Isoflavones have a potential role to play in off-flavour scavenging, with Isoflavone synthase 2 (IFS2) as a key player determining isoflavone accumulation in soybean seeds

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Abstract

Soybean (*Glycine max* (L.) Merr) is a nutrient-rich crop with limited consumer acceptability because of the presence of off-flavour. Owing to the ubiquity of isoflavones in soybean, their inherent antioxidant potential, and inhibitory effect on lipoxygenase activity, their sensorial attribute in mitigating off-flavour is currently being envisaged. We estimated the content and composition of isoflavones in 17 soybean cultivars and established the correlation between isoflavone profile and lipid peroxidation, which was measured in terms of Thiobarbituric acid (TBA) number and carbonyl value. The results showed a significant negative correlation of these parameters with the total isoflavone content and genistein: daidzein ratio. Besides, expression analysis of a key gene involved in isoflavone biosynthesis, i.e., isoflavone synthase (IFS1 and IFS2) in contrasting soybean genotypes during seed development revealed a higher expression of both isoforms in Bragg (High isoflavone) as compared to PUSA 40 (Low isoflavone) at different stages of seed development accompanied by more isoflavone accumulation. The cloning and *in silico* analysis of IFS isoforms revealed a more significant interaction with other biosynthesis enzymes by the IFS2, indicating its more substantial role in isoflavone synthesis. IFS2 overexpression thus can be a valuable target to increase isoflavone biosynthesis, thereby scavenging off-flavour.

Introduction

Soybean is the most widely cultivated legume crop around the globe. Primarily, it is used for edible oil extraction and as animal feed. The various nutrients in soybean make it an excellent candidate for human consumption (Liu 1997). Especially the soy protein is often considered of the highest quality amongst plant proteins due to its high protein digestibility corrected amino acid score (PDCAAS) comparable to animal proteins (Hughes et al. 2011). The presence of beany flavour impedes consumer acceptance of soybean and their products (Nedele et al. 2021). Lipoxygenase (LOX) is a significant contributor to this astringency. LOX is an iron-containing dioxygenase catalysing the oxidation of polyunsaturated fatty acids (PUFAs) with a cis-1, 4-pentadiene structure. As soybean has a very high content of PUFAs and LOX, it is highly susceptible to oxidation. This results in the generation of hydroperoxyl fatty acids. These hydroperoxyl fatty acids then get converted into volatile compounds like hexanal by hydroperoxide lyase (HPL). These compounds are the ones responsible for grassy-beany off-flavour. Inhibition of this off-flavour-producing pathway has been the target to reduce off-flavour. An inverse relation between the amount of vitamin E an potent antioxidant, and Thiobarbituric acid (TBA) number, an indicator of the extent of lipid peroxidation, has been observed, suggesting increased antioxidants content may help improve flavour characteristics (Dahuja and Madaan 2004). Isoflavones are another class of antioxidative secondary metabolites almost exclusively present in the plants belonging to the *Papilionaceae* family, with soybean as the richest source (Abotaleb et al. 2018). Isoflavones impart various human health benefits like prevention of osteoporosis and anticancerous effects due to their structural similarity with female estrogens and antioxidant effects (Křížová et al. 2019; Yamagata 2019). Depending upon the structure, different isoflavones vary in these properties.

Isoflavones are a member of flavonoids where the position of one aromatic ring is shifted. Major isoflavones in soybean are genistein, daidzein and glycitein, present predominantly in the glycosidic forms: genistin, daidzin and glycitin, respectively, with malonyl and acetyl decorations at the 6^{th} position of glucose. Isoflavone biosynthesis takes place via a branch of the phenylpropanoid pathway. Isoflavone synthase (IFS), a cytochrome P450 monooxygenase bound to the endoplasmic reticulum, is a crucial enzyme involved in this pathway. It causes hydroxylation coupled with aryl group migration resulting in shifting of ring position. In soybean, two isoforms (IFS1 and IFS2) of this enzyme are present (Jung et al. 2000).

Reports suggest that isoflavones inhibit the activity of LOX, a principal enzyme involved in off-flavour generation. They act by reducing the active state of ferric iron to ferrous form and function as an antioxidant for their ability to scavenge free radicals (Mahesha et al. 2007; Tsen et al. 2016). Tewari et al (2014) reported an increase in the level of isoflavones upon treatment with low doses of gamma irradiation. Also, they observed a significant decrease in the level of off-flavour determining parameters upon irradiation treatment like LOX activity, carbonyl value and TBA number. The present study focused on studying the role of isoflavones in preventing off-flavour generation in soybean. The variation in the isoflavone profile of 17 Indian soybean cultivars was measured, and their correlation with off-flavour determining parameters was also calculated. In addition, cloning, gene expression analysis of IFS isoforms in developing soybean seeds and *in-silico* analysis were carried out.

Materials And Methods

2.1 HPLC analysis for determining isoflavone content and composition

17 soybean genotypes were selected for estimation of isoflavone content using HPLC. Among these, ten genotypes had yellow-coloured seed coats, while the remaining seven possessed black-coloured seed coats. Seeds were generously procured from Dr S.K. Lal, Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi. Two contrasting varieties were selected for further molecular studies based on the total isoflavone content. Three biological replications were used throughout the experiment. Preparation of sample extract, standard curve and HPLC conditions were performed as per Kumar et al (2010) with slight modifications. The chromatography was performed in a Waters 2695 chromatograph (Waters Corp., Milford, MA, USA) with a 2998 Photodiode array (PDA) detector and C_{18} silica column as the stationary phase. A binary gradient of acetonitrile (ACN) and water was used as the mobile phase with a flow rate of 1.5 ml/min. 13% ACN was used for the initial 1 min. of HPLC separation with the ACN concentration reaching 30% at 20 min. and again reducing to 13% at the end of the run, i.e., 25 min.

2.2 Estimation of TBA number

Upon lipid peroxidation, malondialdehyde (MDA), a degradation product of lipid hydroperoxides, is formed. The level of MDA formed is measured in terms of TBA number. An adduct is formed upon the reaction of MDA with TBA. The adduct gives a pink colour whose absorbance is estimated at 532nm (Ohkawa et al. 1979). Briefly, about 400 mg of overnight soaked soybean seeds were homogenised with 4.0 ml of distilled water followed by centrifugation at 13,000 rpm for 35 minutes to get the clear extract. 0.2 ml of this extract was mixed with 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA and kept in boiling water bath for 60 minutes. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol-pyridine (15:1) were added, and the tubes were shaken vigorously. It was then centrifuged at 4000 rpm for 10 minutes. The pink-coloured organic layer formed at the top was separated from the aqueous phase, and absorbance was taken at 532nm. The standard curve was prepared using tetra ethoxy propane (TEP) ranging from 10 to 70 nmoles, and the level of lipid peroxides was expressed as nmoles of MDA/g fresh weight.

2.2 Assessment of carbonyl value

The carbonyls were estimated using the method described by Henick et al (1954). The 2,4-dinitrophenyl hydrazine reacts with the aldehydes and ketones to form their 2,4-dinitrophenylhydrazone derivatives converted to wine red coloured complex under alkaline conditions by KOH. Carbonyls were determined by measuring the absorbance of the coloured complex at 480 nm. Briefly, the sample extract (0.5 ml) prepared above was mixed with 0.5 ml of 2, 4-dinitrophenylhydrazine (0.05%) and heated in a water bath at 60°C for 30 minutes. After cooling, 1.0 ml of KOH (4%) was added, and a characteristic wine-red colour appeared. Ten minutes after the appearance of the wine-red colour, absorbance was taken at 480nm. Carbonyls were estimated by using the formula ($A = E_{max} \times C \times L$); where A= Absorbance, E max = 2.72 x 10^4 (L mol^{-1} cm^{-1}), C = Concentration (mol L^{-1}), L = Path length (cm).

