



Effect of biotic and abiotic elicitors on isoflavone biosynthesis during seed development and in suspension cultures of soybean (*Glycine max* L.)

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Abstract

The present investigation aimed to look at the effects of biotic and abiotic elicitors during Soybean seed development and cell suspension culture in isoflavones accumulation. The expression levels of four major genes viz., CHS7, CHS8, IFS2, and IFS1 involved in isoflavones biosynthesis during seed developmental stages from R5L–R7 was seen in both MAUS-2 and JS-335 Soybean varieties. The R7 stage showed 1.24-fold upregulation of IFS1 transcript level and considered as the control for Soybean seed development. Both varieties during R6–R8 stages responded differently to the foliar application of 10 μ M SA, 10 μ M MJ and 0.1% *Aspergillus niger*. The *IFS2* transcripts were upregulated by SA at the R7 stage with 5.21- and 4.68-fold in JS-335 and MAUS-2, respectively. *IFS1* expression was significantly increased by *A. niger* treatment at R7 stage with 3.98- and 3.21-fold in MAUS-2 and JS-335, respectively. The expression of CHS7 and CHS8 by 10 μ M SA at R7 level revealed maximum up-regulation of 0.51- and 1.01-fold in MAUS-2; 0.37- and 0.82-fold in JS-335, respectively. In the soybean callus suspension culture, biosynthetic genes were used to validate the effects of elicitor on isoflavones. Both biotic and abiotic treatments contribute to the upregulation of *IFS1* and *IFS2* expression, that in turn, leads to the accumulation of isoflavone in seed development as well as in suspension cultures. These data further suggested that the *IFS2* is the key gene responsible for the isoflavone accumulation during elicitor treatment.

Keywords Augmentation · Callus suspension · Elicitation · Isoflavones · qPCR

Introduction

Isoflavones are polyphenolic secondary plant metabolite found in seedlings, flowers, roots and they are especially abundant in seeds and leaves of Soybean. Within seeds, diverse tissues have the ability to synthesize isoflavones (Dhaubhadel et al. 2003). The soybean development is influenced by multigenic abiotic responses, and highly variable across the plant, organ, tissue and also vary with respect to the environmental conditions (Dhaubhadel et al. 2007; Devi

et al. 2018). Isoflavones are synthesized through the phenylpropanoid pathway branch (Fig. 1) which leads to the synthesis of anthocyanins, lignin, and other significant secondary metabolites (Yu and McGonigle 2005). Phenylalanine ammonia lyase (*PAL*) enzyme is the initial enzyme which converts amino acid L-phenylalanine into cinnamic acid. The first vital enzyme for synthesis of flavonoid is chalcone synthase (*CHS*), which exists as a multigene family in Soybean, though not all copies are expressed at detectable levels in seeds (Dhaubhadel et al. 2007). Other key enzymes involved in the isoflavones synthesis pathway are chalcone reductase (*CHR*), for daidzein and glycitein formation and chalcone isomerase (*CHI*), which converts chalcones to flavanones. On the other hand, the enzyme that exclusively differentiates isoflavone-synthesizing plant species from other species is isoflavone synthase (*IFS*), a cytochrome *P450* monooxygenase, that catalyzes 2,3 aryl ring migration of flavanones to their respective isoflavones (Akashi et al. 1999; Jung et al. 2000). *IFS* is present in two copies, as *IFS1* and *IFS2* in Soybean genome and differ from each other by few amino

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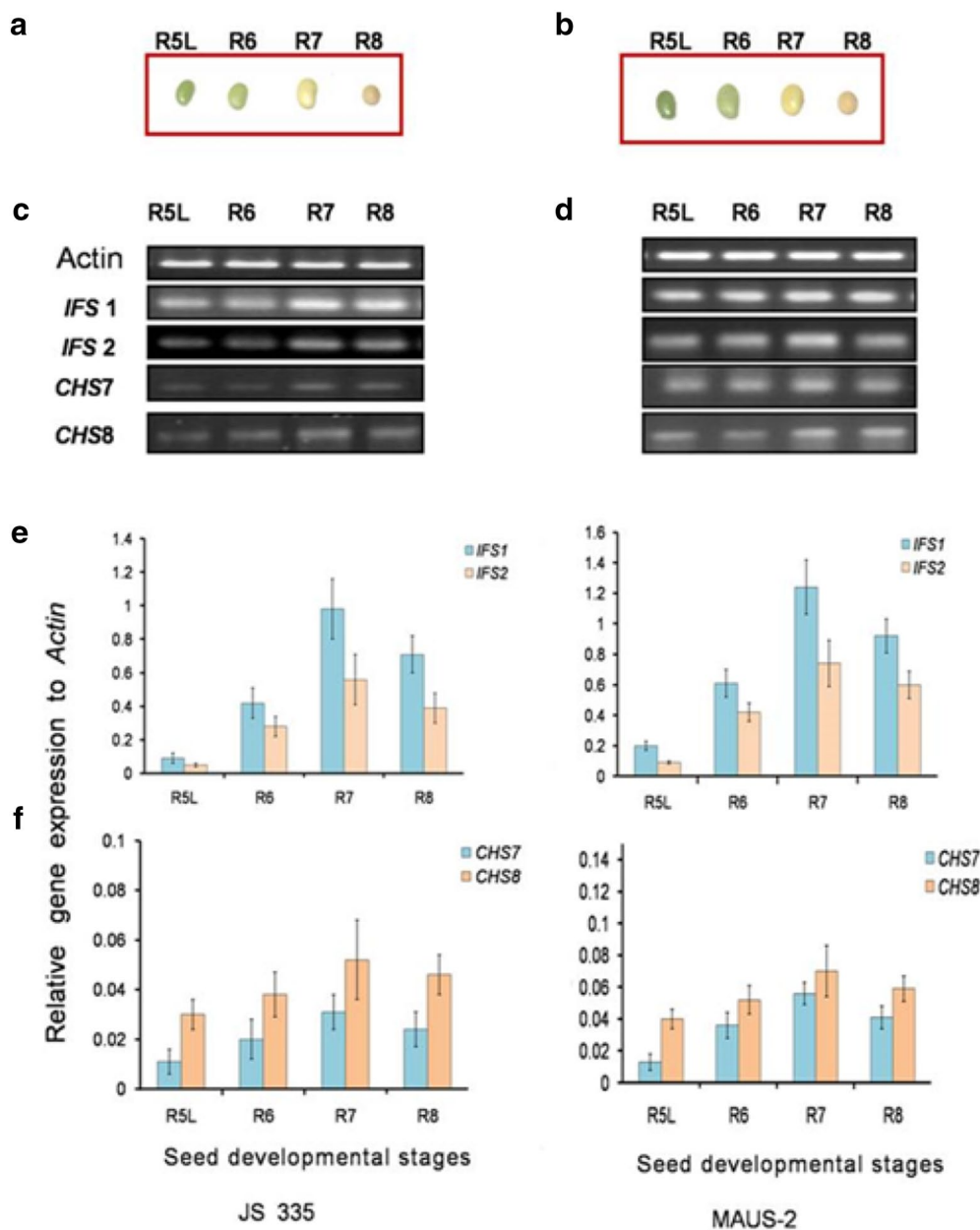


Fig. 1 Expression analysis of isoflavone biosynthetic genes during seed developmental stages in soybean cv. JS-335 and cv. MAUS-2 variety. **a** Seed developmental stages in JS-335. **b** Seed developmental stages in MAUS variety. **c, d** Expression pattern of *Actin*, *IFS1*, *IFS2*, *CHS7* and *CHS8* in JS-335 MAUS-2 variety (semi-quantitative

method). **e** Relative expression pattern of *IFS1* and *IFS2* was normalized to *Actin* in JS-335 and MAUS-2 variety (qPCR). **f** Relative expression pattern of *CHS7* and *CHS8* was normalized to *Actin* in JS-335 and MAUS-2 variety (qPCR). Data shown are the mean \pm SD

acids. They convert naringenin and liquiritigenin to genistein and daidzein, respectively. Both *IFS1* and *IFS2* contribute to the isoflavone level in Soybean seeds and are regulated differentially at the transcriptional level (Cheng et al. 2008). The expression of *IFS2* increases, while the expression of *IFS1* remains constant during late seed developmental stages (Dhaubhadel et al. 2007). Furthermore, only *IFS2* was found to be induced in Soybean transgenic roots and hypocotyls

in response to the attack of pathogens (Subramanian et al. 2004).

