

## Enhancement of Nutritional Quality of Wheat (*Triticum aestivum*) by Metabolic Engineering of Isoflavone Pathway

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### ABSTRACT

Isoflavones are large group of secondary metabolites produced in legumes such as soybeans. They have essential biological functions as nutraceutical and health functions for human. They are involved in plant resistance to biotic and abiotic stresses, symbiotic relationship with nitrogen-fixing organisms and plant competition (allelopathy). In this report, isoflavonoids were expressed in wheat (*Triticum aestivum*) via introducing the key enzymes Isoflavone Synthase (IFS). Transgenic calli induced from wheat immature embryos were propagated and prepared for bombardment. Five gene constructs were prepared; the binary vector (plasmid) pAHC25, 35S-CRC, 35S-IFS, Oleocin-IFS, Oleocin-IFS-CHI and were used for wheat calli transformation. Putative transgenic calli were used to regenerate transgenic wheat plants. Evaluation of recovered transgenic plants was carried out using PCR, southern blotting of PCR products and IFS-specific probe and HPLC analysis of transgenic plant tissue extracts. Genistein and naranigenin were detected in transgenic plants carrying IFS gene, indicating that the introduced IFS was able to use the endogenous substrate from wheat. IFS showed activity under 35S promoter as well as oleocin promoter. The activity of oleocin promoter in monocots provides a good tool to use plant promoters to drive plant gene expression in plants. This also represents promoter compatibility that the cis acting elements of the oleocin promoter represent binding targets for trans acting elements of wheat. Engineering the isoflavone pathway in wheat would lead to enhancement of nutraceutical value of wheat grains and improvement of wheat resistance to diseases.

**Keywords:** Wheat, Transgenic, Isoflavonoids, HPLC, PCR

### 1. INTRODUCTION

Wheat is the world's second largest crop, behind maize and is one of the most important foods for the world. However, wheat and other cereals are poor sources of all human nutrients because they lack many of non-essential micronutrients such as various phytochemicals (Ferreyra *et al.*, 2012; Wang, 2011; Du *et al.*, 2010). Most of these bioactive components are plant secondary metabolites and can be genetically engineered to staple crops such as wheat to improve food quality and develop functional foods. For example, 'Golden rice' was genetically engineered to

enhance the levels of beta-carotene (the precursor to vitamin A) to prevent blindness in developing countries (Ye *et al.*, 2000). Several health promoting components such as human milk proteins in rice (Lonnerdal, 2002), high phytosterol vegetable oil (Venkatramesh *et al.*, 2000), high lycopene in tomatoes (Romer *et al.*, 2000), high flavonoids in tomatoes (Muir *et al.*, 2001; Sevenier *et al.*, 2002) and high isoflavones in soybeans (Jung *et al.*, 2000; Yu *et al.*, 2003) were genetically engineered to produce nutritional and functional food.

Isoflavones are secondary metabolites produced predominately in leguminous plants such as soybean.

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Isoflavones play an important role in plant-microbe interactions and disease resistance response (Arango *et al.*, 2013; Ferrreyra *et al.*, 2012; Mandal *et al.*, 2010). They act as chemoattractants for the rhizobial bacteria and as inducers of *nod* gene expression (Arango *et al.*, 2013; Prasad *et al.*, 2010; Crozier *et al.*, 2009) during the symbiotic relationship between the plant and rhizobial bacteria for the formation of nitrogen-fixing root nodules. The isoflavone daidzein is the precursor to the major phytoalexins including medicarpin and glyceollins, which are produced in alfalfa and soybean, respectively (Ferrreyra *et al.*, 2012; Blount *et al.*, 1992). The isoflavone genistein, the precursor to the phytoalexin kievitone (Mandal *et al.*, 2010; Garcia-Arenal *et al.*, 1978), exhibit antifungal activity (Mandal *et al.*, 2010). There is also evidence for a role for isoflavones in human health as a dietary component (Arango *et al.*, 2013; Ferrreyra *et al.*, 2012).

Isoflavones is associated with health benefits such as decreased risk of heart disease (Arango *et al.*, 2013; Ferrreyra *et al.*, 2012), reduced menopausal symptoms (Ferrreyra *et al.*, 2012; Du *et al.*, 2010) and reduced risk of some hormone-related cancers (Ferrreyra *et al.*, 2012; Du *et al.*, 2010). Various reports indicate that soy products may be helpful both in preventing cancer and in helping to treat it (Arango *et al.*, 2013; Ferrreyra *et al.*, 2012; Prasad *et al.*, 2010).

The active component of soybean that is thought to be primarily responsible for this beneficial action is genistein. The amount of isoflavones in soybean seed can vary up to five fold (Eldridge and Kwolek, 1983) and vary by year, environment and genotype. In addition, isoflavone content in legumes can be stress-induced by pathogenic attack, wounding, high UV light exposure and pollution (Arango *et al.*, 2013; Ferrreyra *et al.*, 2012; Verdan *et al.*, 2011).