2.3 Quantitative expression analysis of isoflavone synthase gene

Using the isoflavone content data estimated above, we selected two extremely contrasting genotypes viz Bragg (High isoflavone) & PUSA 40(Low isoflavone). Real-time expression analysis of IFS1 and IFS2 was performed at 35, 45 and 55 days after flowering (DAF) in contrasting soybean genotypes grown in the open field at ICAR-IARI, New Delhi. RNA was isolated using the Trizol method, and the first strand of cDNA was synthesised from it. All the primers for qPCR (~150bp) were designed using PrimerBlast software available at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The housekeeping gene actin 2/7 was used as an internal control for data normalisation **(S1)**. qPCR was carried out using BioRad CFX96 machine in a reaction volume of 20 ml containing SYBR^{R} Green JumpStart^{TM} Taq ReadyMix^{TM} (*Sigma*), Forward primer (10 mM), reverse primer (10 mM) and ten ng/ml of cDNA using conditions of 94°C for 5 min, then 40 cycles of 94°C for 15s, 55°C for 30 s and 72°C for 45s. The relative expression level was calculated following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Standard errors and standard

deviations were calculated from 3 technical and biological replicates. Seed samples of 35 DAF were considered as the calibrator. The resulting PCR products were also analysed by agarose gel electrophoresis to determine the specificity.

2.4 Cloning of IFS1 and IFS2 cDNA

According to the manufacturer's protocol, total RNA was isolated from soybean seeds using RNAiso plus (TAKARA). IFS1 and IFS2 full-length CDS were amplified from the DS2706 cultivar of soybean. Both forward and reverse primers were designed using PrimerBlast. The amplified PCR products were gel eluted using Quick Guide PCR purification kit (Solgent) and kept for ligation in a pENTR/D-TOPO vector, an entry vector for the Gateway® System, for 30 minutes at 23^{o}C in a 2:1 molar ratio of insert and vector. 3 µl of ligation mix was transformed into the TOP10 chemically competent cell of *E. coli*. Six colonies from each clone were inoculated for plasmid isolation. The confirmation was done by (A) PvuII digestion, (B) PCR and (C) Sanger sequencing.

2.5 *In silico* analysis

The nucleotide and deduced amino acid sequences of IFS1 and IFS2 were compared through NCBI BLAST. The three-dimensional structures of the IFS1 and IFS2 proteins from their amino acid sequence were generated using RoseTTAFold, a deep learning-based structure prediction tool (https://robetta.bakerlab.org). This algorithm is a non-homology-based method which works by pattern detection in protein sequences, interaction amongst the amino acid residues and probable protein tertiary structure, and simultaneously determining everything ((Baek et al. 2021). These predicted models were tested using the MolProbity server (Williams et al. 2018), where the favoured rotamers and Ramachandran plot distribution with ϕ and ψ angles were analysed. After testing predicted structures, the protein-ligand docking analysis was done via the CB-Dock2 tool. It is based on the blind docking principle using AutoDock Vina, and the protein structure was input as a PDB file, whereas the ligand was provided in the SDF format (Liu et al. 2020). The interactions of IFS1 and IFS2 with other proteins were predicted using STRING (Szklarczyk et al. 2015).

Results & Discussion

3.1 Content and composition analysis of isoflavones in soybean genotypes

A large amount of variation exists for isoflavone content and composition in the soybean genotypes (Azam et al. 2020), with malonylated derivatives of genistin and daidzin being the most dominant (Tepavčević et al. 2021). Health benefits (including bioavailability) of these different isoflavones and their lox inhibitors potential could also vary with structural characteristics. Due to the

prevalence of these substituted derivatives, we decided to undertake a comparison of isoflavone aglycones. For this, the acidic hydrolysis of isoflavones was carried out. 17 soybean genotypes were assessed for total isoflavone content and composition to explore its relationship with indices of off-flavour generation (TBA number and carbonyl value) in soybean. Enormous variation was observed amongst cultivars in the total isoflavone content and the content of individual forms of these isoflavones (**Table 1**). The total isoflavone content was expressed as µg/g of soy flour, ranging between 153.53 ± 7.22 (µg/g) for PUSA 40 to 1146.42 ± 43.47 (µg/g) for Bragg (**fig. 1**). Bragg and PUSA 40 were identified as the high and low isoflavone containing genotypes of soybean, respectively and thus selected for gene expression analysis of isoflavone synthase (IFS). Similarly, earlier studies have observed enormous variation in isoflavone content and composition for soybean genotypes grown in different ecoregions (Azam et al. 2020). PK 1042 and PK 416 were identified as the soybean genotypes having a maximum (1.64) and minimum (0.33) Genistein: Daidzein ratio, respectively.

3.2 The assessment of the extent of lipid peroxidation in different soybean genotypes

TBA number is a well-established measure of lipid peroxidation (Ganhão et al. 2011). It detects lipid hydroperoxides produced during this oxidation, and its amount is expressed in nmoles/g of seed. The soybean genotypes tested for TBA number ranged from 46.67 ±4.16 nmol/g for BS1 to 307.33 ± 8.08 nmol/g for UPSL 340 **(Fig. 2).** Similarly, the carbonyl value measures the number of aldehydes and ketone, which is associated with the generation of off-flavour. Its values were expressed in nmoles/g of seed and ranged from 378.95 ± 4.64 nmol/g for EC 109514 to 674.54 ± 4.64 nmol/g for UPSL 340 **(Fig. 3)**. The extent of correlation present between isoflavone content and parameters taken as indices of off-flavour was measured in terms of Pearson's correlation coefficients. The observed value for the correlation coefficient between total isoflavone content and carbonyl value was 0.089, and that of TBA number and total isoflavone content was 0.016 **(Table 2)**. Amongst the various soy isoflavones, genistein has been reported to have the maximum antioxidant activity (Mahesha et al. 2007). Therefore, the correlation of particular forms of isoflavones with these parameters is expected to differ. Hence, the correlation coefficients of off-flavour-determining parameters were also calculated for the genistein and genistein: daidzein ratio. The correlation between genistein content and carbonyl value was -0.12 and between TBA number and genistein content was -0.15. In the genistein: daidzein ratio, the value of the correlation coefficient for TBA number and carbonyl value were -0.41 and -0.42, respectively. The higher antioxidant potential of genistein compared to daidzein can be the reason behind this observation (Ruiz-Larrea et al. 1997). Genistein is also a more potent inhibitor of soybean LOX, with a half inhibitory concentration (IC50) value of 107µM compared to 140µM for daidzein (Mahesha et al. 2007; Vicaş et al. 2011). Previously, Dahuja and Madaan (2004) also observed an inverse relationship between the levels of antioxidant enzymes and the values of off-flavour determining parameters like TBA number and carbonyl value. So, the ability of genistein as an inhibitor of LOX and a general antioxidant seems to be responsible for a reduction in indices of off-flavour generation.