The seed isoflavones are greatly influenced by various environmental stresses during the seed developmental stages (Lozovaya et al. 2005; Kim and Chung 2007; Dhaubhadel et al. 2007). Gutierrez-Gonzalez et al. (2010) have reported that the reduction in seed isoflavone concentration observed during a period of water stress was correlated with the

expression of three key genes involved in isoflavone synthesis (i.e., *CHS7*, *CHS8*, and *IFS2*). Similarly, four key genes involved in isoflavones synthesis (*IFS1*, *IFS2*, *CHS7*, and *CHS8*) of seed isoflavones during seed development (seed maturity stage) were expressed at a higher temperature. But there was no clear correlation between isoflavones concentration and gene expression (Chennupati et al. 2012). A few reports investigated the alteration in the expression pattern of isoflavones biosynthetic genes under the influence of elicitors. Chen et al (2009) investigated gene expression alteration of 14 genes encoding isoflavone in Soybean sprouts (three cultivars), as well as isoflavones concentrations, with chitosan treatment.

Considering the gene expression analysis described above, there is not much information available for the regulation of biosynthesis of isoflavones under the influence of elicitors, mainly during seed development, where isoflavones are actively accumulated. Though there are few reports on the effect of stress factors on isoflavones regulation in *in vitro* cell cultures of *Fabaceae* but there are no reports in Soybean cell cultures. With this, the purpose of present study was to profile the expression of isoflavones biosynthetic genes during seed developmental stages in JS-335 and MAUS-2 varieties and also differential expression analysis of isoflavones biosynthetic genes under the influence of selective elicitors in JS-335 and MAUS-2 variety and callus suspension cultures.

Materials and methods

Chemicals

HPLC standards for daidzin, glycitin, genistin, daidzein, glycitein, and genistein were purchased from Sigma-Aldrich, India. High-performance liquid chromatography (HPLC) grade methanol, acetonitrile, HCl and acetic acid were purchased from Rankem Laboratories, India. Deionized water from Milli-Q (Millipore Co., India) was used for all the extraction and quantification purposes.

Plant material and cultivation

Soybean seeds (*Glycine max* L.) varieties namely, JS-335 and MAUS-2 were collected from AICRP Soybean, Gandhi KrishiVignana Kendra, Bangalore, India. For the present study, uniform size seeds were handpicked and used for isoflavones analysis. To establish *in vitro* seedlings for explants preparation and subsequent callus induction, seeds were subjected to surface sterilization procedure as described by us earlier (Akitha Devi and Giridhar 2014).

Callus induction and suspension culture initiation

The cotyledonary node leaves from three-week-old seedlings of JS-335 variety were used as an explant (9–10 mm) for callus induction on MS medium, supplemented with 9.51 μM 2,4-Dichlorophenoxyacetic acid (2,4-D), 29.64 μM α -Naphthalene acetic acid (NAA), and 1.63 μM kinetin (Kn). The pH was adjusted to 5.8 and autoclaved for 20 min at 121 °C. The cultures were maintained at the light intensity of 57 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (illumination supplied by cool white fluorescent tubes) with a 16 h photoperiod at 25 ± 2 °C. The callus induced from explants were separated and subcultured onto the same medium twice at a 15-day interval to get a friable callus. Suspension cultures were initiated with 200 mg from the established friable callus in 150 mL Erlenmeyer flasks containing 40 mL of MS liquid medium supplemented with 3% sucrose (w/v), growth regulators, 9.51 μM 2,4-D, 29.64 μM α -NAA, and 1.63 μM Kn. Cultures were incubated on a rotary shaker at 100 rpm with 16 h light/8 h dark photoperiod at 25 ± 1 °C. After two subcultures on the same medium at a 4 week interval, cultures were uniform and used for elicitor studies.

Establishment of plants in polyhouse

To establish plants in polyhouse (*in vivo*), the seeds of selective varieties (JS335 and MAUS-2) were then imbibed in double-deionized water for 1 h and then sown in pots containing 6 kg of air-dried red silt loam (fine-silty) at 2-cm depth for germination. Seed at various developmental stages (Table 1, Fig. 2) such as R5L (50d), R6 (65d), R7 (75d), and R8 (88d) was collected from JS-335 variety and R5L (60d), R6 (75d), R7 (85d), and R8 (97d) stage seeds from MAUS-2 variety for analyzing isoflavones content. After harvesting, the samples were immediately frozen in liquid nitrogen and stored at -80 °C for later use.

Elicitor preparation

Biotic elicitors

The biotic elicitor was prepared using fungal culture (Giridhar and Parimalan 2010), *Aspergillus niger* (AN) that was obtained from the Microbiology and Fermentation Technology Department (CSIR-CFTRI). Fresh cultures of AN were grown on potato dextrose agar (PDA) medium (HiMedia, Mumbai) slants and then incubated in the dark at 37 °C for 7 days. Further respective fungal spores were selected for spore suspension preparation in 0.1% sodium lauryl sulfate (w/v) and diluted with sterile distilled water under sterile conditions to obtain a spore density of $\sim 2.5 \times 10^6$ spores mL^{-1} . Afterward, the same spores were then inoculated in 40 mL of potato dextrose broth prepared in 150 mL

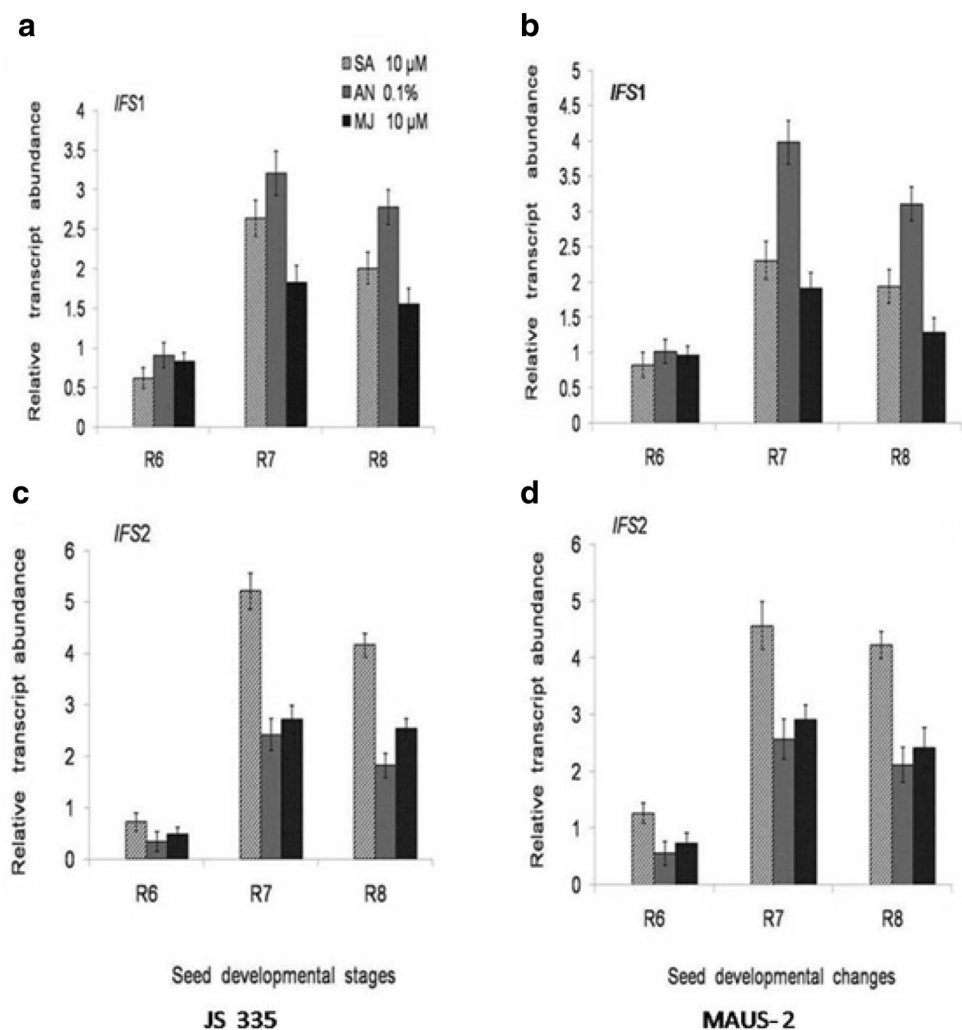
Table 1 Isoflavones concentration in different seed growth stages of selected soybean varieties (micrograms per gram of dry weight) under abiotic elicitor 10 μ M Salicylic acid treatment

