting the flavonol pathway disproportionately. Since naringenin is the substrate for both IFS and flavonoid 3'-hydroxylase (F3'H), the limitation for isoflavonoid synthesis in *Arabidopsis* is at the level of naringenin where partitioning of flux between isoflavonoid and flavonoid biosynthesis pathway occurs. This conclusion was supported by the results where high levels of genistein conjugates were obtained by expressing soybean *IFS* in *Arabidopsis* mutant line *tt3/tt6* that lacks F3'H and flavonol production (Liu et al. 2002). Interestingly, as in soybean, the genistein produced in *Arabidopsis* were conjugated. The conjugated sugars were same as that are conjugated to kaempferol and quercetin but with different position specificity suggesting that conjugation is highly critical for isoflavonoid accumulation. It is possible that engineered isoflavone aglycones may perform as poor substrates for endogenous UGTs and MTs of the host plant, and ultimately get turned over resulting into its reduced accumulation (Liu et al. 2002). Therefore, it is crucially important to confirm the presence of UGTs and MTs that are functionally similar to isoflavonoid specific UGT and MT before plants are selected for metabolic engineering of these metabolites.

The recent efforts on genetic engineering of isoflavonoids have been geared towards introducing and/ or modifying the expression levels of IFS together with other structural genes involved in isoflavonoid biosynthetic pathway (Deavours & Dixon 2005, Lozovaya et al. 2007, Sreevidya et al. 2006, Subramanian et al. 2005) or introducing foreign transcription factors to up-regulate the synthesis of the metabolites (Yu & McGonigle 2005, Yu et al. 2003). One of the *in planta* functions of isoflavones is to serve as signal molecules for rhizobia to establish symbiotic association with legumes for the formation of nitrogen-fixing root nodules (Pueppke 1996, Spaink 2000). There is also a great deal of interest in exploiting this property of isoflavones and transferring the symbiotic nitrogen fixing ability to other crops of economical importance such as rice (Ladha & Reddy 2003, Reddy et al. 2002). Introduction of isoflavonoids in high value crops that normally do not produce these metabolites will reduce the cost of chemical nitrogen fertiliser input as well as may enhance their nutritional value. Introduction of soybean *IFS* gene was sufficient to produce genistein conjugates in rice (Sreevidya et al. 2006). As observed in *Arabidopsis*, tobacco and maize BMS cell line (Liu et al. 2002, Yu et al. 2000), the endogenous UGTs were able to glycosylate genistein in transgenic rice expressing soybean *IFS* gene (*35S-IFS*). Analysis of *35S-IFS* transgenic rice plants was performed for its ability to enhance *nod* gene expression in rhizobia. The results showed that both leaf and root extracts from transgenic *35S-IFS* lines stimulated *nod* gene expression in *Bradyrhizobium japonicum* USDA110 (Sreevidya et al. 2006). It will be of utmost interest if the transgenic *35S-IFS* lines could form nodules that participate in nitrogen fixation. Isoflavonoids also function as phytoalexins in plants. These are defence related compounds produced by the plant upon pathogen infection. Engineering the isoflavonoid pathway in non-legume plants or boosting the production of phytoalexins in legumes may make plants more resistant to pathogens and also reduce the use of chemical pesticide/fungicides. A direct role of isoflavonoids in disease resistance was demonstrated by RNAi silencing of *IFS* expression in soybean which resulted into reduction of the level of isoflavonoid accumulation leading to drastic increase in disease symptoms upon *Phytophthora sojae* infection (Subramanian et al. 2005). The results suggested that increased levels of isoflavonoids may enhance plant innate defense system.