3.3 Quantitative gene expression analysis of IFS and isoflavone profile at three stages of seed development

Isoflavone synthase is the key enzyme of isoflavone biosynthesis responsible for synthesising isoflavone aglycone backbones. There exist two different isoforms of IFS in soybean. In order to determine the relative contribution of these isoforms towards isoflavone accumulation in soybean seeds, we carried out the gene expression analysis of IFS1 and IFS2 in two contrasting soybean genotypes, i.e., Bragg (high isoflavone) & PUSA 40 (low isoflavone) at 3 stages of seed development. Expression profiling suggests that the expression level of IFS1 increased with the advancement in the developmental stage in PUSA 40 **(Fig. 4)**. However, a non-linear and irregular trend was observed in the expression of IFS1 in Bragg; it first increased 2.34-fold from 35 DAF (Days After Flowering) to 45 DAF and then decreased to 2.22-fold at 55 DAF. An increasing trend was observed for IFS2 throughout Bragg's case, but expression level decreased with each developmental stage in PUSA 40. In quantitative terms, an overall 9.34-fold enhancement in the expression of IFS2 was observed in Bragg at 55 DAF compared to its expression at 35 DAF. In comparison, the expression reduced to about 6% of the value at 35 DAF in PUSA 40. These results align with the study by Dhaubhadel et al (2003), who observed the accumulation of IFS2 transcripts in later stages of seed development, but IFS1 expression stayed constant throughout. Gutierrez-Gonzalez et al (2010) also observed a more significant increase in IFS2 expression of up to 20-fold at 70 days after pollination compared to 30 days after pollination during seed development. IFS1, in contrast, only increased 4-fold in the same period. The corresponding levels of the total isoflavone contents at each stage were also determined using HPLC. Total isoflavone content in Bragg remained higher than PUSA 40 at all three stages of seed development.

Further, the total isoflavone content in Bragg showed an increase from 14.07 µg/gm to 78.4 µg/gm at 55 DAF, but in the case of PUSA 40, this increase observed was only about 2.19-fold from 35 DAF to 55 DAF. The expression of IFS2 matched the pattern of isoflavone accumulation in developing soybean seeds. However, no such correlation could be observed with expression levels of IFS1, as the two contrasting cultivars, despite having similar expression levels of IFS1, differed significantly in the isoflavone levels at all the stages of seed development, the difference in the isoflavone being more pronounced at 55 DAF. Thus, IFS2 may directly affect the isoflavone accumulation during the grain filling stage.

3.4 Cloning and *in-silico* analysis of IFS1 and IFS2

The results of qRT PCR analysis suggested a putative role of isoflavone synthase in the accumulation of isoflavones in soybean seeds, which encouraged us to delve deeper into the analysis of isoforms. Based on the sequence information available on NCBI, specific primers were designed for PCR amplification of IFS cDNA genes. The PCR products were run on an agarose gel, and the approximate size of the amplicon was 1566bp. (**Fig. 5a & b**) for both IFS1 and IFS2. The amplicon obtained was then gel eluted and cloned into the pENTR/D-TOPO vector. The cloning of IFS1 & IFS2 was confirmed using plasmid PCR and

restriction digestion (PvuII). PvuII restriction enzyme has two cut positions (174, 812) in the TOPO entry vector. We found a linear band of 2204bp (1566bp insert + 638bp vector) and 1942bp vector backbone in the case of both IFS1 and IFS2 (**Fig. 5c**). The released inserts were then sequenced commercially by the Sanger sequencing method (Chromus Pvt. ltd.). After that, the gene sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank (Accession no. KP843618.1 & KT581120.1). The 1566bp long IFS1 and IFS2 coding sequences showed 92.98% identity to each other with 110 nucleotides difference (**S2**). The amino acid sequence of IFS1 and IFS2 was also aligned, which showed 97.12 % identity to each other with 12 amino acid differences out of 521 amino acid residues (**S3**). The 3D models prepared for both IFS1 and IFS2 using RoseTTAFold showed over 94.8% and 96.5% of the residues in the most favoured region (**Fig. 6 & 7**). The IFS isoforms showed minor differences in their tertiary structures, but their predicted interactions with other proteins differed significantly. In the interaction study done using STRING, IFS2 intriguingly showed a maximum interaction with chalcone isomerase (CHI) with a score of 0.933 (**Fig. 8 & S4**), which was not the case for IFS1. Dastmalchi et al (2016) studied the interactions and subcellular localisation of major enzymes involved in isoflavone biosynthesis. This study's interesting finding was the interaction of Chalcone reductase (*Gm*CHR14) with *Gm*IFS2, but not with *Gm*IFS1. We also got a similar result in the *in-silico* analysis. *Gm*CHR14 is present upstream of the isoflavone synthase in the phenylpropanoid pathway responsible for the biosynthesis of isoflavones. A recent study conducted CRISPR mediated knockdown of IFS1 in which the mutants had nearly all the physiological processes intact, indicating a role of IFS2 (Dinkins et al. 2021). However, a peculiar finding of this study was more effect of IFS1 knockdown on genistein rather than daidzein accumulation, which could explain the more content of daidzein in the Bragg than genistein. It, therefore, appears that IFS2 is more suited for the formation of metabolon (a multi-enzyme complex), which may help in efficient and effective substrate channelling leading to a higher accumulation of isoflavone through this pathway. Thus, over-expression of IFS2 can enhance isoflavone levels.

Conclusions

In this study, the 'Bragg' phenotype was identified to have the highest isoflavone content among the 17 cultivars. The total isoflavone content and genistein: daidzein ratio in these cultivars negatively correlates with off-flavour determining parameters like carbonyl value and TBA number. This inverse relation indicates the role of isoflavones in off-flavour removal due to their antioxidant effect. Thus, a cultivar with higher content of isoflavones might have increased protection against oxidative damage. A higher IFS2 gene expression corresponds to higher isoflavone levels in soybean seeds than IFS1. The cloning and in silico analysis of IFS1 and IFS2 showed more interactions of IFS2 with other proteins in the isoflavone biosynthesis pathway, supported by earlier studies in the same direction. Further investigations of interactions between CHR, IFS isoforms, and other pathway proteins can provide new insights into developing innovative strategies for metabolic engineering. The development of soy plants over-expressing IFS can minimise the genotypic differences allowing us to compare the variation under high isoflavone and low isoflavone conditions with the same genotypic background w.r.t. off-flavour generation.

Declarations

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Conflict of interest statement

The authors declare that they have no conflict of interest in the publication

References

Abotaleb M, Samuel SM, Varghese E, et al (2018) Flavonoids in cancer and apoptosis. Cancers (Basel) 11:. https://doi.org/10.3390/cancers11010028

Azam M, Zhang S, Abdelghany AM, et al (2020) Seed isoflavone profiling of 1168 soybean accessions from major growing ecoregions in China. Food Res Int 130:108957. https://doi.org/10.1016/j.foodres.2019.108957

Baek M, DiMaio F, Anishchenko I, et al (2021) Accurate prediction of protein structures and interactions using a three-track neural network. Science 373:871–876. https://doi.org/10.1126/science.abj8754

Dahuja A, Madaan TR (2004) Off-flavour development in soybeans: comparative role of some antioxidants and related enzymes. J Sci Food Agric 84:547–550. https://doi.org/10.1002/jsfa.1667

Dastmalchi M, Bernards MA, Dhaubhadel S (2016) Twin anchors of the soybean isoflavonoid metabolon: evidence for tethering of the complex to the endoplasmic reticulum by IFS and C4H. Plant J 85:689–706. https://doi.org/10.1111/tpj.13137

Dhaubhadel S, McGarvey BD, Williams R, Gijzen M (2003) Isoflavonoid biosynthesis and accumulation in developing soybean seeds. Plant Mol Biol 53:733–743. https://doi.org/10.1023/B:PLAN.0000023666.30358.ae

Dinkins RD, Hancock J, Coe BL, et al (2021) Isoflavone levels, nodulation and gene expression profiles of a CRISPR/Cas9 deletion mutant in the isoflavone synthase gene of red clover. Plant Cell Rep 40:517–528. https://doi.org/10.1007/s00299-020-02647-4