Variety ^a	Growth stage	Salicylic acid 10 μ M							
		Total daidzein (μ g/g (dw))		Total glycitein (μ g/g (dw))		Total genistein (μ g/g (dw))		Total isoflavone content (μ g/g (dw))	
		<i>C</i>	<i>E</i>	<i>C</i>	<i>E</i>	<i>C</i>	<i>E</i>	<i>C</i>	<i>E</i>
JS-335	R5 (e)	80.6 ^e	108.4 ^g	12.6 ^e	17.39 ^d	50.2 ^e	67.91 ^f	138.4 ^e	193.7 ^g
	R6	105.6 ^{de}	220.55 ^f	40.6 ^{bc}	94.56 ^c	166.9 ^d	393.66 ^{ef}	283.6 ^d	708.77 ^{ef}
	R7 (m)	165.9 ^c	570.45 ^d	37.8 ^{cd}	125.87 ^c	272.2 ^c	922.34 ^d	462.5 ^c	1618.7 ^d
	R8	270.3 ^{ab}	1352.0 ^b	39.5 ^c	199.88 ^b	443.5 ^b	2214.8 ^b	753.5 ^b	3766.6 ^b
MAUS-2	R5 (e)	114.2 ^d	195.24 ^f	17.6 ^d	30.075 ^d	70.3 ^{de}	118.36 ^f	196.3 ^{de}	343.67 ^{fg}
	R6	158.8 ^{cd}	386.56 ^e	65.5 ^a	147.14 ^{bc}	225 ^{cd}	558.05 ^e	376.4 ^{cd}	1091.7 ^e
	R7 (m)	267.9 ^b	1052.3 ^c	49.8 ^b	195.46 ^b	467.8 ^{ab}	1795.3 ^c	780.5 ^{ab}	3043.0 ^c
	R8	359.2 ^a	1939.5 ^a	62.3 ^{ab}	336.42 ^a	626.9 ^a	3386.0 ^a	1048.6 ^a	5661.9 ^a

Different alphabets above the bars indicate a significant difference between the treatment. Treatment showing the same alphabets is not significantly different at p -value 0.05. Values calculated using one way ANOVA followed by Tukey's multiple range test

C Control, *E* Elicitor treatment

Fig. 2 Expression analysis of isoflavones biosynthetic genes during elicitor treatment in soybean varieties. **a, b** Relative transcript abundance of *IFS1* in response to elicitors; SA (10 μ M), *A. niger* (0.1%) and MJ (10 μ M) in JS-335 and MAUS-2 seed developmental stages. **c, d** Relative transcript abundance of *IFS2* in response to elicitors; SA (10 μ M), *A. niger* (0.1%) and MJ (10 μ M) in JS-335 and MAUS-2 seed developmental stages



Erlenmeyer conical flasks and then incubated for 10 days at dark. Subsequently, incubated cultures were autoclaved and mycelium was separated from the culture broth using filtration, and fresh weight was recorded. The aqueous extract was prepared by homogenization. The extract was then filtered (using Whatman no. 1 filter paper) and set aside as the stock solution. From the stock, broad working concentration (fungal mycelium wet weight in 1 L of distilled water) range of fungal mycelial extract was prepared in sterile water and used for conducting elicitor experiments. Similarly, *Rhizopus oligosporus* was cultures and mycelial extract was prepared in the same manner as explained for *A. niger*. Chitosan (Sigma-Aldrich, India) was prepared by dissolving 1% (w/v) in 0.5% acetic acid solution, and then adjusted the final volume to 100 mL using distilled water and then the pH adjusted to 5.8 with NaOH.

Abiotic elicitors

Stock solutions of salicylic acid (SA) was prepared by dissolving in sterile distilled water, filter-sterilized and diluted to different concentrations. Methyl jasmonate (MJ) (Sigma Chemical Co., St. Louis, USA) was dissolved in 70% (v/v) ethanol and prepared as a stock solution. Further dilution was done using deionized water. Solution was filtered through a microfilter (0.2 mm) before being dispensed into cell culture at various concentrations. Control cultures were treated with sterile water was added instead of elicitor.

Foliar application of elicitors in in vivo *Glycine max* plants

Germinated soybean seedlings were maintained for control and treatment as quadruples at the Plant Cell Biotechnology Department's polyhouse. Initial studies were conducted with different concentrations of SA (2.5, 5, 10, 25 and 50 μM), MJ (2.5, 5, 10, 25 and 50 μM) and AN (0.05, 0.1, 0.5, 1 and 2) for the evaluation of isoflavones in the seeds (data not shown). Among them, 10 μM SA, 10 μM MJ and 0.1% AN were selected for the present study according to initial results. All the elicitors were sprayed onto the fully opened flower of JS-335 and MAUS-2 plants between 10 and 11 AM. Respective biotic elicitor (*A. niger*) and abiotic elicitors

(Salicylic acid-SA and methyl jasmonate-MJ) was administered on the flowers for the evaluation of isoflavones production in the seeds and control plants were sprayed with an equal quantity of distilled H_2O . Both treated and control seeds were harvested from various developmental stages such as R5,R6, R7 and R8 from both JS-335 and MAUS-2 varieties. For gene expression studies, the harvested Soybean seeds were frozen immediately and stored at -80°C for later use.

Elicitor treatment in callus suspension cultures

Biotic and abiotic elicitors were added to *G. max* callus suspension culture with concentration of SA (2.5, 5.0 and 10 μM), MJ (2.5, 5.0 and 10 μM) and *A. niger*-AN (0.1, 0.5 and 1.0%), *R. oligosporus* (0.1, 0.5 and 1.0%) and chitosan (2.0, 5.0 and 10 μg) for the evaluation of isoflavones. Culture with equal volumes of water without elicitor was maintained as a control. Cultures were harvested at 12 to 24th days (for isoflavones) after the addition of elicitors. The experiment was repeated thrice for the analysis of growth parameters and metabolites. To study the gene expression, suspension cultures were harvested on the 18th day based on the peak levels of isoflavones content and frozen immediately and stored at -80°C (Akitha Devi and Giridhar 2014). Expression of genes selected viz., CHS7, CHS8, IFS1, and IFS2 from the isoflavones biosynthetic pathway were quantified by qRT-PCR. The gene specific primers were designed using the Integrated DNA technology (IDT) online software and synthesized from IDT (Belgium). The list of gene-specific primers used is shown in Table 2.