Several metabolic engineering attempts are made to modify the level of isoflavonoids in soybean seeds for the enhancement of the nutritional value of the seed. Expression of several phenylpropanid genes such as *PAL*, *CHS*, *CHI* and *IFS* was performed in different combinations that resulted in change in the isoflavonoid levels in soybean seeds (Yu et al. 2003, Zernova et al. 2009). However, the most significant level of increase in isoflavonoid in seeds was achieved by introducing the maize C1 and R transcription factors in soybean (Yu et al. 2003). Isolated from maize, C1 is an R2R3 MYB transcription factor that requires an R MYC co-factor for its function in anthocyanin production (Grotewold et al. 1998). A chimeric protein CRC was produced, in which R was inserted between the DNA binding and activation domains of C1, and introduced into soybean. The transgenic soybean lines overexpressing chimeric CRC produced significantly higher levels (up to four times) of isoflavonoids than that of wild-type soybean seeds. The increase in isoflavonoid levels in transgenic soybean CRC lines was achieved when the complete flow of substrate was diverted to the isoflavonoid pathway by blocking the expression of flavonoid biosynthetic genes (Yu et al. 2003). Realising the complexity of phenylpropanoid pathway, a better understanding of complete biosynthetic pathway and the associated branch pathways, their regulation and cross connection with branch pathways is crucial for metabolic engineering of isoflavonoids.

#### 4. Regulation of isoflavonoid biosynthesis in soybean seeds

Since soybean seed is the main source of isoflavonoids for human consumption, much research is focussed on understanding the biosynthesis and accumulation of these compounds in seeds. As shown in Fig. 4, the accumulation of isoflavonoids in soybean embryos increases as seeds approach towards maturity [50-70 days after pollination (DAP)]. After 70 days of pollination, soybean seed slowly starts losing water, shrinks in size and goes into dormant condition. The level of seed isoflavonoids reaches to maximum level in mature dry seeds (Dhaubhadel et al. 2003). The measurements of accumulation of isoflavonoids in soybean seeds (Fig. 4) were performed by harvesting seeds from field

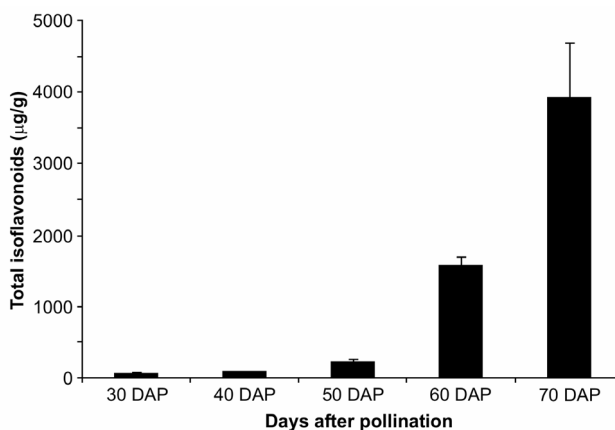


Fig. 4. Total isoflavonoid content in developing soybean embryos measured by HPLC. Data are mean values from three independent experiments. DAP- days after pollination

grown soybean plants at various stages of seed development and separating the embryos from seed coat for isoflavonoids extraction. The embryo extracts were hydrolysed to convert malonyl-derivatives to their corresponding glycosides and levels of six different isoflavonoids (daidzein, glycitein, genistein, daidzin, glycitin and genistin) were measured by high-performance liquid chromatography (HPLC).

For the detail analysis of seed isoflavonoid content, soybean cv Harosoy63 was grown under controlled condition. Flowers were tagged on the first day of pollination and seeds harvested every day starting from 40 DAP to 75 DAP for isoflavonoid measurement by HPLC. The results suggested that glycitein and its derivative level remained constant from early to late seed maturity in soybean. However, both daidzein and genistein and their conjugates accumulation increased during seed development suggesting that it is the synthesis and accumulation of daidzein and genistein and their conjugates that contribute to the increased accumulation of isoflavonoids in seeds during the later stages of seed development (Fig 5).

It has been demonstrated in the past that many plant natural products are often transported from the site of synthesis to the site of accumulation. Examples include nicotine in tobacco which is biosynthesized in roots, then translocated to leaves and finally accumulated in central vacuole of leaves by a novel multidrug and toxic compound extrusion (MATE)-type transporter, Nt-JAT1 (Hashimoto & Yamada 2003, Shitan et al. 2009, Shoji et al. 2000). Similarly glucosinolates are synthesized in leaves and translocated to seeds during development in *Tropaeolus majus* (Lykkesfeldt & Moller 1993). A carrier mediated system has been proposed for accumulation of glucosinolates in developing rapeseed embryos (Chen & Halkier 2000, Gijzen et al. 1989). To investigate if similar pattern of transport and accumulation occurs in soybean seed isoflavonoids, the ability of soybean embryos to synthesize isoflavonoids were assessed and maternal effect on seed isoflavonoids was determined (Dhaubhadel et al. 2003). The precursor feeding [<sup>14</sup>C Phe] experiment demonstrated that together with leaf and pod tissues, soybean embryos were also able to incorporate radiolabelled Phe into isoflavonoids demonstrating the ability of embryos to synthesize these compounds within the tissue. In addition, the *in vitro* uptake study and determination of maternal effect on seed isoflavonoid content revealed that there may be involvement of transport mechanism in the accumulation of isoflavonoids in soybean seeds. A significantly large amount of isoflavonoids were also found in leaf and stem tissues which may ultimately get channelled into the sink tissues such as seeds. The study demonstrated that soybean embryos have an ability to synthesize isoflavonoids, however, isoflavonoids synthesized in maternal tissues may contribute to the total seed isoflavonoid accumulation at maturity (Dhaubhadel et al. 2003).

As discussed previously, the majority of seed isoflavonoids are accumulated in conjugated form (Fig. 3). The soybean seed isoflavone aglycones are predominantly present in their malonylglycoside form followed by glycoside derivatives. These conjugation processes are catalyzed by MT and UGT, respectively. Involvement of UGT and MT in isoflavonoid biosynthesis was demonstrated previously, however, the genes encoding these enzymes were identified only recently. Two independent studies, using functional genomics approach and homology based strategy, identified *GmMT7/GmIF7MaT* as isoflavonoid specific malonyltransferase (Dhaubhadel et al. 2008, Suzuki et al. 2007). Heterologously produced *GmMT7/GmIF7MaT* was able to transfer malonyl group to isoflavone glycosides.

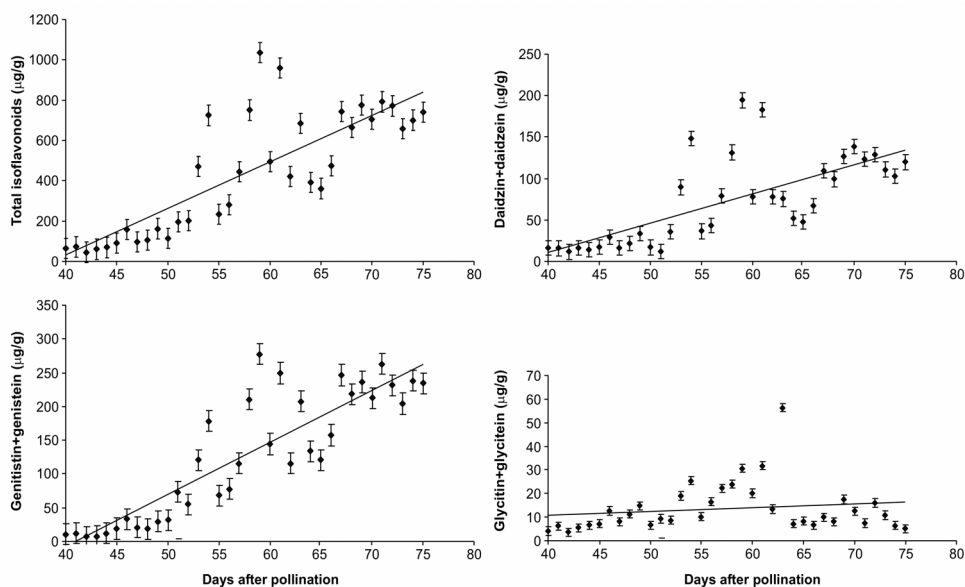


Fig. 5. Detail accumulation pattern of isoflavonoids in soybean seeds measured by HPLC. Data are mean values from two independent experiments. Numbers indicate days after pollination.

The recombinant GmMT7 was used to produce radiolabelled [ $^{14}\text{C}$ ] malonylgenistin and malonyldaidzin which were subsequently used in the feeding experiment to investigate if externally supplied isoflavonoids can accumulate in embryos or not. The results obtained were similar to when [ $^{14}\text{C}$ ] labelled Phe, a precursor for isoflavone synthesis, was used. As shown in Fig. 6, a soybean branch containing developing pods and leaves were taken and [ $^{14}\text{C}$ ] labelled Phe was fed through the stem for 24 h at 24°C followed by autoradiography. Incorporation of precursors to isoflavonoids was induced by cold treatment of the soybean plant at 4°C for 3 days before feeding radiolabelled isoflavonoids or precursors. The results suggested that both [ $^{14}\text{C}$ ] labelled Phe and [ $^{14}\text{C}$ ] labelled isoflavonoids were transported to mature leaves and embryos. The HPLC analysis was performed to confirm the incorporation of [ $^{14}\text{C}$ ] Phe into isoflavonoids (data not shown). The  $^{14}\text{C}$  signal in embryos was only detected in opened pods (Fig. 6A) but not in closed pods. Furthermore, a high amount of transport was observed in mature embryos compared to young embryos (Fig. 6B). These data support the long distance transport of isoflavonoids to the site of accumulation as observed in case of many other secondary metabolites. Identification of the specific transporter involved in accumulation of isoflavonoids in soybean seed will help in understanding how these metabolites are accumulated in the sink tissues as well as may aid in metabolic engineering of these compounds in seeds.

A global gene expression analysis during embryo development was performed in soybean where gene expression profiles of two soybean cultivars (RCAT Angora-high isoflavonoid cultivar and Harovinton-low isoflavonoid cultivar) that contrasted in seed isoflavonoid content were compared (Dhaubhadel et al. 2007). Since isoflavonoid level is influenced by environmental factors (Eldridge & Kwolek 1983, Tsukamoto et al. 1995, Vyn et al. 2002), the



analysis consisted of the samples collected from two different locations grown at two different years. Gene expression analysis was performed using soybean cDNA microarray (Vodkin et al. 2004). In deed, environmental effects on the gene expression of the developing seeds were large and surpassed cultivar specific differences. Among 5910 genes that are differentially expressed in one of the five stages of embryos included in the study,

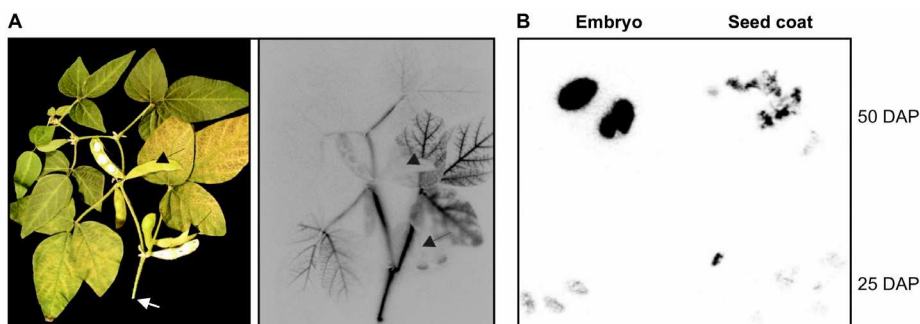


Fig. 6. Transport of  $[^{14}\text{C}]$  isoflavonoids in the sink tissues. A.  $[^{14}\text{C}]$  Phe was fed to a detached soybean branch containing leaf, developing pod and flowers for 24 h followed by autoradiography. The white arrow indicates stem part where feeding was performed. B. Autoradiography of dissected embryos (25 and 50 DAP) harvested from A. DAP-days after pollination.

*CHS7* and *CHS8* expression were higher in RCAT-Angora compared to Harovinton. The expression profiles of these two genes correlated well with the isoflavonoid accumulation in seed during development suggesting a critical role for *CHS7* and *CHS8* genes in seed isoflavonoid synthesis. Both coding sequences and 5' upstream regions of *CHS7* and *CHS8* genes are closely related compared to their similarities with other members of the *CHS* gene family (Yi et al. 2010). RNAi gene silencing of *CHS8* gene in soybean hairy roots resulted in reduced transcript levels of both *CHS7* and *CHS8* genes which led to reduced isoflavonoids in the roots. *In silico* analysis of promoter regions of *CHS7* and *CHS8* identified several *cis*-regulatory factors that are common to both the genes, however, some unique motifs were also identified for each these gene. From the promoter analysis it has been speculated that *CHS7* may be involved in induced isoflavonoid synthesis whereas the role for *CHS8* possibly in isoflavonoid synthesis under normal condition. However, these speculations have to be verified experimentally (Yi et al. 2010).

The transcriptional regulation of phenylpropanoid pathway is one of the most extensively studied regulatory pathways in plants (Grotewold 2005, 2008, Ni et al. 1996, Weissshaar & Jenkins 1998). Even though several different groups of transcription factors such as MYBs, bHLH, WRKY, MADS box and WD40 have been shown to regulate the pathway in a coordinated manner (Gonzalez et al. 2008, Hartmann et al. 2005, Ramsay & Glover 2005), MYB transcription factors are largely studied (Jin & Martin 1999) and have been involved in majority of steps in flavonoid biosynthetic pathway. In *Arabidopsis*, an R2R3 MYB transcription factor regulates *CHS* gene expression thereby affecting the flavonoid composition (Mehrtens et al. 2005, Stracke et al. 2007). The maize *C1* gene and *Arabidopsis* *PAP1* gene belong to MYB transcription factor family and both of them regulate anthocyanin biosynthesis (Cone et al. 1986, Martin & Paz-Ares 1997). *C1* is an R2R3 MYB

that requires a bHLH containing MYC co-factor R for its function (Grotewold et al. 1998). In the presence of R, C1 recognizes "CAACCACC" motif present in the *cis*-acting regions of the phenylpropanoid genes and activates the entire pathway (Grotewold et al. 2000). For metabolic engineering of isoflavonoids, a chimeric protein CRC was introduced in soybean under a seed-specific phaseolin promoter. Transgenic soybean plants overexpressing CRC gene produced four times more isoflavonoids in seeds compared to wild type soybean (Yu et al. 2003). This effect was only observed when transcription of flavanone 3-hydroxylase was suppressed to block anthocyanin branch of the pathway and total flux of substrates was diverted to isoflavonoid biosynthesis which suggested a strong competition for the precursor substrates by different branches of phenylpropanoid pathway. The expression levels of many phenylpropanoid genes such as *PAL*, *C4H*, *CHR*, *DFR* including *CHS* was increased in CRC transgenic soybean seeds (Yu et al. 2003). These results along with others in *Arabidopsis*, maize and several other plants have shown the regulation of *CHS* gene by MYB transcription factors (Sablowski et al. 1994, Solano et al. 1995, Stracke et al. 2007). Recently, a functional genomic approach was used to search for transcription factor that regulates expression of *CHS8* gene and isoflavonoid synthesis. The study identified GmMYB176, an R1 MYB transcription factor, as a regulator for *CHS8* expression (Yi et al. 2010). Most of the plant MYBs and those that are involved in phenylpropanoid pathway, consist of R2 and R3 repeats (Romero et al. 1998) and belong to the R2R3 MYB group. Some MYB-like proteins with R1 repeats have also been identified from several plant species (Baranowskij et al. 1994, Feldbrugge et al. 1994, Rubio-Somoza et al. 2006, Wang et al. 1997). However, GmMYB176 is the only R1 MYB transcription factor identified so far which is involved in plant secondary metabolism. GmMYB176 recognizes a 23 bp motif containing TAGT(T/A)(A/T) sequence within the *CHS8* promoter and binds with it for *CHS8* gene regulation. The RNAi silencing of *GmMYB176* in soybean hairy roots reduced the level of isoflavonoids in the transgenic roots demonstrating the functional role of GmMYB176 in isoflavonoid biosynthesis. The GmMYB176 silenced transgenic hairy roots showed reduced transcript levels of *GmMYB176*, *CHS8* and *IFS* genes. However, overexpression of GmMYB176 in soybean hairy roots neither increased expression levels of isoflavonoid biosynthetic genes nor the metabolite level. The results suggested that the regulation of *CHS8* gene by GmMYB176 may involve requirement for additional co-factors for its function in root pointing towards a cooperative and combinatorial mechanism of gene regulation. Eukaryotic gene expression is generally regulated by multi-protein complex which involves combinatorial action of several transcription factors. Such interactome include protein-protein interactions, as well as protein-DNA interactions (Libault et al. 2009). An example of combinatorial regulation in plants is the direct interaction between two distinct classes of transcription factors, C1/P1 and R/B, required for the biosynthesis of anthocyanin in maize (Goff et al. 1992, Goff et al. 1990). Identification of GmMYB176 interactome will highlight the complexity of isoflavonoid regulation and regulation of other metabolic processes associated with it.

Even though GmMYB176 is localized in nucleus, it does not possess a nuclear localization signal. It was suspected that a protein-protein interaction may be involved in the localization of GmMYB176 in nucleus (Yi et al. 2010). Sequence analysis predicted presence of pST binding sites within GmMYB176, to which 14-3-3 protein may bind thereby assisting in its nuclear localization. Deletion of one of the predicted 14-3-3 protein site within GmMYB176 changed its subcellular localization suggesting that 14-3-3 may regulate the nuclear localization of GmMYB176. In plants, 14-3-3s are thought to be involved in a variety of signaling processes (Bai et al. 2007, Paul et al. 2008, Schoonheim et al. 2009, Sehnke et al.

2002, Xu & Shi 2006). They regulate activities of a wide array of target proteins via protein-protein interactions which involves binding with phosphoserine/phosphothreonine residues in the target proteins (Muslin et al. 1996, Yaffe et al. 1997) or by some unknown mechanism (Aitken 2006, Fu et al. 2000). Using Bimolecular Florescent Complementation and targeted yeast two-hybrid assays, it has been shown that GmMYB176 interacts with soybean 14-3-3 protein, SGF14d (Dhaubhadel & Li 2010). Soybean genome contains 18 14-3-3 genes among which 16 are transcribed (Li & Dhaubhadel 2010). Identification of 14-3-3 specific for GmMYB176 regulation/localization will provide a deeper understanding of isoflavonoid synthesis and regulation in soybean.

## 5. Future research

Although the therapeutic medicinal properties associated with consumption of soy isoflavonoids and their role in plant-microbe interaction including nitrogen fixation and defence mechanism is well established and substantial amount of efforts already focussed towards the metabolic engineering of isoflavonoids in legumes and nonlegumes plants, the task of manipulating the levels of isoflavonoids has been an enormous challenge. The major problems in this endeavour are the complexity of trait (Gutierrez-Gonzalez et al. 2010) and its inter-connection with other major branches of the phenylpropanoid pathway. The individual genes involved in isoflavonoid biosynthetic pathway are well known for several decades and regulation of these genes are starting to be understood. The availability of soybean genome sequence makes it possible to identify the members of each gene family that are involved in isoflavonoid specific pathway. The future research should gear towards identifying the regulators for isoflavonoid specific genes and their regulons in the pathway, determining how different branches of the phenylpropanoid pathway are interconnected, and how the metabolon is formed. The knowledge generated will allow us to modify the approach necessary for efficient metabolic engineering of these compounds for human health or for plant protection and nitrogen fixation in crops of agronomic importance.

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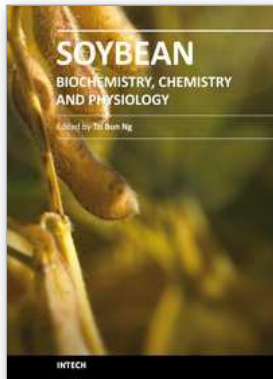
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Phone: +86-21-62489820  
Fax: +86-21-62489821

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