Ganhão R, Estévez M, Morcuende D (2011) Suitability of the TBA method for assessing lipid oxidation in a meat system with added phenolic-rich materials. Food Chem 126:772–778. https://doi.org/10.1016/j.foodchem.2010.11.064

Gutierrez-Gonzalez JJ, Guttikonda SK, Tran L-SP, et al (2010) Differential expression of isoflavone biosynthetic genes in soybean during water deficits. Plant Cell Physiol 51:936–948. https://doi.org/10.1093/pcp/pcq065

Henick AS, Benca MF, Mitchell JH (1954) Estimating carbonyl compounds in rancid fats and foods. J Am Oil Chem Soc 31:88–91. https://doi.org/10.1007/BF02612488

Hughes GJ, Ryan DJ, Mukherjea R, Schasteen CS (2011) Protein digestibility-corrected amino acid scores (PDCAAS) for soy protein isolates and concentrate: criteria for evaluation. J Agric Food Chem 59:12707–12712. https://doi.org/10.1021/jf203220v

Jung W, Yu O, Lau SM, et al (2000) Identification and expression of isoflavone synthase, the key enzyme for biosynthesis of isoflavones in legumes. Nat Biotechnol 18:208–212. https://doi.org/10.1038/72671

Křížová L, Dadáková K, Kašparovská J, Kašparovský T (2019) Isoflavones. Molecules 24:. https://doi.org/10.3390/molecules24061076

Kumar V, Rani A, Dixit AK, et al (2010) A comparative assessment of total phenolic content, ferric reducing-anti-oxidative power, free radical-scavenging activity, vitamin C and isoflavones content in soybean with varying seed coat colour. Food Res Int 43:323–328. https://doi.org/10.1016/j.foodres.2009.10.019

Liu K (1997) Chemistry and nutritional value of soybean components. In: Soybeans. Springer US, Boston, MA, pp 25–113

Liu Y, Grimm M, Dai W-T, et al (2020) CB-Dock: a web server for cavity detection-guided protein-ligand blind docking. Acta Pharmacol Sin 41:138–144. https://doi.org/10.1038/s41401-019-0228-6

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Mahesha HG, Singh SA, Rao AGA (2007) Inhibition of lipoxygenase by soy isoflavones: evidence of isoflavones as redox inhibitors. Arch Biochem Biophys 461:176–185. https://doi.org/10.1016/j.abb.2007.02.013

Nedele A-K, Gross S, Rigling M, Zhang Y (2021) Reduction of green off-flavor compounds: Comparison of key odorants during fermentation of soy drink with Lycoperdon pyriforme. Food Chem 334:127591. https://doi.org/10.1016/j.foodchem.2020.127591

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95:351–358. https://doi.org/10.1016/0003-2697(79)90738-3

Ruiz-Larrea MB, Mohan AR, Paganga G, et al (1997) Antioxidant activity of phytoestrogenic isoflavones. Free Radic Res 26:63–70

Szklarczyk D, Franceschini A, Wyder S, et al (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43:D447-52. https://doi.org/10.1093/nar/gku1003

Tepavčević V, Cvejić J, Poša M, et al (2021) Classification and discrimination of soybean (Glycine max (L.) Merr.) genotypes based on their isoflavone content. Journal of Food Composition and Analysis 95:103670. https://doi.org/10.1016/j.jfca.2020.103670

Tewari K, Kumari S, Vinutha T, et al (2014) Gamma irradiation induces reduction in the off-flavour generation in soybean through enhancement of its antioxidant potential. J Radioanal Nucl Chem 303:2041–2051. https://doi.org/10.1007/s10967-014-3803-9

Tsen SY, Tan XY, Tan YM, et al (2016) Relative Inhibitions of 5-Lipoxygenase and Myeloperoxidase and Free-Radical Scavenging Activities of Daidzein, Dihydrodaidzein, and Equol. J Med Food 19:543–548. https://doi.org/10.1089/jmf.2015.3557

Vicaş SI, Chedea VS, Socaciu C (2011) Inhibitory effects of isoflavones on soybean lipoxygenase-1 activity. J Food Biochem 35:613–627. https://doi.org/10.1111/j.1745-4514.2010.00405.x

Yamagata K (2019) Soy isoflavones inhibit endothelial cell dysfunction and prevent cardiovascular disease. J Cardiovasc Pharmacol 74:201–209. https://doi.org/10.1097/FJC.0000000000000708

Tables

Table 1: Isoflavone content and composition of the 17 soybean genotypes

	Variety	Genistein (µg/gm)	Daidzein (µg/gm)	Total Isoflavones (µg/gm)	Genistein: Daidzein Ratio
Yellow Seed Coat	PK 1042	410.69 ± 42.81^{c}	661.65 ± 19.5^{a}	1072.34 ± 60.19^{b}	1.61
	BRAGG	600.26 ± 32.71^{b}	546.16 ± 11.77^{b}	1146.42 ± 43.47^{a}	0.91
	PUSA 16	159.33 ± 8.61^{i}	78.99 ± 3.8^{k}	238.32 ± 12.34^{l}	0.5
	PUSA 22	212.85 ± 3.86^{g}	118.87 ± 5.33^{j}	331.72 ± 9.17^{jk}	0.56
	PUSA 24	333.13 ± 27.72^{de}	244.73 ± 2.26^{e}	577.86 ± 27.43^{e}	0.73
	PUSA 37	185.19 ± 9.69^{ghi}	150.88 ± 2.33^{hi}	336.07 ± 10.84^{ij}	0.81
	PUSA 40	108.39 ± 3.2^{j}	45.14 ± 4.19^{l}	153.53 ± 7.22^{m}	0.42
	PUSA 9814	246.78 ± 3.92^{f}	156.76 ± 2.87^{h}	403.54 ± 5.88^{h}	0.64
	PK 416	301.75 ± 30.4^{e}	91.32 ± 8.21^{k}	393.07 ± 22.32^{h}	0.3
	SL 525	625.16 ± 59.02^{a}	378.05 ± 15.32^{c}	1003.21 ± 67.59^{c}	0.6
Black seed coat	BS 1	276.90 ± 2.75^{f}	371.17 ± 31.6^{c}	648.07 ± 34.28^{d}	1.34
	AMSS 34	248.22 ± 2.74^{f}	117.96 ± 10.39^{j}	366.19 ± 12.09^{i}	0.48
	EC 109514	263.12 ± 14.2^{f}	317.96 ± 3.95^{d}	581.08 ± 17.89^{ef}	1.21
	EC 114526	329.24 ± 39.75^{d}	203.74 ± 3.68^{f}	532.98 ± 43.33^{f}	0.62
	UPSL 340	176.51 ± 6.81^{hi}	124.83 ± 4.44^{j}	301.34 ± 11.23^{k}	0.71
	UPSL 785	272 ± 6.34^{f}	179.21 ± 5.7^{g}	451.21 ± 1.69^{g}	0.66
	UPSL 19	201.12 ± 6.1^{gh}	150.64 ± 6.1^{i}	351.76 ± 4.05^{ij}	0.75

An average of 3 biological replicates ± standard deviation (SD) are presented here. Duncan's multiple range tests (DMRT) was performed to check the statistical difference for the isoflavone content at 5% level of significance ($p<0.05$).