Isoflavones extraction

The Soybean isoflavones were extracted from seeds and callus as described by Sakthivelu et al. (2008) with minor modifications. 2 g of Soybean seed with the seed coat was mixed with 2 mL of 0.1 N HCl and 10 mL of acetonitrile (ACN) in a 125 mL screw-top flask, stirred for 2 h at room temperature, and filtered through Whatman no. 42 filter paper. The filtrate was dried in a vacuum evaporator and re-dissolved in 10 mL of 80% HPLC grade methanol and re-dissolved

Table 2 Primer pairs used in quantitative RT-PCR

Gene	Enzyme name	Forward primer (5'-3')	Reverse primer(5'-3')
<i>CHS7</i>	Chalcone synthase 7	AACCCACCAAACCGTGTGTGAT	CTTGTCACACATGCGCTGAAAT
<i>CHS8</i>	Chalcone synthase 8	GCACACCTTCATTTCAACCTC	ACATGCGCTGGAATTTCTCT
<i>IFS1</i>	Isoflavone synthase 1	GGGCCCTCAAGGACAAATA	CTGCGATGGCAAGACACTAC
<i>IFS2</i>	Isoflavone synthase 2	AAACCAAGGACGAGAACACG	TGGCCACTGAGCTATCATAG
<i>Actin</i>	<i>Actin</i>	TCCCAGTATTGTTGGCCGA	TTCCATGTCATCCCAGTTGCT

samples were filtered through 0.45 µm filter unit (Cameo 13 N syringe-filter, nylon).

Extraction of isoflavones from callus cultures: Callus mass (400 mg) from each treatment was ground finely and then extracted with 2 mL concentrated HCl and 10 mL ethanol in a boiling water bath for 2 h using a standard method (Vyn et al. 2002). The resultant suspensions were then cooled and centrifuged at 111800 g for 10 min, and the supernatant was further filtered by a syringe filter (Whatmann 0.5 µm, 13 mm diameter).

HPLC analysis

The HPLC analysis was conducted as described by Sakthivelu et al. (2008). The Shimadzu LC 20-AD high-pressure liquid chromatograph equipped with a dual pump and a UV detector (model SPD-10A) was used to separate, identify, and quantify isoflavones from the samples. Separation of isoflavones was achieved by a Bondapak C18 reversed phase HPLC column (150 mm × 4.6 mm and 5 µm internal diameter), and 20 µL samples were injected using a Rheodyne 7125 injector. A linear HPLC gradient was used with solvent A (0.1% glacial acetic acid and 5% acetonitrile in water) and solvent B (0.1% glacial acetic acid in acetonitrile). HPLC program Solvent B was initiated from 10 to 14% B over 10 min, then increased to 20% over 2 min, maintained at 20% for 8 min continued to increase to 70% over 10 min, maintained at 70% for 3 min and then returned to 10% at the end of the 34 min running time. The flow rate of the solvent was kept at 1 mL/min. The wavelength of the ultraviolet (UV) detector was set at 260 nm. Solvent ratios were expressed on a volume basis.

Calibration curves were obtained for each standard with high linearity ($r > 0.995$), by plotting the standard concentration as a function of the peak area, obtained from HPLC

analyses with 20 µL injections. Triplicate injections were analysed for each concentration of standards.

RNA isolation and cDNA synthesis

Total RNA was isolated from 100 mg samples with TRIZOL reagent as per manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Prior to DNase, the RNA concentration and quality was determined using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA), and the total RNA purity was determined with A260/A230 and A260/A280 ratios. The first-strand cDNA synthesis was carried out using 1 µg of DNase-treated total RNA in 20 µl reaction mix was performed using the GeNei™ M-MuLV RT-PCR Kit (GeNei, Bangalore, India).

Quantitative real-time PCR (qPCR) analysis

qPCR was carried out in a 10 µl reaction volume, which includes 2 µl of diluted cDNA, each primer at 5 µM, and 5 µl of 2X SsoFastEvaGreenSupermix (Bio-Rad Inc., CA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Inc., CA, USA). The qPCR program steps included an initial denaturation step of 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. A melt curve was obtained from 65 to 95 °C, with an increment of 0.5 °C at every 5 s. To normalize the gene expression, *Actin* was used as an internal control. Relative gene expression was calculated according to a $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The fold change in each target gene was compared with a control, which was set to 1 and also the qPCR was done in triplicates.

Table 3 Isoflavones concentration in different seed growth stages of selected soybean varieties (micrograms per gram of dry weight) under abiotic elicitor 10 µM Methyl jasmonate treatment

Variety ^a	Growth stage	Methyl jasmonate 10 µM							
		Total daidzein (µg/g (dw))		Total glycitein (µg/g (dw))		Total genistein (µg/g (dw))		Total isoflavone content (µg/g (dw))	
		<i>C</i>	<i>E</i>	<i>C</i>	<i>E</i>	<i>C</i>	<i>E</i>	<i>C</i>	<i>E</i>
JS-335	R5 (e)	80.6 ^e	88.44 ^f	12.6 ^e	13.75 ^d	50.2 ^e	55.395 ^e	138.4 ^e	157.59 ^e
	R6	105.6 ^{de}	159.65 ^f	40.6 ^{bc}	73.795 ^{cd}	166.9 ^d	262.66 ^{de}	283.6 ^d	496.1 ^{de}
	R7 (m)	165.9 ^c	456.17 ^d	37.8 ^{cd}	105.48 ^{bc}	272.2 ^c	709.68 ^c	462.5 ^c	1271.3 ^c
	R8	270.3 ^{ab}	1012.4 ^b	39.5 ^c	148.13 ^b	443.5 ^b	1663.4 ^b	753.5 ^b	2823.9 ^b
MAUS-2	R5 (e)	114.2 ^d	132.19 ^f	17.6 ^d	20.82 ^d	70.3 ^{de}	82.185 ^{de}	196.3 ^{de}	235.19 ^e
	R6	158.8 ^{cd}	264.94 ^e	65.5 ^a	123.13 ^{bc}	225 ^{cd}	387.58 ^d	376.4 ^{cd}	775.64 ^d
	R7 (m)	267.9 ^b	861.76 ^c	49.8 ^b	153.54 ^{ab}	467.8 ^{ab}	1482.5 ^b	780.5 ^{ab}	2497.8 ^b
	R8	359.2 ^a	1496.1 ^a	62.3 ^{ab}	218.8 ^a	626.9 ^a	2448.7 ^a	1048.6 ^a	4163.5 ^a

Different alphabets above the bars indicate a significant difference between the treatment. Treatment showing the same alphabets is not significantly different at p -value 0.05. Values calculated using one way ANOVA followed by Tukey's multiple range test

C Control, *E* Elicitor treatment

Statistical analysis

Isoflavones analysis in Soybean seed developmental stages by HPLC was performed in triplicates. The data were analysed statistically by SPSS 17.0 using one-way analysis of variance (ANOVA), and the difference between the means of the sample was analysed by the least significant difference (LSD) test at a probability level of 0.05.

Results and discussion

The isoflavones content (on a dry weight basis) of control seeds (untreated) is found to be maximum in MAUS-2 soybean variety, wherein the highest levels of daidzein (359.2 µg/g), glycitein (62.3 µg/g) and genistein (626.9 µg/g) were recorded, respectively. A positive correlation of total isoflavones (TI) content and growth stages was found during the control seed development (Table 1). These results obtained in our study were further supported by the report of Kim and Chung (2007), who noticed an elevation in TI content during the growth advancement stages (R5–R8). Significant rise in total isoflavones concentration was observed for JS-335 (from 138.4 to 753.5 µg/g which is 5.4-fold) and MAUS-2 (from 196.3 to 1048.6 µg/g which is 5.3-fold), respectively, from R5 to R8 stage. In early R5 stage, MAUS-2 had the maximum total isoflavones (196.3 µg/g), total daidzein (114.2 µg/g), total glycitein (17.6 µg/g) and total genistein (70.3 µg/g). However, JS-335 had the lowest level of total isoflavones (138.4 µg/g), total daidzein (80.6 µg/g), total glycitein (12.6 µg/g) and total genistein (50.2 µg/g). The same trend was observed in R6, and R7, until complete maturity, where the level of most isoflavones forms was at the highest in MAUS-2 than JS-335.

The R6 and R7 stage seed of MAUS-2 showed an increase of 2.8 and 1.3 fold TI in comparison to matured seeds. The data presented in Table 3 showed a maximum level of isoflavones forms were present between R5 and R6 stages, where the minimal increase of isoflavones forms was found between R7 stage and complete maturity.