Other studies to enhance the nutrition quality of plant products have been successful. The carotenoid (antioxidant) concentration has been enhanced in *B. napus* seeds via suppressing the *BnDETI* gene (Wei *et al.*, 2009). The *Arabidopsis* MYB regulatory gene *AtPAP1* (*Production of Anthocyanin Pigment 1*) was overexpressed in *B. napus* (canola) for the production of anthocyanin and developed the "Purple Canola" which has antioxidant activity about four fold higher in its leaves (Li *et al.*, 2010).

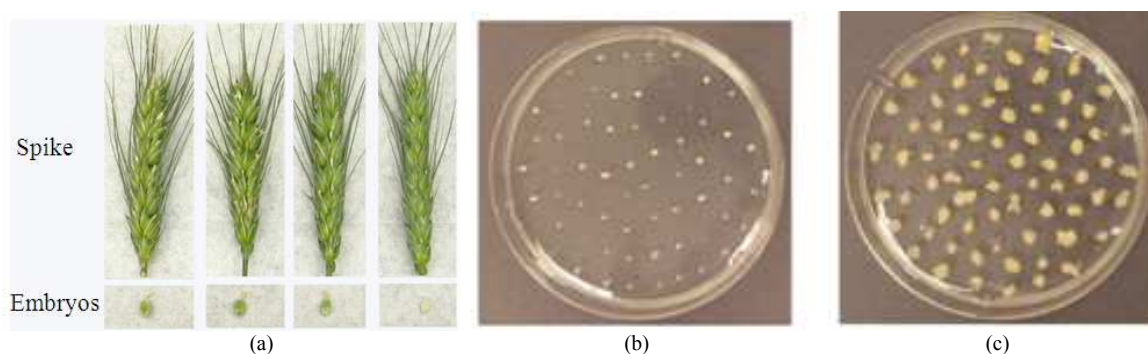
Cloning of IFS gene provided the opportunity for metabolic engineering to influence isoflavone synthesis in legumes and non-legumes (Yu *et al.*, 2000; 2003; Liu *et al.*, 2002; 2007; Sreevidya *et al.*, 2006; Li *et al.*,

2011). Expression of IFS gene in *Arabidopsis* using a strong constitutive promoter resulted in the production of genistein. *Arabidopsis* does not have IFS and does not make isoflavones, but it does produce the IFS substrate naringenin, which is normally involved in the production of anthocyanins and condensed tannins (Ferrreyra *et al.*, 2012; Rimando *et al.*, 2012). In the same study, genistein and daidzein were expressed in tobacco (*Nicotiana tabacum*) plants and maize (*zea mays*) cell system (Rimando *et al.*, 2012). Soybean IFS was overexpressed in soybean to increase isoflavones in its seeds (Yu *et al.*, 2003). Also, IFS from soybean was used to produce transgenic *Brassica napus* plants (Li *et al.*, 2011). The isoflavone genistein was expressed in tobacco, lettuce and petunia (Liu *et al.*, 2007). This number of studies demonstrated the feasibility of metabolic engineering of IFS for the production of isoflavones in agronomically important plants. In cereal plants, isoflavone metabolic pathway was engineered in rice to produce these novel compounds and induce nodulation genes (Zhang *et al.*, 2009). Wheat, which does not produce isoflavones, is one of the major food staples of humans. Synthesis of isoflavones in wheat may enhance its nutritional value and consequently reduce the chronic diseases such as cardiovascular disease in human (Arango *et al.*, 2013). In this study, we engineered wheat to produce isoflavones using IFS gene under 35S as well as soybean oleocin promoters.

## 2. MATERIALS AND METHODS

### 2.1. Callus Induction of Wheat Immature Embryos

Wheat varieties were planted and maintained in the green house. Calli were initiated from immature embryos of wheat varieties (**Fig. 1a and b**). Immature seeds were surface sterilized in 70% (v/v) ethanol for 1 min and subsequently in 1.0% (w/v) sodium hypochlorite for 15 min. They were rinsed three times with sterilized water. Immature embryos were isolated and cultured on MS medium modified for wheat callus induction (Murashige and Skoog, 1962) solidified with 2.5 g L<sup>-1</sup> phytagel (Sigma, St Louis, MO) containing MS vitamins, 150 mg L<sup>-1</sup> asparagine, 2 mg L<sup>-1</sup> 2,4-D, 100 mg L<sup>-1</sup> myo-inositol, 20 g L<sup>-1</sup> sucrose. The cultures were incubated in the dark at 24°C. After 3 weeks of culture, embryogenic calli derived from immature embryos were propagated twice on the same media and conditions to prepare enough calli for bombardment.



**Fig. 1.** (a) Wheat (*Triticum aestivum*) spikes at 14 days of anthesis and the corresponding embryos used for isolation of immature embryos for callus induction; (b): Wheat (*Triticum aestivum*) immature embryos on MS media for callus induction; (c): Calli induced from immature wheat (*Triticum aestivum*) embryos on MS media

## 2.2. Preparation of Gene Constructs

IFS gene constructs were kindly provided by Dr. Oliver Yu (Danforth Plant Science Center). He also provided the DNA plasmid pAHC25 (Christensen *et al.*, 1992). pAHC25 consists of the *uidA* gene encoding  $\beta$ -Glucuronidase (GUS) and the *bar* gene encoding Phosphinothricin Acetyltransferase (PAT) that inactivates Phosphinothricin (PPT), active ingredient of the herbicide glufosinate. Transcription of *uidA* and *bar* genes is under the control of the maize ubiquitin Ubi1 promoter (Christensen *et al.*, 1992). The transcription factor CRC was used under the control of 35S promoter to enhance the expression of the transferred genes and other genes in the phenylpropanoid pathway. These constructs were transformed into *E. coli* and maintained for plasmid isolation for bombardment (Fig. 2).

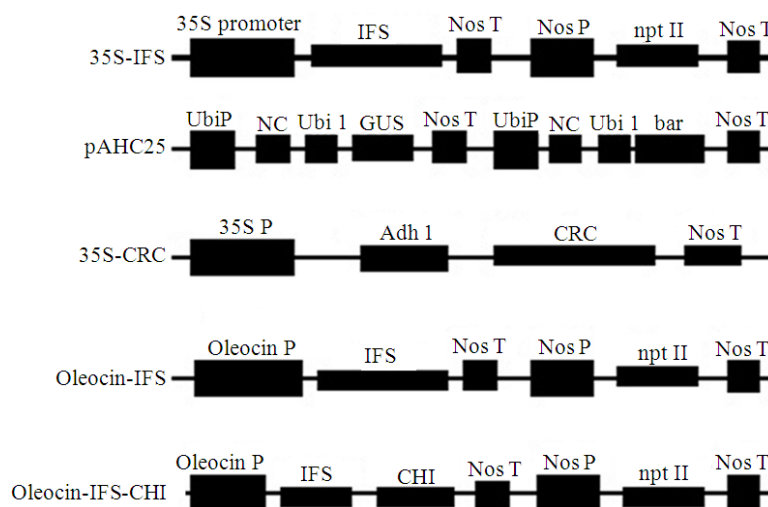
## 2.3. Particle Bombardment

Microcalli were placed in the center of 9 cm diameter Petri dishes containing MS medium solidified with 2.5 g L<sup>-1</sup> phytagel, supplemented with 0.4 M mannitol for 4 h. The microcalli were immediately subjected to microprojectile bombardment with the Biolistic Particle Delivery PDS-1000/He system (Bio-Rad). Gold particles (1.0  $\mu$ m) were coated with the plasmid DNA as per the published procedure (Manoharan and Dahleen, 2002). Three milligrams (3 mg of 1.0  $\mu$ m diameter) of gold particles was sterilized in 100  $\mu$ L absolute ethanol for 2 min with vortexing. After washing with sterile water twice, particles were resuspended in 100  $\mu$ L sterile water. The mixture was prepared as follows: 100

$\mu$ L of particle suspension were mixed with 5  $\mu$ L (1  $\mu$ g  $\mu$ L<sup>-1</sup>) of plasmid DNA, 50  $\mu$ L CaCl<sub>2</sub> (2.5 M) and 20  $\mu$ L spermidine (0.1 M). The particle/DNA suspension was vortexed and left for 10 min at room temperature. The DNA coated particles were pelleted by centrifugation at 10,000 rpm for 10 sec. The supernatant was completely removed and discarded. The pellet was then resuspended in 60  $\mu$ L absolute ethanol. Particle/DNA mixture (8  $\mu$ L) was placed in the center of microcarrier. The target cells were placed at a distance of 8 cm from the screen and directly covered with a baffle (500  $\mu$ m stainless steel mesh). Calli on each plate were bombarded once at 1300 psi. Different combinations of gene constructs were used in the bombardment of wheat calli (Table 1).

## 2.4. Propagation and Testing of Transgenic Calli

Following bombardment, calli were kept on the osmotic media for 16 more hours and then transferred to MS media for 2 days for recovery. Bombarded calli were transferred to selection media containing 3 mg L<sup>-1</sup> biolaphos. Calli were subcultured twice on the same selection media in the dark. Samples of transgenic calli were analyzed by PCR. Genomic DNA was isolated from transgenic calli according to Dellaporta *et al.* (1983). IFS-specific primers (Forward: ATGTTGCTGGAAGCTTGCCT, Reverse: GCGCTTGGGCCATAACCCT) were used to amplify 500 bp of IFS gene in the putative transgenic calli. Amplified products were electrophoresed on 1.0% agarose-ethidium bromide gels and observed under UV light.



**Fig. 2.** Molecular map of gene constructs that were used in bombardment of wheat calli

**Table 1.** Summary of gene construct combinations used in transformation of wheat calli. More details about these constructs are showed in **Fig. 2**

35S-BAR	35S-CRC	35S-IFS	O-IFS	O-IFS-CHI
+	+			
+	+	+		
+	+		+	
+	+			+

## 2.5. Regeneration of Transgenic Plantlets

Survived transgenic calli were transferred to regeneration media (same MS with 0.2 mg L<sup>-1</sup> 2,4,D; MMS0.2) supplemented with 3 mg L<sup>-1</sup> bioalaphos for two-three weeks or shoot emergence, then transferred to MMS0 (same MS with 0 mg L<sup>-1</sup> 2,4,D) supplemented with 1 mg/L bioalaphos for another two weeks or root emergence in the light (fluorescent light (3.1-5.5 µmol/m<sup>2</sup> per second) at (24-26°C, 16/8 h day/night). Green shoots usually develop within 2-3 weeks on the regeneration medium. The regenerated shoots were transferred to rooting medium (half-strength MS) with 3 mg L<sup>-1</sup> bioalaphos. Fully developed plants were transferred to peat pellets and then to pots in the greenhouse (21-26°C, 16/8 h (day/night) photoperiod with natural lighting supplanted with sodium vapor lamps). Transgenic plants were tested by PCR using the same IFS-specific primers and conditions above.

## 2.6. Molecular Characterization of Transgenic Plants

### 2.6.1. RNA Analysis

Expression of IFS gene at transcriptional level was confirmed by reverse transcription followed by PCR (RT-PCR). Total RNA was isolated from transgenic

plants using TRI REAGENT (Molecular Research Center). Reverse specific primer (Reverse: GCGCTTTGGGCCATAACCCT) for IFS was used to synthesize the first strand of cDNA. Specific forward and reverse primers for IFS (Forward: ATGTTGCTGGAACCTGCACT, reverse: GCGCTTTGGGCCATAACCCT) were used to amplify 500 bp diagnostic fragments of IFS transgene to confirm its transcription.

### 2.6.2. Southern Blotting

Southern blotting was carried out according to Hedgcoth *et al.* (2002). Five µL of PCR product was separated on agarose and transferred to a positively charged Nylon membrane. A DNA probe of the IFS coding region was prepared by PCR using the same IFS-specific primers that were used in PCR amplification (Forward: ATGTTGCTGGAACCTGCACT, Reverse:GCGCTTTGGGCCATAACCCT). The probe was labeled with digoxigenin-11-dUTP (Rathburn *et al.*, 1993) for chemiluminescent detection following hybridization of a Southern blot.

### 2.6.3. HPLC of Isoflavones

For isoflavone quantification and analysis, plant tissues were ground in liquid nitrogen, extracted with



80% (v/v) methanol at 100 mg  $\text{ML}^{-1}$  and filtered through syringe filters. To hydrolyze any possible isoflavone conjugates, 3 mL of 1 N HCl were added to 1 mL of the extract and the samples were incubated at 95°C for 2 h, followed by extraction using 1 mL ethyl acetate. The HPLC conditions were similar to that previously used by Jung *et al.* (2000). Samples were assayed on HPLC Agilent Model 1100 system on a Phenomenex Luna C18 column (4.6×250 mm, 10 $\mu$ ), separated by using a 12 min linear gradient from 20% methanol/80% 50 mM ammonium acetate (pH 5.9) to 100% methanol at a flow rate of 2 mL  $\text{min}^{-1}$  with oven temperature of 35°C and injection volume of 10  $\mu\text{L}$ . Genistein and naringenin were monitored by A290. For calibration, authentic compounds were dissolved in ethanol and used as standards. Peak areas were converted to micrograms.

### 3. RESULTS

Wheat immature embryos (**Fig. 1b**) were excised from wheat inflorescence 14 days after anthesis (**Fig. 1a**) and used for callus induction on MS media. Calli were propagated and prepared for bombardement (**Fig. 1c**)

Five gene constructs were used in bombardement of wheat calli **Fig. 2**. The binary vector pOY204, which contains a chimeric gene consisting of the cauliflower mosaic virus 35S promoter, the soybean IFS1 coding region and the nopaline synthase terminator (*Nos* 39), together with an *nptII* gene cassette for kanamycin selection in plants (Jung *et al.*, 2000). Plasmid pAHC25 (Christensen *et al.*, 1992). It contains the *uidA* gene encoding  $\beta$ -Glucuronidase (GUS) and the *bar* gene encoding Phosphinothricin Acetyltransferase (PAT) that inactivates Phosphinothricin (PPT), active ingredient of the herbicide glufosinate. Transcriptions of *uidA* and *bar* genes are under the control of the maize ubiquitin *Ubi1* promoter (Christensen *et al.*, 1992). One construct of the transcription factor CRC which include 35S promoter, Adh1 intron 6, CRC coding region and *Nos* terminator was used to enhance the expression of the transferred genes (Yu *et al.*, 2000). The construct Oleocin-IFS contains the IFS gene under the control of soybean oleocin promoter, *Nos* terminator and *NptII* gene under the control of *Nos* promoter and *Nos* terminator. The construct Oleocin-IFS-CHI contains a chimeric gene of IFS1 and CHI from soybean under the control of oleocin promoter, *Nos* terminator and *NptII* gene under the control of *Nos* promoter and *Nos* terminator.

#### 3.1. Callus Transformation and Transgenic Plants Regeneration

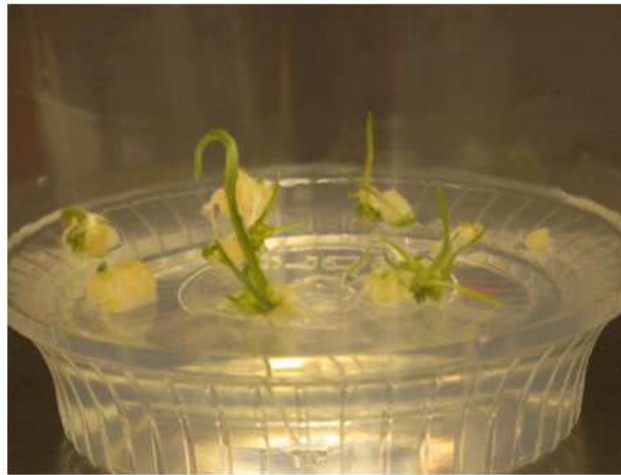
Combinations of the five constructs described above were used in transforming wheat calli (**Table 1**). Many putative transgenic calli were recovered and propagated. A number of transgenic plantlets were regenerated, grown and maintained (**Fig. 3**).

#### 3.2. Molecular Characterization of Transgenic Plants

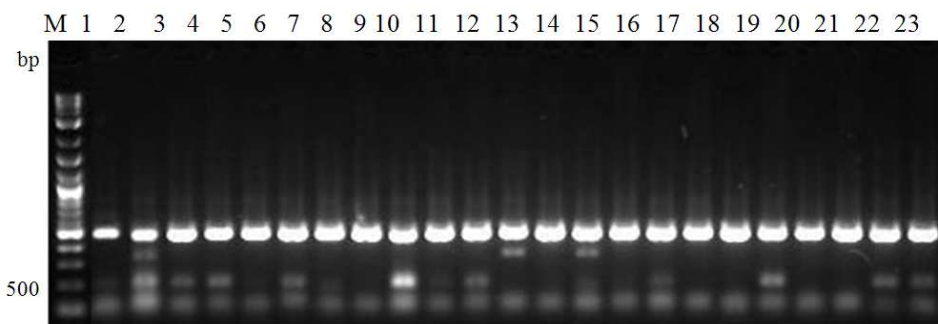
Specific primers of IFS gene were used to amplify DNA fragment of 500 bp of the 5' end of IFS gene. Many transgenic plants were tested using RT-PCR for transcription of the IFS gene. Forty three positive transgenic plants (**Table 2**) were tested of which 23 are shown in **Fig. 4**. Southern blotting was carried out using the PCR product amplified from transgenic plants and IFS-specific DNA probe. Thirty six transgenic plants of which 11 transgenic lines (1,3,4,6,9,10,11,14,17,18,21) gave strong signal on southern blot (**Fig. 5**), three plants (5,8,16) gave weak signal and 7 plants (2,7,12,13,15,19,20) gave no signal. Only plants with strong signals were used as transgenic lines and used for more analysis (**Fig. 5**).

#### 3.3. Progeny Analyses of Transgenic Plants

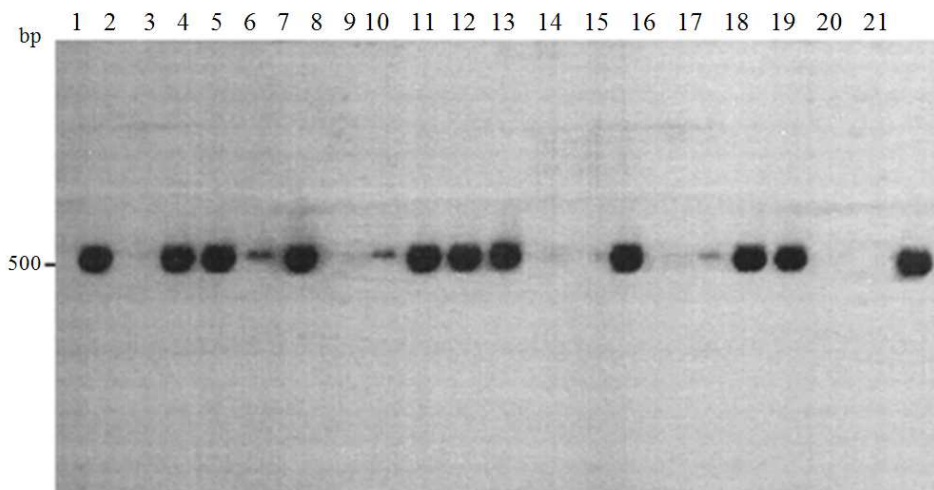
Concentrations of naringenin and genistein varied in the recovered transgenic plant seeds. There were some low and high expressers of the IFS gene in wheat plants under both 35S and oleocin promoters. Genistein showed high accumulation in some transgenic plant lines (**Table 2**). This indicates the high activity of the exogenous IFS in turning over the endogenous naringenin into genistein competing for naringenin conversion into anthocyanins. The seeds of low genistein accumulators showed normal color. Only constructs 35S-IFS and O-IFS showed genistein, whereas it was not detected in O-IFS-CHI transgenic seeds. It seems the chimeric gene IFS-CHI was not functional in wheat or metabolic channeling by the endogenous CHI and IFS was more competitive than the heterologous ones. The tested lines showed various concentrations of genistein. Naringenin concentrations range was 0.7 - 3.54  $\mu\text{g mL}^{-1}$  in 35S-IFS plants, whereas its range was 1.13-3.13  $\mu\text{g mL}^{-1}$  in the O-IFS plants. Its average was 2.20 and 2.58  $\mu\text{g mL}^{-1}$  in 35S-IFS and O-IFS plants consecutively. Genistein concentrations ranged from 4.8  $\mu\text{g mL}^{-1}$  to 35.14  $\mu\text{g mL}^{-1}$  in 35S-IFS plants, whereas it ranged 9.81-44.61 in the O-IFS plants. Its average was 20.64 and 29.29 in 35S and O-IFS plants consecutively (**Table 2 and Fig. 6**).



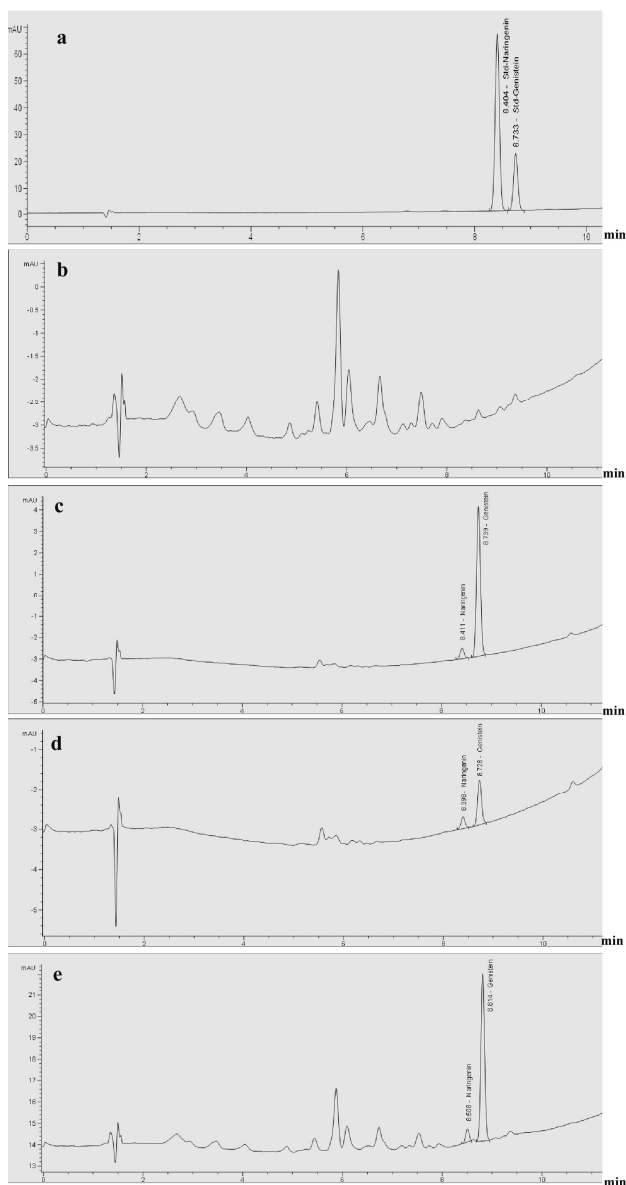
**Fig. 3.** Regenerated shoots of transgenic wheat plantlet on regeneration medium



**Fig. 4.** RT-PCR amplification of 500 bp from IFS gene from 23 wheat transgenic plants



**Fig. 5.** Southern blot analysis of PCR products of representative transgenic plants



**Fig. 6.** HPLC estimation of naringenin and genestein of (a) standard mix (naringenin and genestein); (b) negative control; (c), (d), (e), representative transgenic plants

**Table 2.** Summary of recovered transgenic wheat plants and their characteristics

	Construct		Total
	35S-IFS	O-IFS	
No of transgenic plants	29	14	43
No of transgenic plants tested by Southern	20	16	36
No of transgenic plants tested by HPLC	14	9	23
Naringenin concentration (average, µg/mL)	2.20	2.58	-
Naringenin range, µg/mL	0.7-3.54	1.13-3.13	-
Genestein concentration (average, µg/mL)	20.64	29.29	-
Genestein range, µg/mL	4.80-35.14	9.81-44.61	-

#### 4. DISCUSSION

Wheat was genetically engineered by the key enzyme of isoflavone synthase under the control of 35S promoter and soybean oleocin promoter. Direct introduction of DNA constructs into wheat calli derived from immature embryos was used. Transgenic plants were able to produce and accumulate genistein in wheat seeds giving some essential indications. First, the heterologous IFS was active in the wheat background and was able to use wheat endogenous substrate of naringenin. Second, the oleocin promoter was active in wheat seeds. This promoter is seed-specific promoter from soybean. Its activity in wheat seeds is very interesting because it limits its expression to the seed tissue and can be used in similar studies in the future to drive the expression of other genes to replace viral promoter like 35S. In this study, it showed a leaky expression in some plant lines, especially in leaves (data not shown). Third, it provides the first report on the expression of isoflavone in wheat (second largest stable crop for food and feed after maize) in the world. Genistein accumulation varied greatly among our transgenic plants. Up to 9 fold differences in the accumulation of genistein in wheat seeds expressing soybean IFS under the control of 35S as well as oleocin promoter were detected. Also, the IFS in some transgenic lines are more competitive to the F3H gene for the naringenin substrate to the final product genistein. Other lines did not show this high competitiveness and they are considered low expressers of the transgene.

The low level of genistein has been discussed in several previous studies. This could be due to different scenarios. Metabolic channelling has been reported to be one of the reasons. It has been accepted that the series of enzymes in flavonoid/isoflavonoid pathway form into an enzyme complex called metabolon to improve its catalytic activity by channeling intermediates from upstream to downstream enzymes (Jorgensen *et al.*, 2005; Yu *et al.*, 2000). Therefore, the availability of the substrate for the foreign enzyme would be limited because it does not fit into the metabolon of the host enzymes or activity of other upstream enzymes in the metabolon is lower turning less substrate over for the IFS transgene (Liu *et al.*, 2007). In our study, the CRC gene was used to activate other enzymes in the pathway to overcome the later problem (Yu *et al.*, 2000; 2003), yet transgenic lines varied in the production and accumulation of genistein. In addition, the competition of IFS and F3H for the naringenin substrate may vary in individual transgenic lines leading to different levels of

genistein. It was reported that, cosuppression of F3H in soybean did not cause an increase of total isoflavone level in seeds (Yu *et al.*, 2003). However, cosuppression of F3H resulted in enhancement of genistein content when maize transcription factor CRC was used to activate the anthocyanin pathway. C1 and R transcription factors were used to increase the expression of isoflavone levels in soybean seeds (*Glycine max*) by metabolic engineering of the complex phenylpropanoid biosynthetic pathway. In this study, genistein was decreased and daidzein was increased with a small overall increase in total isoflavone levels. C1/R expression and cosuppression of flavanone 3-hydroxylase to block the anthocyanin branch of the pathway led to higher levels of isoflavones. This study concluded that, gene activation driven by transcription factor in conjunction with suppression of a competing pathway provided a successful strategy to enhance accumulation of isoflavones in soybean seed (Yu *et al.*, 2003). Moreover, the low level of genistein in some transgenic lines could be explained by the low level of naringenin substrate (Sreevidya *et al.*, 2006) or its channeling to flavonol biosynthesis. In our study, we detected varied level of naringenin (0.7 to 3.54  $\mu\text{g mL}^{-1}$ ), while genistein level ranged from 4.8 to 44.61  $\mu\text{g mL}^{-1}$  supporting the later scenario. Also, transgenic lines may have limited ability to store high level of genistein or turn it over to the glycosylated (stored) form.

Application of metabolic engineering of isoflavones is becoming more interesting and successful toward changing the nutritional and pharmaceutical value in many plant species. Several successful attempts have been done to engineer the isoflavonoids branch of phenylpropanoid into various plant groups including tomato (Shih *et al.*, 2008), rice (Sreevidya *et al.*, 2006), Arabidopsis (Liu *et al.*, 2002) and oilseed rape *B. napus* (Li *et al.*, 2011), dicots (Yu *et al.*, 2000; Liu *et al.*, 2007; Li *et al.*, 2011), monocots (Sreevidya *et al.*, 2006). Heterologous IFS enzyme from soybean was introduced in various non-legume dicot and monocot systems to evaluate its ability to interact with the endogenous phenylpropanoid pathway, which provides the substrate for IFS and produces genistein in several plant tissue systems. In tobacco (*Nicotiana tabacum*) genistein was increased in floral tissue that synthesizes anthocyanins, relative to leaves. In a monocot cell system (maize BMS cells), IFS was able to produce genistein when introduced a transcription factor regulating genes of the anthocyanin pathway. The cells were able to produce the isoflavone daidzein when the chalcone reductase gene was introduced. The genistein produced in



tobacco, *Arabidopsis* and maize cells was present in conjugated forms, meaning that endogenous enzymes were capable of recognizing genistein as a substrate (Yu *et al.*, 2000). The isoflavone genistein was produced in various tissues of *Arabidopsis thaliana* after the introduction of soybean isoflavone synthase (Jung *et al.*, 2000). Isoflavone pathway was engineered in rice using soybean IFS. Generated transgenic plants were evaluated using PCR and southern blot analysis. IFS expression was documented by estimation of genistein in rice tissues. The results of this study confirmed that the IFS gene is active in transgenic rice plants. Genistein was present in the glycoside form, which means that endogenous glycosyl-transferases were able to recognize genistein as a substrate (Sreevidya *et al.*, 2006). *Brassica napus* was engineered by the introduction of exogenous soybean IFS (*Glycine max*). The soybean IFS gene (GmIFS2) was introduced into *B. napus* plants, which was able to direct the synthesis and accumulation of genistein derivatives in leaves up to 0.72 mg/g dw. These results indicated that the heterologous GmIFS2 enzyme was able to use the *B. napus* naringenin as a substrate to produce genistein in oilseed rape (Li *et al.*, 2011). Our results are consistent with other successful studies to express soybean IFS in rice (Sreevidya *et al.*, 2006), tomato (Shih *et al.*, 2008), *Brassica napus* (Li *et al.*, 2011) and *Arabidopsis* (Liu *et al.*, 2002) tobacco (*Nicotiana tabacum*) and maize (*zea mays*) cell system (Yu *et al.*, 2000), soybean seeds (Yu *et al.*, 2003), lettuce and petunia (Liu *et al.*, 2007). In addition, a unique achievement was confirmed in this study that is using the oleocin promoter (plant promoter) to drive the plant gene IFS and getting the same level of gene expression under the control of the strong 35S promoter used in all previous studies.

Soybean IFS was introduced into *Arabidopsis* to generate transgenic plants in which the F3H gene (tt6/tt3 mutant) and HFR are impaired. This resulted in increased level of genistein (31-169 nmoL/g FW). F3H shares substrate naringenin with IFS and can compete for the same substrate. This could represent a limiting factor for genistein biosynthesis in transgenic plants (Liu *et al.*, 2002). In our study, transfer of IFS from soybean into wheat (*Triticum aestivum*) under the control of 35S promoter led to the accumulation of genistein in seeds, whereas the same gene expression under the oleocin promoter was limited to seeds with traces in some transgenic lines in other tissues. These results suggest that the exogenous soybean IFS was able to use the endogenous substrate naringenin from wheat. This gives the chance to enhance the nutraceutical value of

wheat grains through metabolic engineering of isoflavone branch in wheat.

According to our results and other studies, fluctuations in the IFS activity was very clear. Therefore, each transgenic line must be considered as an independent case because each one has been developed from a transformation and genomic insertion incident. Each insertion could be different regarding to the position of insertion of the gene and the microgenetic environment as well as the epigenome as a whole. Epigenome could modulate the expression of the transgene ranging from enhancing to silencing and anywhere in between. On the other hand, the heterologous enzyme has to compete with other endogenous enzymes that turn genistein over to other components in the phenylpropanoid pathway. The F3H competes for the naringenin and converts it to the final product anthocyanin (Yu *et al.*, 2003).

## 5. CONCLUSION

The isoflavone pathway was engineered in wheat plant under the control of 35S and oleocin promoter. The introduced IFS was able to use the endogenous wheat substrate to produce genistein and naringenin under both 35S promoter as well as oleocin promoter. The activity of oleocin promoter in monocots provides a good tool to use plant promoters to drive plant gene expression in plants. This represents promoter compatibility among *cis acting* elements of the oleocin promoter and *trans acting* elements of wheat at the gene regulation level. Engineering the isoflavone pathway in wheat will help to the improvement of nutraceutical value of wheat grains and increase its resistance to diseases.

## 6. ACKNOWLEDGEMENT

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