Table 2: Correlation coefficients between different variables viz. including isoflavones, genistein, Genistein: Daidzein (G:D) ratio, Carbonyl value and TBA number in soybean genotypes

Isoflavones	1				
Genistein	0.95	1			
G:D ratio	0.59	0.80	1		
Carbonyl Value	0.09	-0.12	-0.42	1	
TBA Number	0.02	-0.16	-0.41	0.76	1
	Isoflavones	**Genistein**	**G:D ratio**	**Carbonyl Value**	**TBA Number**

Figures

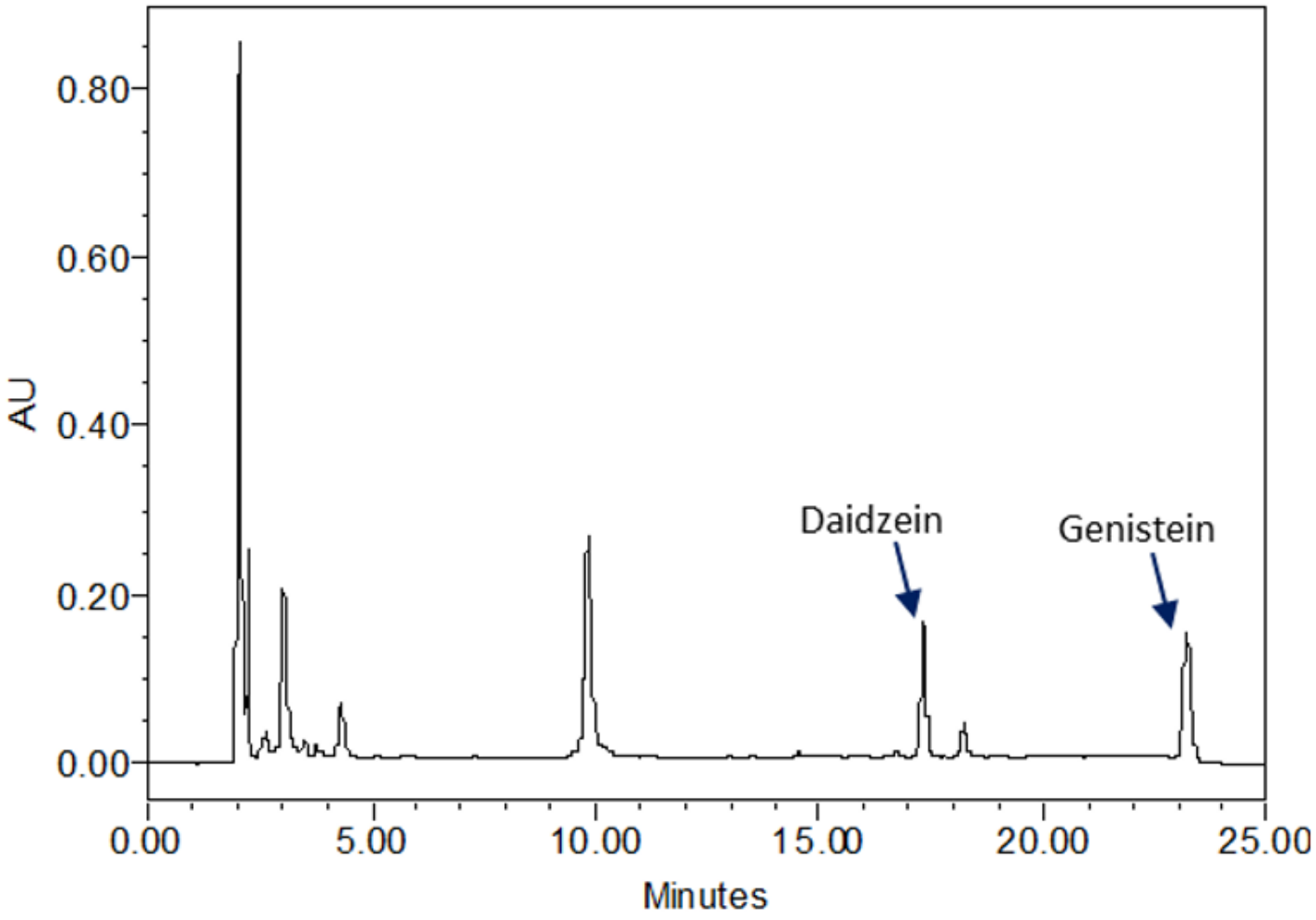

Figure 1

A representative chromatogram of soybean variety (PK1042) for isoflavone estimation. Peaks corresponding to major isoflavones i.e., daidzein and genistein are labelled.

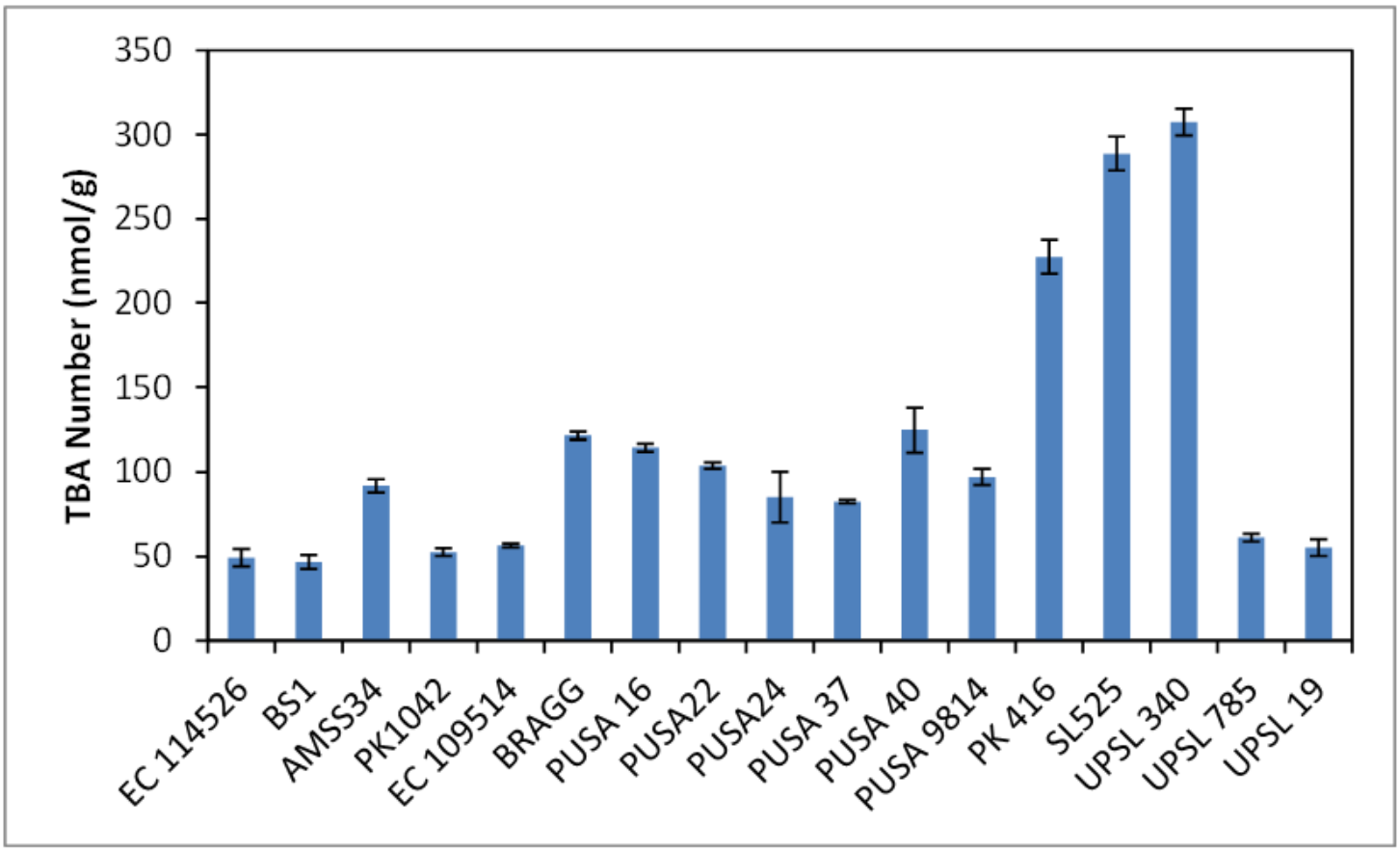

Figure 2

Determination of TBA number (An Index of Lipid Peroxidation) in 17 soybean genotypes. Values presented are the calculated averages of 3 replicates ± standard deviation (SD).