Influence of elicitor treatment on isoflavones content of soybean seeds

Biotic elicitor *A. niger* at 0.1% triggered total isoflavone content of MAUS-2 seeds by 3.1, 3.6 and 5.9 folds compared to control R6 (1166.84 µg/g DW), R7 (2809.8 µg/g DW) and R8 (6186.74 µg/g DW) stage seeds (Table 4). However, the response was slightly less for 10 µM SA treatment followed by 10 µM MJ elicitation. The overall total isoflavone content of JS-335 seeds was significantly low compared to that of MAUS-2 at respective elicitor treatment. Interestingly, for

Table 4 Isoflavones concentration in different seed growth stages of selected soybean varieties (micrograms per gram of dry weight) under biotic elicitor 0.1% *Aspergillus niger* treatment

Variety ^a	Growth stage	Aspergillus niger 0.1%			Total daidzein (µg/g (dw))			Total glycitein (µg/g (dw))			Total genistein (µg/g (dw))			Total isoflavone content (µg/g (dw))		
		E			C			E			C			E		
		C	E	E	C	E	E	C	E	E	C	E	E	C	E	E
JS-335	R5 (e)	80.6 ^c	59.94 ^g	61.31 ^f	12.6 ^e	108.29 ^c	180.62 ^b	50.2 ^e	44.895 ^f	138.4 ^e	166.14 ^g					
	R6	105.6 ^{de}	276.99 ^{ef}	108.29 ^c	40.6 ^{bc}	120.98 ^{bc}	70.3 ^{de}	166.9 ^d	409.71 ^e	283.6 ^d	794.99 ^f					
	R7 (m)	165.9 ^e	531.665 ^d	120.98 ^{bc}	37.8 ^{cd}	180.62 ^b	225 ^{cd}	272.2 ^c	828.16 ^d	462.5 ^c	1480.80 ^d					
	R8	270.3 ^{ab}	1305.865 ^b	180.62 ^b	39.5 ^c	23.32 ^f	467.8 ^{ab}	443.5 ^b	1980.36 ^b	753.5 ^b	3466.85 ^b					
MAUS-2	R5 (e)	114.2 ^d	156.185 ^{fg}	23.32 ^f	17.6 ^d	179.12 ^b	70.3 ^{de}	70.3 ^{de}	94.68 ^f	196.3 ^{de}	274.19 ^g					
	R6	158.8 ^{cd}	408.44 ^d	179.12 ^b	65.5 ^a	179.12 ^b	225 ^{cd}	225 ^{cd}	580.71 ^e	376.4 ^{cd}	1166.27 ^e					
	R7 (m)	267.9 ^b	961.26 ^c	179.54 ^b	49.8 ^b	179.54 ^b	467.8 ^{ab}	467.8 ^{ab}	1668.5 ^c	780.5 ^{ab}	2809.3 ^c					
	R8	359.2 ^a	2860.0 ^a	48.8 ^a	62.3 ^{ab}	48.8 ^a	626.9 ^a	626.9 ^a	2841.5 ^a	1048.6 ^a	6186.3 ^a					

Different letters are significant among cultivars ($p < 0.05$). C Control, E Elicitor treatment

total isoflavones content 10 μM SA showed highest performance (2.8, 3.2 and 4.6 folds compared to control) that to that of 0.1% AN (2.5, 3.5 and 5 folds compared to control) followed by MJ (1.75, 2.75 and 3.75 folds compared to control) as in case of JS-335 seeds at R6 to R8 stages. The trend for total daidzein, total glycitein and total genistein under respective elicitor treatments were appears to be same to that of total isoflavones content.

Differential expression of isoflavone biosynthetic genes during seed developmental stages in *G. max*

Soybean seeds are the major source of isoflavones for human consumption, most of the research has been focused on understanding the biosynthesis and the accumulation of these isoflavones in seeds (Dhaubhadel et al. 2007). In the present study, two varieties, JS-335 (widely cultivated) and MAUS-2 (high isoflavonoid content) were selected to analyse the differential expression of isoflavones biosynthetic genes during late seed developmental stages (R5–R8). Expressions of major genes of isoflavones biosynthesis, such as *CHS7*, *CHS8*, *IFS1* and *IFS2* were primarily analysed using qPCR. Actin was selected as a house-keeping gene in the study due to its stability in the gene expression (Chen et al. 2009).

The seed developmental stages, R5L (late stage), R6, R7 and R8 of JS-335 and MAUS-2, are depicted in Fig. 1a, b, respectively. The expression pattern of *CHS7*, *CHS8*, *IFS1*, and *IFS2* were analysed by qPCR in seed developmental stage of both varieties (Fig. 1e, f). Increase in the progression of four genes expression in the order of *CHS7*, *CHS8*, *IFS1*, and *IFS2* during developmental stages from R5L–R7 was observed in both the varieties. (Fig. 1e, f). Among the varieties, the up-regulation of 1.24-fold was observed in *IFS1* expression at the R7 stage of MAUS-2 (Fig. 1e). In JS-335 variety, the expression of *IFS1* (0.98-fold) reaches maximum at the R7 stage, followed by *IFS2* (0.56-fold). Besides, there was a decline in the expression of all the four genes in the matured seeds (R8) (Fig. 1e, f). Likewise, in MAUS-2 variety, there was a stepwise increase in the expression level from R5L to R7 seed developmental changes (Fig. 1e, f) and the expression level was maximum for *IFS1*, followed by *IFS2*, *CHS8* and *CHS7*. An up-regulation of *CHS7*, *CHS8*, *IFS1* and *IFS2* expression was observed at R7 stage of MAUS-2 than JS-335 variety (Fig. 1e, f).

The up-regulation of genes during the R7 developmental stage revealed that the accumulation of isoflavones in Soybean embryos increases as seeds matures. After R7, Soybean seed slowly starts losing water, shrinks its size and attains dormant condition. The level of seed isoflavones reaches the maximum level at mature seeds (Dhaubhadel et al. 2003).

CHS7 and *CHS8* expression in the seed developmental stages were higher in MAUS-2 and JS-335, suggested that these two genes have a critical role in the accumulation of isoflavones. The same phenomenon is observed in gene expression analysis during the embryo development in Soybean. Where the 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). The highest level of *IFS1* and *IFS2* expression pattern was observed in MAUS-2 than JS335 variety and this confirms the higher accumulation of total isoflavones in MAUS-2. Though, the earlier studies have reported that the higher *IFS1* expression was observed in the root and seed coat and *IFS2* was expressed in embryos and pods, and also in elicitor-treated or pathogen-challenged tissues (Dhaubadhal et al. 2003).

Differential expression of isoflavones biosynthetic genes under the influence of elicitors in *G. max* developing seeds