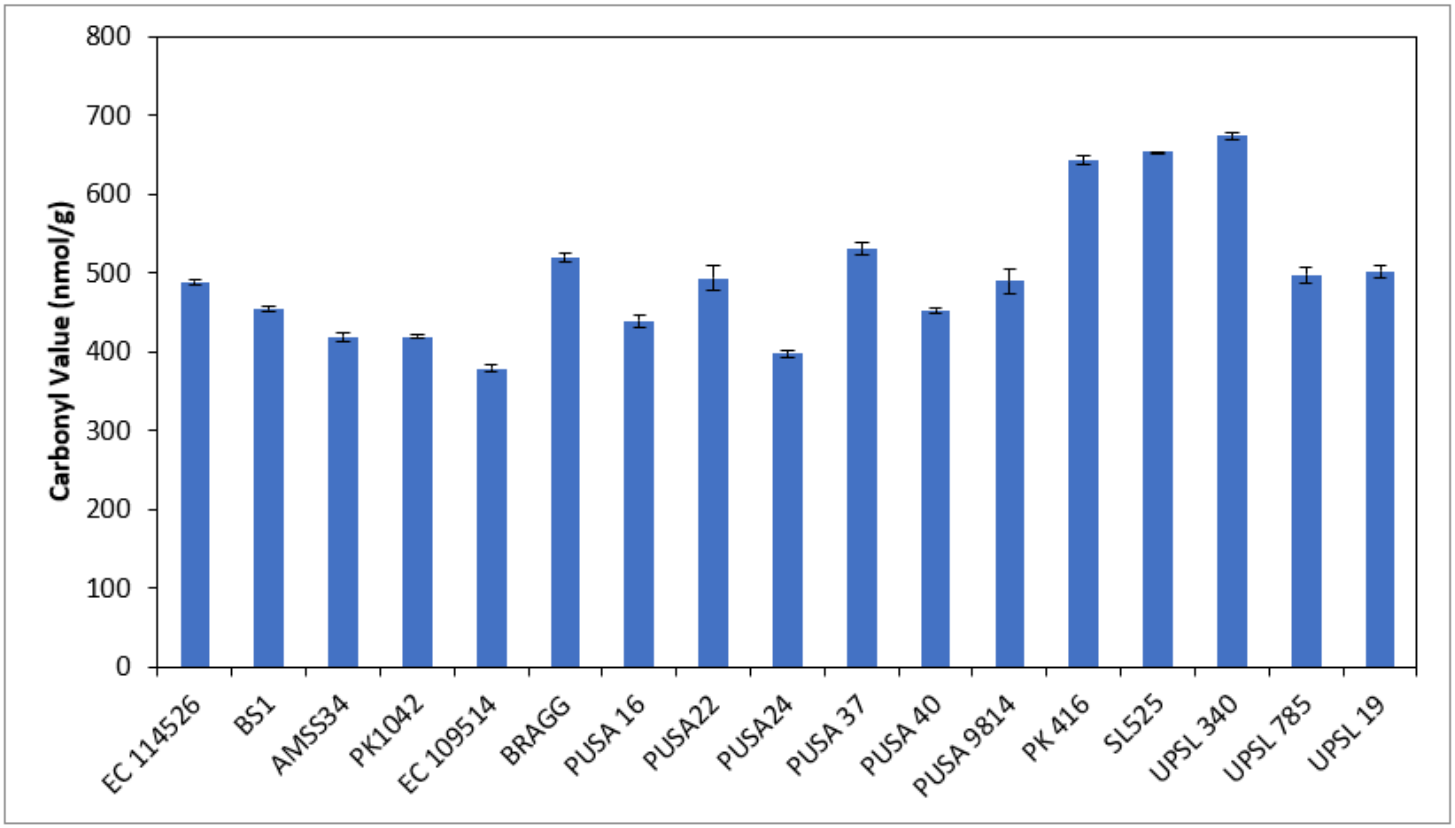

Figure 3

Carbonyl Value estimation in of in 17 soybean genotypes. Values presented are the calculated averages of 3 replicates ± standard deviation (SD).

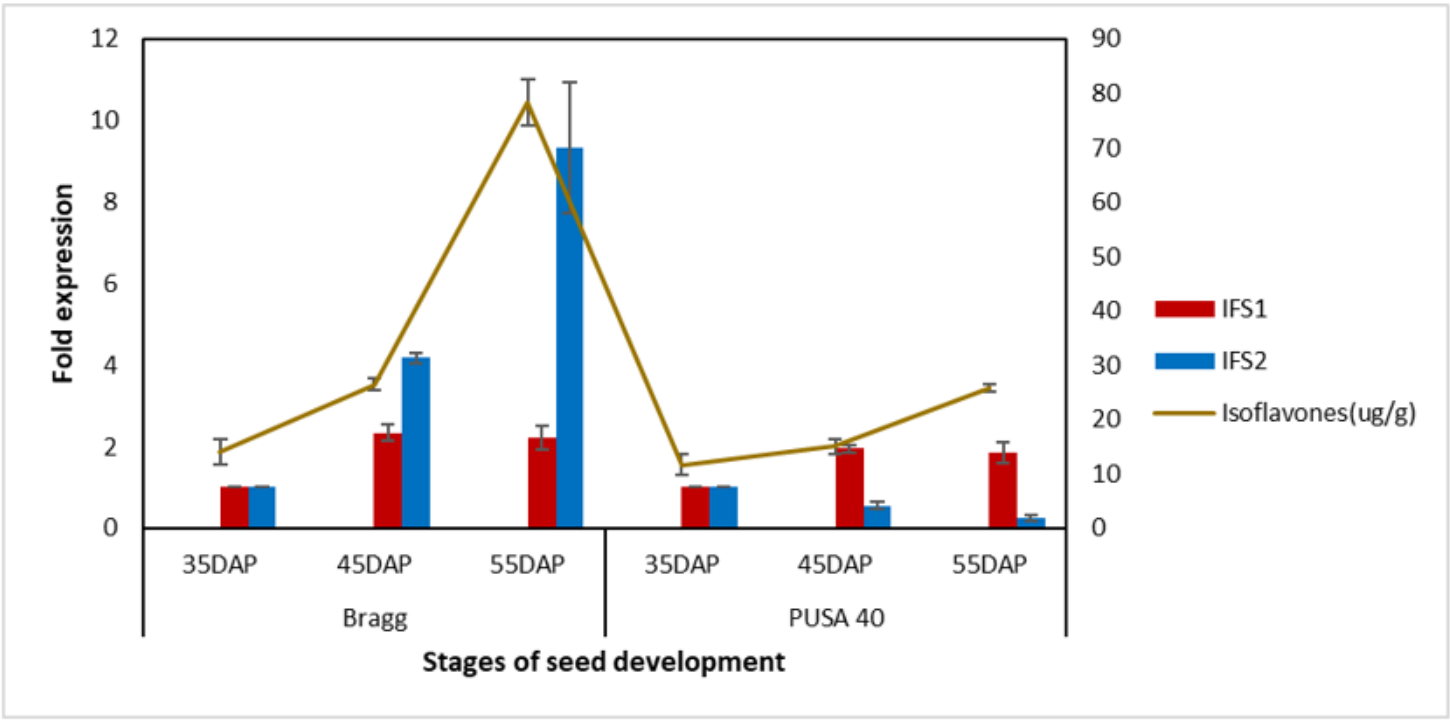

Figure 4

Variation in the Isoflavone synthase (IFS1&2) gene expression and corresponding isoflavone accumulation in contrasting (Bragg = High isoflavone content and PUSA 40 = Low isoflavone content) soybean genotypes at three stages of seed development. Values presented are the calculated averages of 3 replicates ± standard deviation (SD).

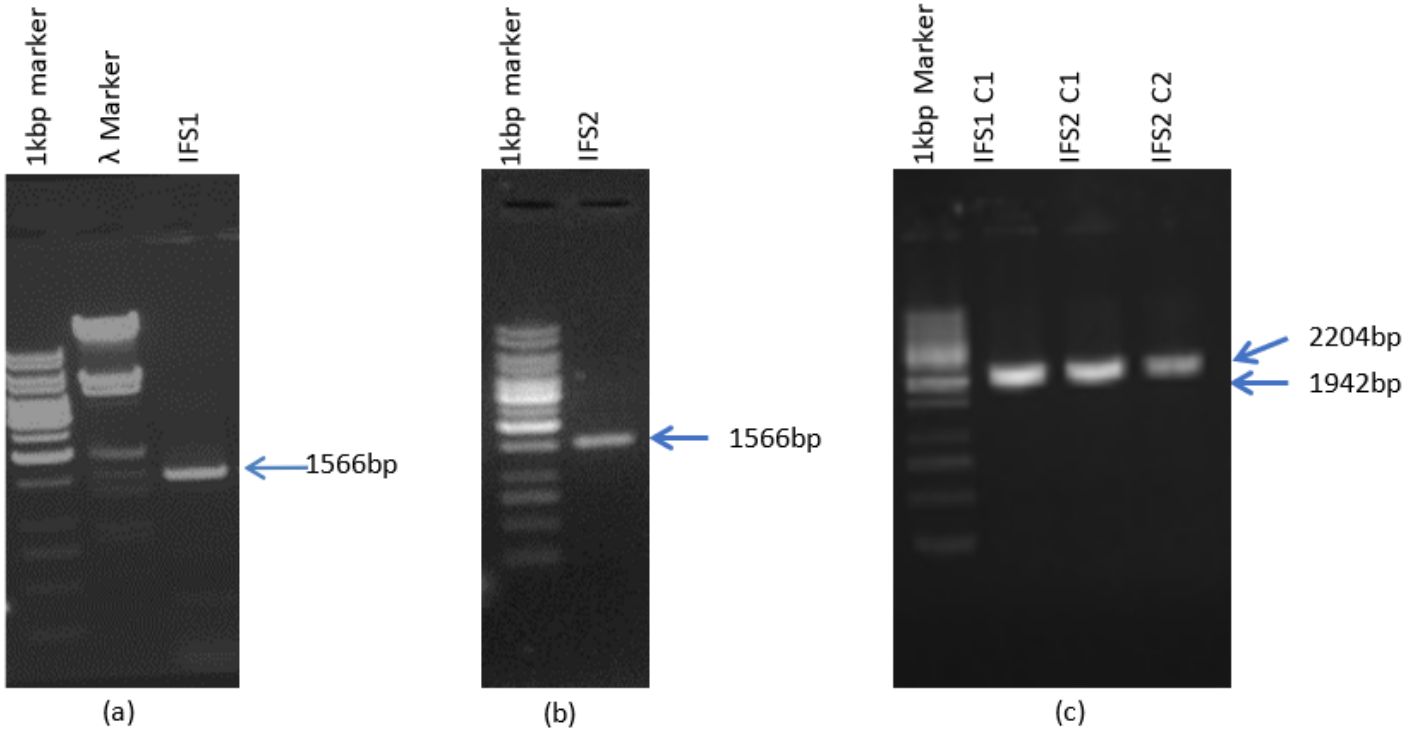

Figure 5

PCR amplification of IFS1 (a) and IFS2 transcript (b). PvuII digestion products (2204bp and 1942 bp) of IFS1 & IFS2 cloned into the pENTR-D-TOPO vector (c).

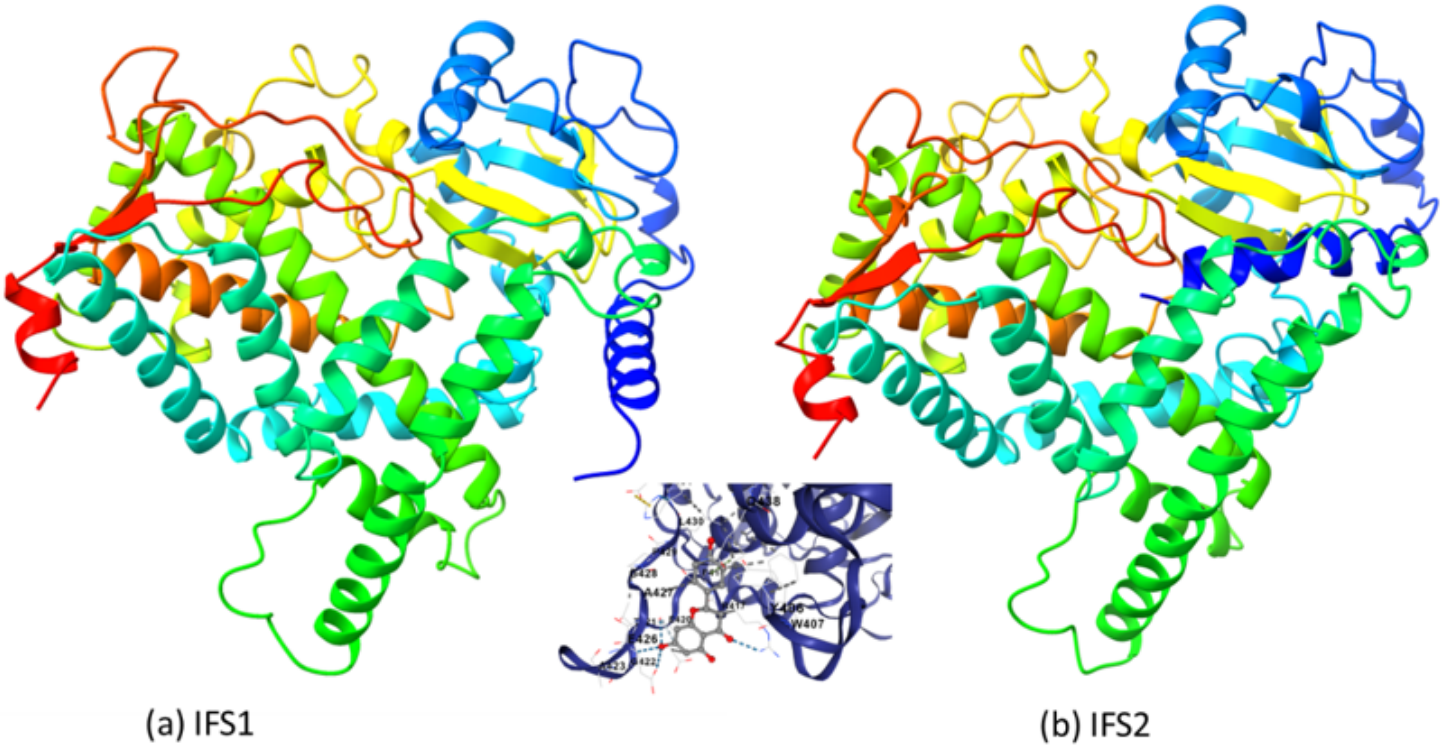

Figure 6

3D structure of IFS1 (a) and IFS2 (b) generated using RoseTTAFold. Also shown is the naringenin (a substrate for genistein formation) bound at the binding site of IFS2.