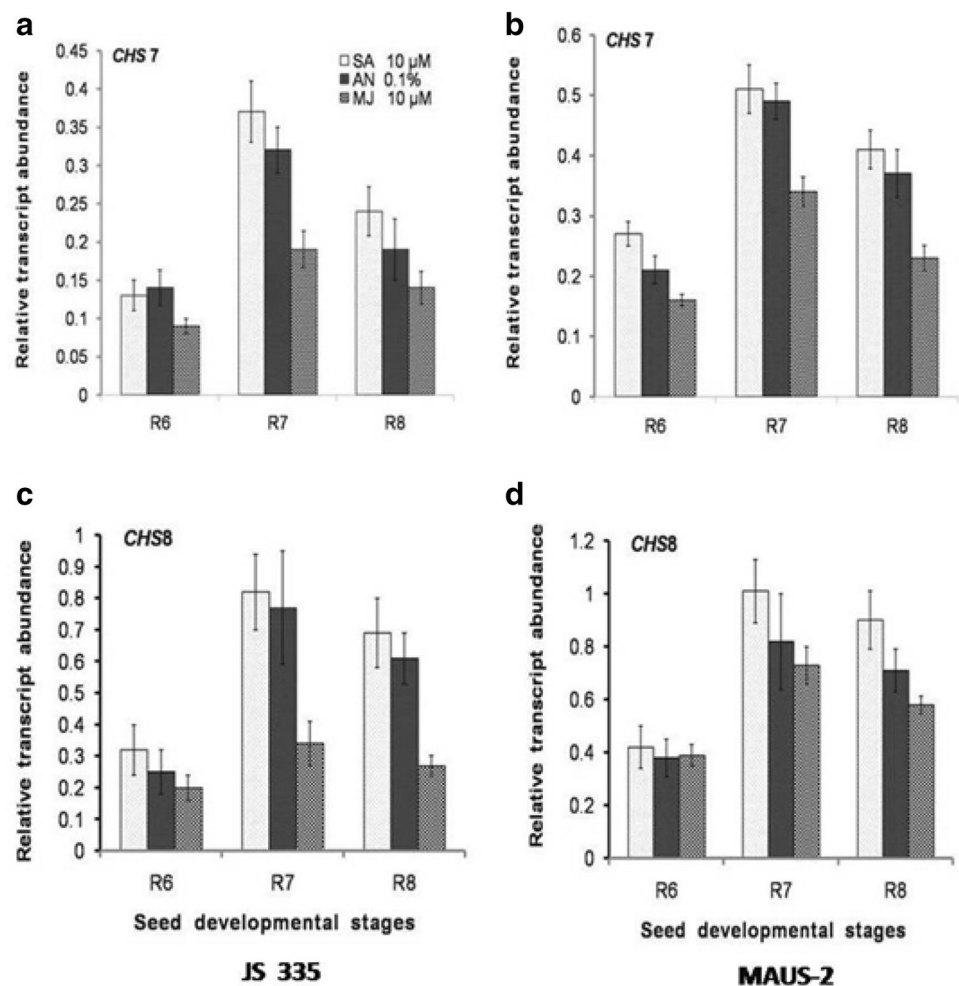
To study the correlation of isoflavones accumulation and regulation of isoflavones biosynthesis which occur during elicitor treatment, the expression profiling of major genes were investigated. The phenylpropanoid pathway genes for qPCR analysis were chosen on the basis of earlier literature (Chen et al. 2009; Gutierrez-Gonzalez et al. 2010; Chenunapati et al. 2012).

The key genes that encode enzymes leading to isoflavones production were selected for qPCR analysis. Each primer set designed to amplify a specific gene under the optimized qPCR conditions and the amplicon was sequenced. The sequence identity was confirmed through BLAST. The BLAST showed 100% homology for *CHS7*, *CHS8*, *IFS2* except for *IFS1*, which of showed 99.9% homology in JS-335 variety. The sequence of *IFS1* has been submitted to the NCBI database (Accession No. JZ845698).

The expression level of four key genes (*CHS7*, *CHS8*, *IFS1*, and *IFS2*) were investigated after foliar application of selected abiotic (SA 10 μM and MJ 10 μM) and biotic (*A. niger* 0.1%) elicitors in two Soybean varieties (JS-335 and MAUS-2) during R6–R8 stages (Figs. 2, 3). Both the varieties have responded differently under the influence of elicitors. *IFS2* expression was up-regulated under the influence of SA, at R7 stage with 5.21-fold and 4.68 fold in JS-335 and MAUS-2, respectively (Fig. 2c, d). However, *IFS1* abundant expression was observed due to the *A. niger* treatment at R7 stage with 3.98- and 3.21-fold in MAUS-2 and JS-335, respectively (Fig. 2a, b).

Expression of *CHS7* and *CHS8* showed maximum up-regulation at R7 stage in presence of SA 10 μM , 0.51-fold and 1.01-fold in MAUS-2 variety (Fig. 3b, d). At R7 stage

Fig. 3 Expression analysis of isoflavones biosynthetic genes (*CHS7* and *CHS8*) during elicitor treatment in Soybean varieties. **a, b** Relative transcript abundance of *CHS7* in response to elicitors; SA (10 μ M), *A. niger* (0.1%) and MJ (10 μ M) in JS-335 and MAUS-2 seed developmental stages. **c, d** Relative transcript abundance of *CHS8* in response to elicitors; SA (10 μ M), *A. niger* (0.1%) and MJ (10 μ M) in JS-335 and MAUS-2 seed developmental stages



of JS-335 variety, the expression of *CHS7* and *CHS8* were upregulated with a fold increase of 0.37 and 0.82-fold (SA 10 μ M) (Fig. 3a, c). Similar to the differential expression pattern observed during seed development, there is a stepwise increase in the expression of *CHS7*, *CHS8*, *IFS1*, and *IFS2* from R6 to R7 followed by a decline in R8 in both varieties.

Several researchers have studied the expression pattern of isoflavones biosynthetic genes in various biotic, environmental and physical stresses in Soybean (Gutierrez-Gonzalez et al. 2010; Chennupati et al. 2012). Gutierrez-Gonzalez et al. (2010) reported that the reduction in seed isoflavones concentration observed during a period of water stress was correlated with the expression of three key genes involved in isoflavones synthesis (*CHS7*, *CHS8*, and *IFS2*). Chennupati et al. (2012), stated that the effect of high-temperature stress leads to Soybean isoflavones accumulation during the seed development, as well as the expression of four key genes involved in isoflavones synthesis (*IFS1*, *IFS2*, *CHS7*, and *CHS8*). However, the results suggested that there was no clear correlation between isoflavones concentration and gene expression.

A very few reports have explained the alteration in the expression pattern of isoflavones biosynthetic genes under the influence of elicitors. This is the first report that investigated the regulation of the genes encoding key enzymes of isoflavones biosynthesis by SA, MJ and *A. niger* treatments, leading to the gene induction in Indian Soybean cultivars. Previously, Chen et al. (2009) investigated the gene expression alteration of 14 genes encoding isoflavones in Soybean sprouts (three cultivars), as well as isoflavone concentrations, following treatment with chitosan. In general, MJ and SA are the key signaling molecules, modulating several physiological events such as defense response to the environmental stresses in plants (Creelman and Mullet 1997; Draper 1997). Significant evidences have showed that the exogenous application of MJ has led to the increase of various classes of secondary metabolites in several plants (Modolo et al. 2002; Wei 2010). As suggested by earlier reports, the use of MJ activates the genes of phenylpropanoid (PP) pathway (Dixon and Paiva 1995). The various expression of *IFS* genes (*IFS1* and *IFS2*) is responsible for the changes in the level of isoflavones in

seeds under the elicitor treatment (Dhaubhadel et al. 2007; Cheng et al. 2008). Overall, the results of the present correlate isoflavones augmentation and up-regulation of biosynthetic genes under the elicitor treatments.

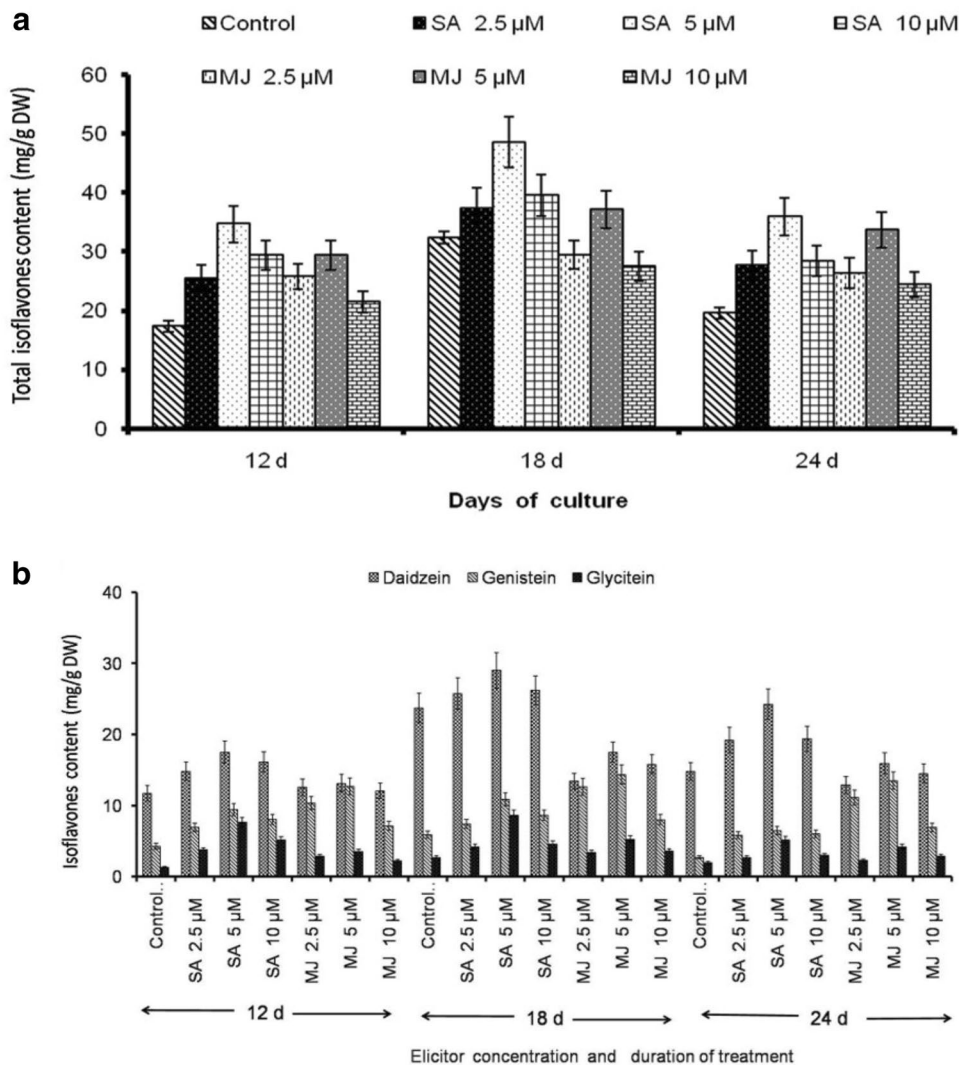
The expression of IFS1 (relative transcript abundance) was prominent for 0.1% AN treatment in both JS-335 and MAUS-2 varieties followed by 10 μM SA and 10 μM MJ at R6 to R8 stages. But, IFS2 expression was more for SA treatment followed by MJ and AN at R6 to R8 stage of seed development in both JS-335 and MAUS-2 varieties. It is quite interesting to note that, both CHS7 and CHS8 genes transcript abundance was more under SA treatment followed by AN and MJ elicitor treatments in both soybean varieties at R6-R8 stages. A glance at all these selected genes expression pattern at different stages of seeds development and their total isoflavones content appears to be having similar trend. As the IFS1 and IFS2 genes expression was very high compared to CHS7 and CHS8, they might be having

a pivotal role in overall isoflavones content changes. The overall, relative transcript abundance of CHS7 and CHS8 was quite low compared to IFS1 and IFS2 genes; however, the difference was moderate among the three elicitors viz, SA, MJ and AN.

Influence of elicitors on isoflavones content of in vitro cell suspension cultures of *G. max*

All the elicitors significantly influenced isoflavones content in callus suspension cultures at different time intervals when compared to control. Highest isoflavones content was found in 18th day old cultures. Abiotic elicitors SA and MJ positively influenced the TI content than biotic elicitor *A. niger*. This is obviously evident from the results that amongst the various elicitors, TI content was considerably high in SA supplemented cultures than control. SA (5 μM) treated cultures showed a maximum TI content (48.55 ± 3.89 mg/g

Fig. 4 a Influence of abiotic elicitors SA and MJ on total isoflavones content and b. Effect on daidzein, genistein and glycitein content in callus suspension cultures of *G.max*. Values are means ± S.D with significant at $p < 0.05$



DW) (Fig. 4a) that includes daidzein (23.74 ± 2.07 mg/g DW), genistein (5.92 ± 0.48 mg/g DW) and glycitein (2.71 ± 0.24 mg/g DW) (Fig. 4b). Therefore, an increase of 39% TI than control (32.37 ± 2.93 mg/g DW) cultures were examined (Fig. 4a). Lower concentration of SA significantly influences the TI content and higher concentration of SA ($10 \mu\text{M}$) mimic the enhancement. In contrast, a remarkable increase in the isoflavones content was exhibited with an increase in MJ concentration. This was evident with a rise of 13.72% and 27.45% in TI content in $5 \mu\text{M}$ and $10 \mu\text{M}$ MJ, respectively (Fig. 4a). Results of the present study suggested that the elicitor concentration, incubation time as well as growth conditions were crucial for elicitation strategy as per earlier reports (Gueven and Knorr 2011; Sivanandhan et al. 2013; Theboral et al. 2014). Still the results were in contrast with earlier reports that MJ was more efficient than SA for the isoflavones accumulation soybean callus suspension cultures (Gueven and Knorr 2011; Theboral et al. 2014).

The various biotic elicitors, 0.1% *A. niger* treated cultures showed an increase of 27.63% than control (Fig. 5a). The TI content in *A. niger* treated cultures were 42.75 ± 4.12 mg/g DW that includes daidzein (16.31 ± 1.68 mg/g DW), genistein (19.26 ± 1.54 mg/g DW) and glycitein (7.18 ± 0.58 mg/g DW) (Fig. 5b). In contrast to abiotic elicitors, *A. niger* and chitosan positively influence the genistein content. Also, it is evident from the study that the lower concentration of *A. niger* and *R. oligosporus* has an augmenting effect than the higher concentration. All concentration of chitosan excluding $10 \mu\text{g}$, negatively influence the TI content. (Komaraiah et al. 2002) reported that *Rhizopus* and *Aspergillus* sp. elicit twofold to threefold plumbagin production in *Plumbago rosea* L. suspension cultures. In the present study, we found that the *A. niger* elicitor proved to be better for the induction of isoflavones than *R. oligosporus*.

Fig. 5 a Influence of biotic elicitors on total isoflavones content. b Effect on daidzein, genistein and glycitein content in callus suspension cultures of *G.max*. Values are means \pm S.D with significant at $p < 0.05$

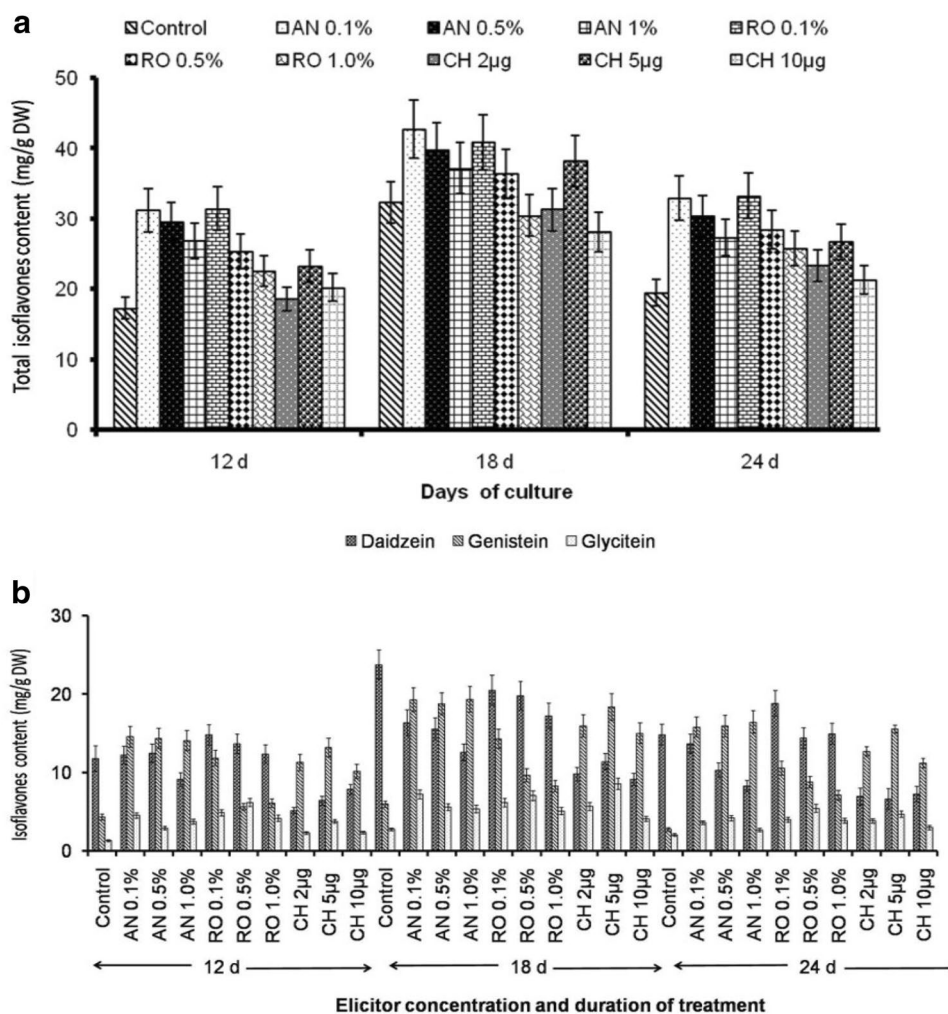
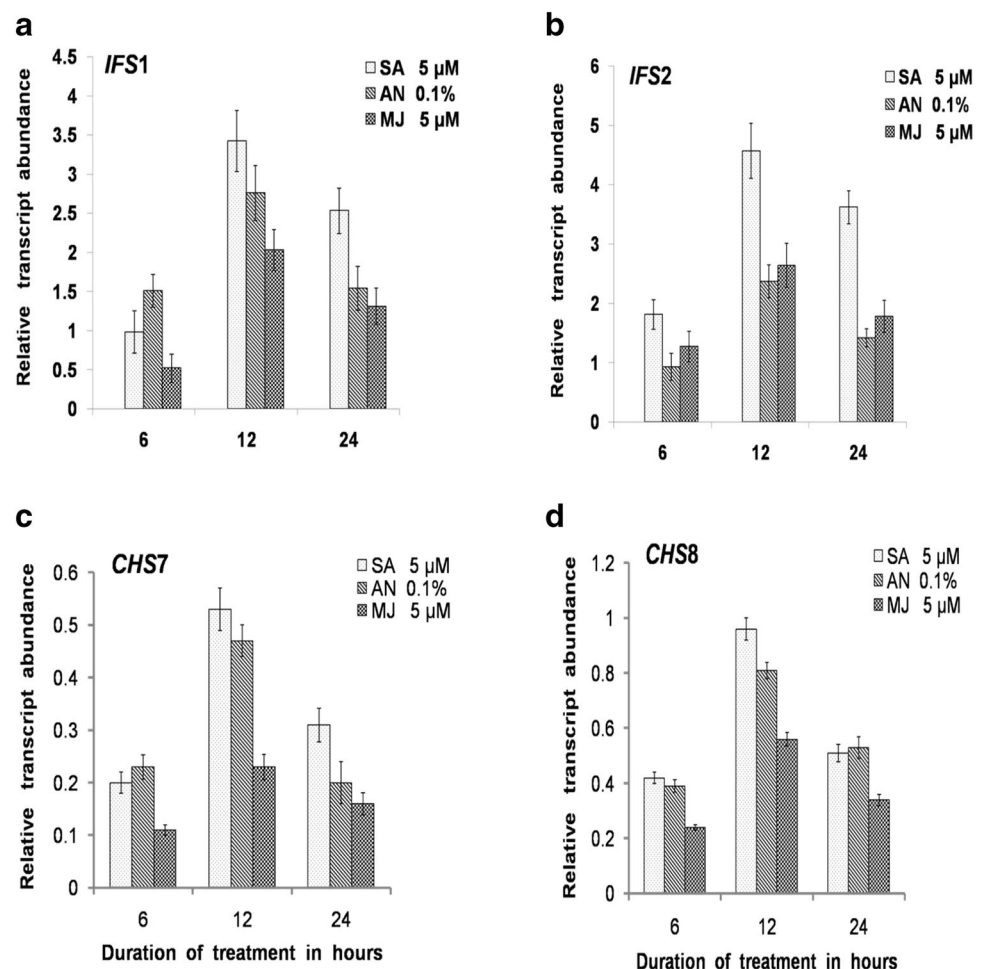


Fig. 6 Expression analysis of isoflavones biosynthetic genes during elicitor treatment in JS-335 *G. max* callus suspension cultures. **a** Relative transcript abundance of **a** IFS1 **b** IFS2 **c** CHS7 **d** CHS8 in response to elicitors SA (5 μ M), *A. niger* (0.1%) and MJ (5 μ M) treatment



Differential expression of isoflavones biosynthetic genes under the influence of elicitors in in vitro cultures of *G. max*

In the present investigation, accumulation of isoflavones was correlated with the expression of isoflavones biosynthetic genes during elicitor treatment in callus suspension cultures of JS-335 by qPCR. To determine the optimum elicitors, concentration for isoflavones augmentation and its influence on isoflavones gene expression were investigated. The up-regulation of isoflavones biosynthetic genes during elicitation in in vitro cultures was due to the elicitors or other environmental factors. On the basis of preliminary experiments carried out in this regard, the best concentration of abiotic elicitors SA (5 μ M), MJ (5 μ M) and biotic elicitor *A. niger* (0.1%) were selected.

Production of the isoflavones was maximum on the 18-day-old callus suspension culture in control as well as elicitor-treated cultures. On this basis, the expression of four genes *CHS7*, *CHS8*, *IFS1* and *IFS2* were quantified on the 18-day-old culture at 6 h, 12 h, and 24 h intervals. Expression of four genes was detected at all the three intervals, wherein

the up-regulation was maximum at 12 h for all the genes and the treatments (Fig. 6). The *IFS2* and *IFS1* expression were up-regulated to the maximum, with 4.57- and 2.76-fold with SA 5 μ M (12 h), *A. niger* 0.1% (12 h), respectively (Fig. 6a, b). A maximum up-regulation of 0.53-fold and 0.96-fold was observed in *CHS7* and *CHS8* under the SA 5 μ M (12 h) treatment (Fig. 6c, d). The results also revealed that the up-regulation of isoflavones biosynthetic genes was correlated with the isoflavones accumulation under the influence of elicitors in callus suspension cultures. Furthermore, yeast extract elicits the accumulation of isoflavones via elevated level of L-phenylalanine ammonia lyase and chalcone synthase expression in the callus suspension of *Medicago truncatula* (Suzuki et al., 2005). In contrast, long-term drought stress in Soybean plants has been established to result in the down-regulation of *IFS2* gene coinciding with a decrease in isoflavone content (Gutierrez-Gonzalez et al. 2010). Similarly, drought stress exhibits profound influence on variations in physiological responses and isoflavone content in soybean (Akitha Devi and Gridhar 2015). Various biotic and abiotic elicitors are reported to be efficient in triggering metabolites

production in soybean suspension culture as shown recently for folic acid (Akitha devi et al. 2018).

Conclusion

In the present study, improvement of isoflavones content in Soybean seeds as well as callus suspension cultures was achieved in various elicitors' treatment. Expression of *IFS1*, *IFS2*, *CHS7*, *CHS8* differentially expressed during the seed development and selected biotic and abiotic elicitors in selected two seed varieties, is first of its kind and no such reports are known. Present studies unraveled significant expression levels of both *IFS1* and *IFS2* under stress, that is, a good sign for obtaining enhanced levels of isoflavones. With respect to the developed callus suspension cultures of soybean, the elicitor mediated differential expression of *IFS1*, *IFS2* and *CHS* along with the three type of isoflavones augmentation under respective elicitor treatment in JS-335 which is the commercially important soybean variety. This outcome could be useful in the future to look at the functionality of identified regulatory genes involved in isoflavones biosynthetic pathway.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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