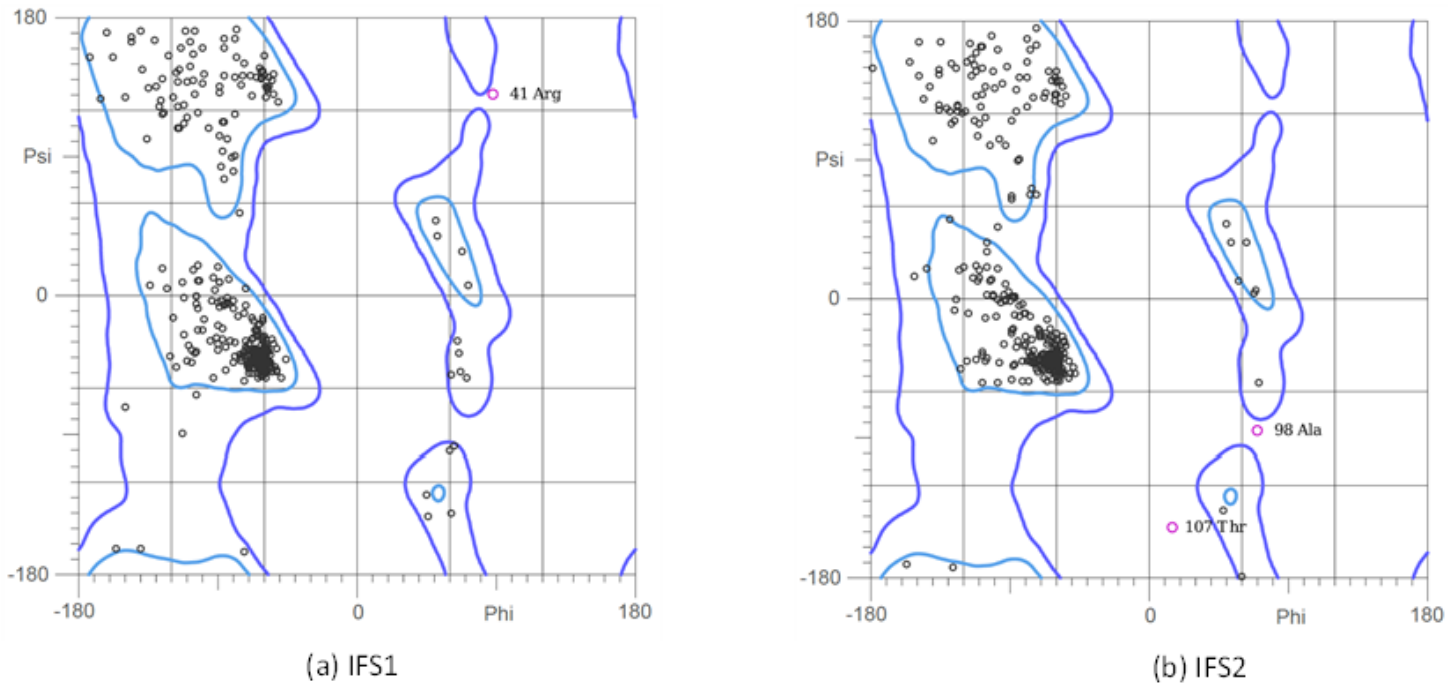

Figure 7

Ramachandran plot generated on the MolProbity server for IFS1 (a) and IFS2 (b) 3D models.

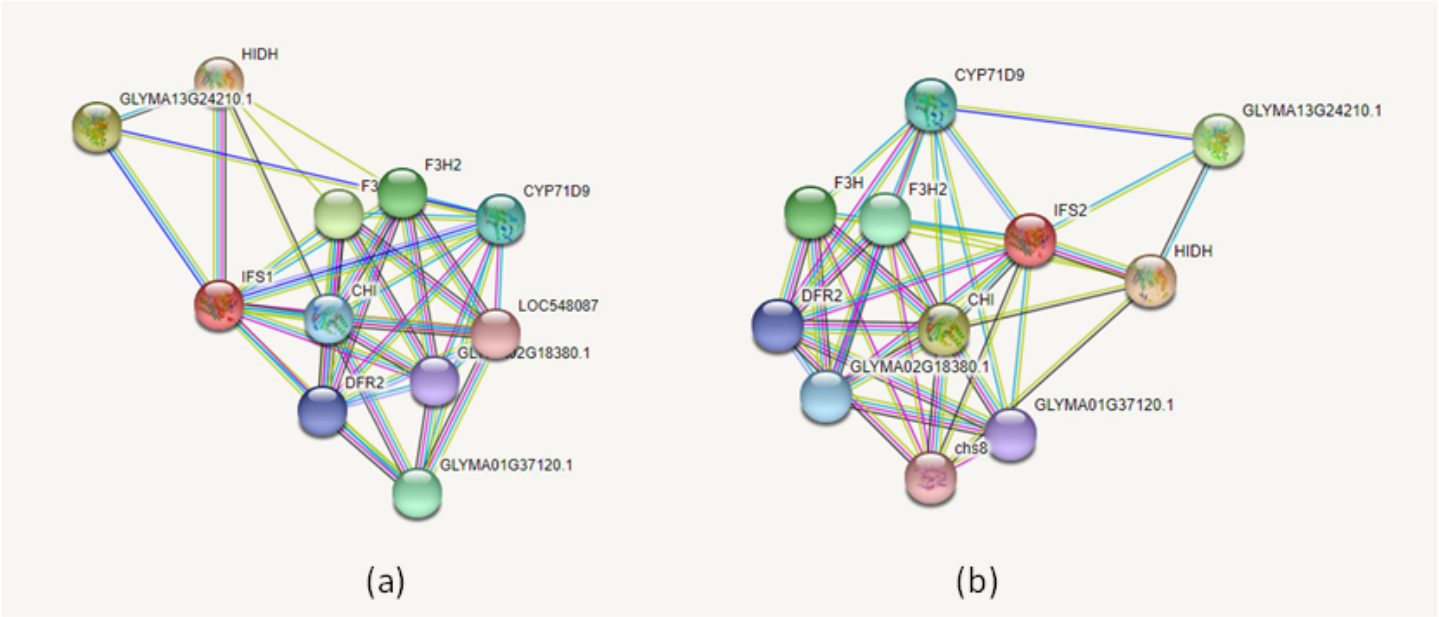

Figure 8

Protein-protein interactions for (a) IFS1 (b) IFS2 prepared using STRING [IFS1: Isoflavone synthase 1; IFS2: Isoflavone synthase 2; HIDH 2-Hydroxyisoflavone dehydratase; GLYMA13G24210.1: Uncharacterised (SAM-binding methyltransferase superfamily); F3H: Flavanone 3-Hydroxylase; F3H2:

Uncharacterised (Flavanone 3-Hydroxylase); CHI: Chalcone-flavanone Isomerase; DFR2: Dihydroflavonol-4-reductase; details in **S4**]

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx