**GmFNSII-Controlled Soybean Flavone Metabolism Responds to Abiotic Stresses and Regulates Plant Salt Tolerance**

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Flavones and about 700 flavone glycosides, including the aglycone structures, O- and C-glycosides, have been isolated recently (Williams and Grayer 2004). The major flavone aglycones, including apigenin, luteolin and 7,4'-dihydroxyflavone (DHF), have been well known for their function as defense compounds, signaling molecules and regulators of gene expression during pathogen attack (Martens and Mithöfer 2005). As secondary metabolites involved in abiotic and biotic stresses, flavones play important and specific roles in plant adaptation to environmental challenge and protection of photosynthetic tissues from oxidative damage (Harborne and Williams 2000).

Introduction

Flavones are a major category of flavonoids in most plant tissues, play multiple roles in plant–environment interactions. In our study, the expression of the two soybean flavone synthase genes, *GmFNSII-1* and *GmFNSII-2*, was significantly increased by methyl jasmonate (MeJA), glucose, mannitol and NaCl treatment, which were also found to increase flavone aglycone accumulation in *Glycine max* (L.) Merrill. In the *GmFNSII-1* promoter, a specific CGTCA motif in the region (−979 bp to −806 bp) involved in the MeJA response was identified. Promoter deletion analysis of *GmFNSII-2* revealed the presence of osmotic-responsive (−1,143 bp to −767 bp) and glucose-repressive sequence elements (−767 bp to −475 bp), which strongly supported the hypothesis that glucose induces soybean flavone production by acting as both an osmotic factor and a sugar signaling molecule simultaneously. Silencing of the *GmFNSII* gene clearly reduced the production of flavone aglycones (apigenin, luteolin and 7,4'-dihydroxyflavone) in hairy roots. The *GmFNSII-RNAi* (RNA interference) roots that had a reduced level of flavones accompanied by more malondialdehyde and H2O2 accumulation were more sensitive to salt stress compared with those of the control, and we concluded that flavones, as antioxidants, are associated with salt tolerance.

**Keywords:** Flavone aglycones ● Glucose ● Methyl jasomate ● Promoter deletion analysis ● Salt tolerance ● Soybean flavone synthase genes.

**Abbreviations:** DAB, 3,3'-diaminobenzidine; DFR, dihydroflavonol reductase; DHF, 7,4'-dihydroxyflavone; FNSII, flavone synthase II; GUS, β-glucuronidase; JA, jasmonate; LDOX, leucoanthocyanidin dioxygenase; MDA, malondialdehyde; MeJA, methyl jasmonate; MS, Murashige and Skoog; 12-OPDA, 12-oxo-phytodienoic acid; ROS, reactive oxygen species; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; UF3GT, UDP-Glc:flavonoid 3-O-glucosyltransferase.

Flavones and about 700 flavone glycosides, including the aglycone structures, O- and C-glycosides, have been isolated recently (Williams and Grayer 2004). The major flavone aglycones, including apigenin, luteolin and 7,4'-dihydroxyflavone (DHF), have been well known for their function as defense compounds, signaling molecules and regulators of gene expression during pathogen attack (Martens and Mithöfer 2005). As secondary metabolites involved in abiotic and biotic stresses, flavones play important and specific roles in plant adaptation to environmental challenge and protection of photosynthetic tissues from oxidative damage (Harborne and Williams 2000).

Jasmonates (JAs), the stress-related phytohormones, have been shown to enhance the amounts of flavonoid in plants through inducing the expression of several genes involved in plant flavonoid biosynthesis (Tamari et al. 1995, Wasternack 2007, Pauwels et al. 2009, Shan et al. 2009, Fliegmann et al. 2010). The transcript levels of the soybean isoflavone synthase genes *IFS1* and *IFS2* were induced by JA, and a significant increase of *IFS1* promoter activity stimulated by JA was detected in transgenic Arabidopsis roots (Subramanian et al. 2004). A study on *Medicago truncatula* flavone synthase genes (*MtFNSII-1* and *MtFNSII-2*) revealed that the expression of *MtFNSII-2* promoter–β-glucuronidase (GUS) but not *MtFNSII-1* promoter–reporter was elevated in transgenic *M. truncatula* roots by methyl jasmonate (MeJA) treatment (Zhang et al. 2007). The promoter of *PtrCHS4*, a key enzyme in flavonoid biosynthesis in *Populus trichoarpa*, contained two conserved G-box motifs (CACCGT) that were predominantly responsible for JA induction (Sun et al. 2011). In soybean cell cultures, the activity of flavone synthase II (FNSII) was enhanced significantly by JA (Fliegmann et al. 2010), although JA-responsive motifs in its promoter remained undefined.

Many studies have shown the flavonoid accumulation in plants induced by sugar. In this physiological process, sugar seemed not only to be a nutrient source and an inducer of osmotic stress, but also acted as a signaling molecule to activate/repress flavonoid biosynthesis (Villadsen and Smith 2004, Teng et al. 2005, Solfanelli et al. 2006). Soybean FNSII was reported to be induced by glucose due to osmotic stress.
Flavone induction and salt tolerance in soybean

Flavonoids enhanced salinity tolerance by maintaining reactive oxygen species (ROS) homeostasis and mitigating oxidative damage (Blokhina et al. 2003, Wahid and Ghazanfar 2006, Chutipaijit et al. 2009). Soil salinity is a leading limiting factor in soybean production by affecting soybean germination, growth, nutrient composition and yield (Essa 2002, Tunçturk et al. 2008). High soil salinity promoted production of ROS, and consequently resulted in oxidative damage to proteins, DNA and lipids (Zhu 2001). ROS homeostasis was tightly controlled by ROS-generating and scavenging systems (Mittler et al. 2004). Apigenin (4',5,7-trihydroxyflavone), an abundant flavone aglycone widely distributed in plants, has been shown to inhibit lipid peroxidation, eliminate free radicals and enhance endogenous antioxidant defense (Heim et al. 2002, Prince Vijeya Singh et al. 2004). Studies on transgenic plants indicated that overexpression of oxidative protection enzymes or other stress regulatory transcription factors improved plant tolerance to abiotic stresses (Dai et al. 2007, Yang et al. 2009, Ganesan et al. 2012, Yang et al. 2012).

GmFNSII, a membrane-bound Cyt P450 monooxygenase, converts flavanones directly into flavones. Two soybean FNSII genes, GmFNSII-1 (CYP93B16) and GmFNSII-2, have been identified (Fliegmann et al. 2010, Jiang et al. 2010). The function of these two genes in abiotic stress responses remains unclear. Here, we studied expression patterns of the two genes and profiles of the flavone aglycone content under MeJA, glucose, mannitol and NaCl treatments. We characterized upstream regions that were responsible for GmFNSII promoter activity and identified glucose- and MeJA-responsive elements in GmFNSII-1 and GmFNSII-2 promoter regions. Furthermore, we found that GmFNSII-silenced soybean seedlings were more sensitive to salt stress due to the elevated oxidative production [hydrogen peroxide (H$_2$O$_2$) and malondialdehyde (MDA)] in the roots and the accumulation of flavones enhanced plant salinity tolerance via the alleviation of oxidative damage in soybean roots.

Results

Promoters of GmFNSII genes are activated by multiple abiotic stimuli in soybean hairy roots

To get a glimpse into the expression of GmFNSII genes in response to abiotic stimuli, the −1,672 bp GmFNSII-1 promoter and −1,143 bp GmFNSII-2 promoter were separately constructed into a GUS reporter. GUS activities were analyzed in transgenic soybean hairy roots treated with MeJA (100 µM), glucose (0.4 M), mannitol (0.4 M) and NaCl (100 mM). As shown in Fig. 1, both promoter–GUS reporters were clearly inducible by these treatments. The induction patterns of the two reporters were similar and also variable. While mannitol produced similar GUS activity in the two promoters, the MeJA, glucose and NaCl treatments induced greater GUS expression in the GmFNSII-1 promoter–reporter. These findings were further validated by GUS histochemical assays (Supplementary Fig. S1a, b). The promoter–reporter experiment suggests that GmFNSII genes are involved in abiotic responses in soybean.

The accumulation of GmFNSII genes and flavone aglycones is stimulated by MeJA

To address the roles of GmFNSII genes in plant abiotic responses, we first determined their expression by MeJA modulation. In a real-time PCR analysis, the transcript levels of GmFNSII-1 and GmFNSII-2 in different tissues of soybean seedlings were found to be simultaneously induced by 100 µM MeJA treatment. However, the induction patterns of the two genes were not exactly identical across samples. The peak levels of GmFNSII-1 and GmFNSII-2 appeared at 1 h after MeJA treatment in leaves (2.79- and 1.97-fold, respectively, compared with ACTIN) and roots (0.35- and 0.32-fold, respectively); while their highest induction (1.43- and 0.97-fold, respectively) in stems occurred at 4 h after the treatment (Fig. 2a).

Meanwhile, the transcript level of GmFNSII-1 was higher than that of GmFNSII-2 in control and treated soybean tissues, whereas the expression of GmFNSII-2 was more sensitive to the MeJA treatment in soybean roots and stems (the transcript level normalized to control for GmFNSII-1 vs. GmFNSII-2: 176 vs. 372 in 1 h treated roots; 93 vs. 331 in 4 h treated stems). The content of flavone aglycones (DHF, luteolin and apigenin) in different tissues was determined after 4 h treatment with 100 µM MeJA using a HPLC method (Fig. 2b). As shown in Fig. 2b, the content of flavone aglycones was predominantly accumulated in the soybean leaves and stems. This accumulation was consistent with GmFNSII transcript levels. Along with the up-regulation of GmFNSII genes, the level of flavone aglycones was concurrently increased in all of the treated
samples. It is noteworthy that roots had the greatest increase (3.6-fold) in the MeJA-induced production of these compounds.

**MeJA cis-elements in the GmFNSII-1 promoter are responsible for the MeJA response**

To characterize the MeJA-responsive region in the promoter of *GmFNSII-1* ([P1]), two successive deletions of the promoter were created according to the cis-element prediction ([Fig. 3a](#)). These promoter–reporter constructs were introduced into soybean hairy roots through transformation to generate composite soybean seedlings that contained wild-type shoots with transgenic roots. There was a significant increase in MeJA-induced GUS activity of the −1,672 bp promoter–reporter at 24 h after treatment, and the −979 bp truncated promoter–reporter (P1D1) still maintained 85% induction ([Fig. 3a](#)). However, further deletion of the promoter to −806 bp (P1D2) completely abrogated the ability of the promoter to respond to the MeJA treatment ([Fig. 3a](#)). Therefore, a primary MeJA-responsive sequence was mapped to the region between −979 to −806 bp in the *GmFNSII-1* promoter. Consistent with this finding, in GUS histochemical assays ([Fig. 3b–d](#)), the darkest staining was present in P1 (*GmFNSII-1* promoter) transgenic hairy roots under 100 μM MeJA treatment ([Fig. 3b](#)), whereas P1D2 counterparts were only lightly stained in the same condition ([Fig. 3d](#)). These GUS reporter experiments indicate that the −806 bp promoter (P1D2) only retains basal activity of the *GmFNSII-1* promoter ([Fig. 3a–d](#)).

To investigate whether the predicted MeJA-responsive motif (CGTCA) in the promoter sequence (from −979 to −806 bp) of *GmFNSII-1* was responsible for MeJA induction, we substituted the consensus sequence with TCGAC to generate a *GmFNSII-1* promoter mutant. We transformed this *GmFNSII-1* promoter mutant reporter into soybean hairy roots for GUS histochemical assay ([Figs. 3e, f](#)). We found that there were no differences in the GUS staining between the mutant and normal *GmFNSII-1* promoter–reporters under normal condition; however, the roots with the mutant reporter showed much lighter staining after MeJA treatment, revealing a reduced activity. The result indicates that the CGTCA motif is a bona fide MeJA cis-element.

**The differential tissue expression of GmFNSII genes induced by glucose and mannitol**

In order to investigate whether the osmotic effect or sugar-specific effect is related to the function of glucose on flavone accumulation, the non-metabolized mannitol was used to create an osmotic pressure gradient in the system.
The transcript levels of \textit{GmFNSII-1} and \textit{GmFNSII-2} in roots, stems and leaves at soybean seedling stage V1 were detected by real-time PCR under 0.4 M glucose and 0.4 M mannitol stresses. Following the treatments, expression of \textit{GmFNSII-1} and \textit{GmFNSII-2} was increased by both glucose and mannitol and reached the highest level at 2–4 h after treatment in all tissues, with the highest expression levels found in leaves (Fig. 4a, b). Interestingly, mannitol appeared to be a more potent inducer for \textit{GmFNSII} expression than glucose; the peak levels of \textit{GmFNSII-1} (mannitol vs. glucose treatment) were 101- vs. 2.6-fold, 304- vs. 2.9-fold and 71- vs. 62-fold induction in leaves, stems and roots, respectively (Fig. 4a).

Similar expression patterns were also found for \textit{GmFNSII-2} during the two treatments (Fig. 4b). These results were also consistent with the GUS activity assay in \textit{GmFNSII–GUS} transgenic hairy roots under glucose and mannitol stresses (Fig. 1; Supplementary Fig. S1). The flavone aglycone contents were measured after 2 h treatments with 0.4 M glucose and 0.4 M mannitol (Fig. 4c). The highest levels were observed in leaves following both glucose and mannitol treatments, consistent with the expression of \textit{GmFNSII} mRNA. The flavone aglycone contents in roots (3.55 μg g⁻¹ FW), stems (6.97 μg g⁻¹ FW) and leaves (9.62 μg g⁻¹ FW) after mannitol treatment were 5.4-, 3.7- and 5.7-fold higher than those of the controls. Glucose resulted in a 3.3-fold (roots, 2.17 μg g⁻¹ FW), 2.7-fold (stems, 5.02 μg g⁻¹ FW) and 2.2-fold (leaves, 5.02 μg g⁻¹ FW) increase in flavone aglycone contents after 2 h treatment.

The motifs responsive to glucose are present in the \textit{GmFNSII-2} promoter

By analyzing a set of truncated \textit{GmFNSII-2} promoters (P2) (Fig. 5a), we found that transgenic hairy roots undergoing glucose stress for 24 h showed a sharp reduction to 58% in GUS activity when the promoter was deleted from /C0/1,143 bp (P2, \textit{GmFNSII-2} promoter) to /C0/767 bp (P2D1). Interestingly, the smallest promoter–reporter (/C0/475 bp, P2D2) revealed a higher GUS activity than the larger one, P2D1. These results suggest that \textit{GmFNSII-2} promoter probably contains both positive (between /C0/1,143 and /C0/767 bp) and negative regulatory regions (between /C0/767 bp and /C0/475 bp) involved in glucose response. Based on a bioinformatic analysis, a dehydration-responsive element (/C0/804 bp) and a sugar repression element (/C0/714 bp) were predicted in the \textit{GmFNSII-2} promoter. Our data obtained from the GUS histochemical assays (Fig. 5b–d) supported this
prediction. Under 0.4 M glucose stress, the GUS accumulation was markedly decreased to a lower level with the deletion of the promoter sequence (from −1,143 bp to −767 bp), as indicated by the staining results in the transgenic roots of P2 and P2D1 (Fig. 5b, c). Consistent with the observation in the GUS promoter assay, the GUS staining was restored in glucose-stressed P2D2 transformants to a level similar to that of P2 roots (Fig. 5b, d). Despite the discrepancy in activities among the GmFNSII-2 truncated promoters in glucose treatment, the GUS levels were comparable among these promoter-reporters.
under normal condition (Fig. 5b–d). Since there are dehydration-responsive and sugar-repressive regions in the promoter of \textit{GmFNSII-2}, we speculate that the glucose can enhance flavone levels through its osmotic function and glucose also probably regulated flavone synthesis through the action on the sugar-repressive element.

Flavone production is associated with salt tolerance in soybean

To determine flavone production in salt-stressed soybean, we treated soybean seedlings with different concentrations of NaCl for 24 h. In the treated soybean leaves, stems and roots, the expression of \textit{GmFNSII-1} and \textit{GmFNSII-2} and the flavone aglycone content were significantly increased (Fig. 6a, b). The transcript levels of \textit{GmFNSII-1} and \textit{GmFNSII-2} peaked in 150 mM NaCl-treated leaves (0.26 and 0.07 compared with \textit{ACTIN}, respectively), whereas the induction was not further enhanced by 200 mM NaCl, probably attributable to the severely wilted leaves caused by salt stress. The highest mRNA levels in stems (0.11 and 0.02 compared with \textit{ACTIN}, respectively) and roots (\textit{GmFNSII-1}, 0.11, compared with \textit{ACTIN}) were detected in the 200 mM NaCl treatment. Under the 100 mM NaCl treatment, we found that the flavone aglycone content (1.92 μg g⁻¹ FW) in roots was significant higher than that of the control (0.65 μg g⁻¹ FW), and the highest flavone production (2.63 μg g⁻¹ FW) was observed in the 150 mM NaCl treatment (Fig. 6b). The peak levels of flavone aglycone in leaves and stems were found in the 200 mM NaCl treatment.

To determine the role of flavone aglycones in soybean salt tolerance, we silenced the expression of \textit{GmFNSII} genes in soybean hairy roots through an RNA interference (RNAi) approach, and generated composite plants containing transgenic hairy roots and untransformed shoots. To evaluate off-target effects of the \textit{GmFNSII}-silencing cassette, genes homologous to \textit{GmFNSII} were searched in the available databases. \textit{Cyt P450} (Glyma11g15330) identified from SoyBase (http://soybase.org/) shares 56% and 55% identity at nucleotide level with \textit{GmFNSII-1} and \textit{GmFNSII-2}, respectively. However, the transcript of this gene was undetectable using quantitative real-time PCR, and the 322 bp target sequence in the \textit{GmFNSII-RNAi} construct barely matched any region of \textit{Cyt P450}; therefore the off-target effect of \textit{GmFNSII-RNAi} on this gene was not studied further.
We found that *GmFNSII*-1 and *GmFNSII*-2 were efficiently depleted by RNAi to an undetectable level in both salt-treated and untreated plants (Fig. 7a). It was also found that *GmFNSII*-RNAi hairy roots had a lower level of flavone aglycones (DHF, luteolin and apigenin) when compared with the vector control and wild-type roots under 100 mM NaCl treatment for 3 d (Fig. 7b, c). In contrast, the flavone aglycone content was not significantly altered in the untreated wild-type roots compared with the vector control (data not shown). To determine the growth inhibition effect of salt stress, we used 100 mM NaCl to treat vector control and *GmFNSII*-RNAi composite plants for 2 weeks, as 200 mM NaCl severely inhibited the growth of soybean seedlings and 50 mM NaCl treatment could not phenomenally distinguish control and RNAi plants. The growth of *GmFNSII*-RNAi seedlings rather than controls was found to be more severely inhibited by the stress, as indicated by a much slower growth rate of *GmFNSII*-RNAi seedlings (Fig. 7d, e).

**Flavone accumulation reduces peroxide level in salt stress**

Membrane lipid peroxidation is a physiological index for the loss of membrane integrity resulting from oxidative damage (Shalata et al. 2001). The accumulation of lipid peroxidation (represented by MDA) in the roots of wild-type and vector control seedlings was significantly lower than that in the *GmFNSII*-RNAi roots under salt stress (Fig. 8a). 3,3’-Diaminobenzidine (DAB) staining (Fig. 8b, c) revealed that accumulation of H$_2$O$_2$ in the roots of *GmFNSII*-RNAi seedlings was higher than that of wild-type and vector control roots under 100 mM NaCl stress, because *GmFNSII*-RNAi samples had a darker reddish-brown coloration representing a higher H$_2$O$_2$ concentration. This result suggests that flavone aglycone accumulation in soybean roots can alleviate oxidative damage induced by salt stress.

**Discussion**

A growing body of evidence shows that MeJA regulates flavone biosynthesis in plants, such as petunia corollas (Tamari et al. 1995), *M. truncatula* (Zhang et al. 2007) and soybean (Gundlach et al. 1992). MeJA pre-treatment increased the flavonoid level, which most probably in turn contributed to the improved adaptation of plants to salinity stress (Tsonev et al. 1998, Walia et al. 2007, Yoon et al. 2009). In our study, both *GmFNSII*-1 and *GmFNSII*-2 showed a MeJA-dependent induction (Figs. 1, 2a; Supplementary Fig. S1). It has been reported that MeJA-responsive regulatory elements (CGTCA motifs) were commonly present in the promoters of some genes and were responsible for their MeJA induction, such as *Sm4CL2* (Jin et al. 2012), *PgD1* (Germain et al. 2012) and *VER2* (Feng et al. 2009). Our promoter mapping analysis of *GmFNSII*-1...
revealed a positive MeJA response element located between /C0 979 bp and /C0 806 bp. Promoter deletion or mutation of the consensus motif compromised the promoter-driving GUS activity in response to MeJA. These results provided novel evidence for the direct function of jasmonates in soybean flavonoid biosynthesis and enhanced our understanding of the mechanism underlying abiotic-induced production of flavone aglycone.

As a group of plant secondary compounds induced by environmental stresses, flavonoids acted as major antioxidants to prevent cellular damage from ROS attack (Heim et al. 2002). Proanthocyanidins were known for their anti-oxidation ability in improving Arabidopsis seed germination under high oxidative stress (Jia et al. 2012). Flavones, probably as ROS scavengers, enhanced salt tolerance in sugarcane (Wahid and Ghazanfar 2006). It was also reported that there was a higher accumulation of MDA and H2O2 in salt-sensitive but not salt-tolerant rice cultivars (Khan and Panda 2008). These findings suggest that H2O2 and MDA can be used as indicators for ROS damage in plant cells, and a high level of these two compounds represents a severe ROS lesion. Consistent with this notion, we indeed found that the impaired flavone production resulted in a marked growth inhibition accompanied by a significant increase of H2O2 and MDA in salt-stressed GmFNSII-RNAi soybean seedlings.

Induction of GmFNSII genes is stimulated by many abiotic factors. Our gene silencing experiments confirmed the need for these two stress-responsive genes in accumulation of flavonoids that can relieve plant oxidative damages caused by abiotic stresses. Other studies demonstrated that overexpression of oxidative stress (Redha et al. 2012). Flavones, probably as ROS scavengers, enhanced salt tolerance in sugarcane (Wahid and Ghazanfar 2006). It was also reported that there was a higher accumulation of MDA and H2O2 in salt-sensitive but not salt-tolerant rice cultivars (Khan and Panda 2008). These findings suggest that H2O2 and MDA can be used as indicators for ROS damage in plant cells, and a high level of these two compounds represents a severe ROS lesion. Consistent with this notion, we indeed found that the impaired flavone production resulted in a marked growth inhibition accompanied by a significant increase of H2O2 and MDA in salt-stressed GmFNSII-RNAi soybean seedlings.

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some stress-induced genes altered stress tolerance (Dai et al. 2007, Ganesan et al. 2012). Likewise, we surmised that genetically engineered plants overexpressing GmFNSII genes should have a similar effect on plant abiotic tolerance due to an expected increase in flavone content. To our surprise, transgenic hairy roots overexpressing GmFNSII genes did not show a significant alternation in salt tolerance (data not shown). Since the solo action of GmFNSII genes seems not to be sufficient to increase salt tolerance, in spite of these facts, we believe that GmFNSII genes are critical and indispensable for salt tolerance based on our gene silencing data. To understand completely the intricate mechanism of GmFNSII-mediated salt tolerance, further study is needed in the future.

GmFNSII-1 and GmFNSII-2 share very high identity at the nucleic acid (95%) and amino acid level (93%) and their encoding proteins showed similar enzymatic function to convert (2S)-naringenin into apiigenin in yeast cells (Jiang et al. 2010). However, the expression of GmFNSII-1 and GmFNSII-2 has been shown to be differentially regulated in different tissues under field and glucose stress conditions (Jiang et al. 2010). Our results also revealed that their expression patterns were similar but not exactly identical in soybean under MeJA, glucose, mannitol and NaCl stresses. Prediction analysis revealed some specific putative cis-elements present in the promoters of the two genes, some of which were present as single elements, while others occurred several times. The detailed comparison (according to the PLACE and PlantCARE database) revealed that the number of repeated motifs varied in the two promoters (Supplementary Table S1). There were many potential regulatory elements associated with stress-related responses found in the GmFNSII-1 and GmFNSII-2 promoter region. Both of the promoters contained an ACCTATERD1 binding site (response to dehydration), an A-box motif (sugar repression), a GT1 binding site (light-responsive element), a W-box (salicylic acid-responsive element) and NODCON2GM (nodulation-responsive element). There were some specific elements only in the GmFNSII-1 promoter, such as a CGTCA-motif that has been demonstrated to be a MeJA cis-acting regulatory element (Song and Wang 2009). On the other hand, the ERE motif related to ethylene response (Montgomery et al. 1993) was only found in the GmFNSII-2 promoter. Promoter analysis also led to the discovery of 12 and eight MYB transcription factor binding sites in the GmFNSII-1 and GmFNSII-2 promoters, respectively, some of which have been found to play a role in flavonoid synthesis. For example, the MYBPLANT motif, a plant MYB binding site, was found in promoters of phenylpropanoid biosynthetic genes such as PAL, CHI and DFR (Sablowski et al. 1994). These discrepancies are a possible reason for the different expression of the two genes in response to various stimuli.

In conclusion, we found that MeJA induced production of flavones in soybean seedlings, which is consistent with the identification of a MeJA-responsive motif in the GmFNSII-1 promoter. The production of flavones in soybean seedlings was possibly affected by the dual function of glucose: as an osmotic stressor and a signaling molecule. A dehydration-responsive element (positive) and a sugar repression element (negative) were identified in the GmFNSII-2 promoter by promoter deletion analysis. Under MeJA, glucose and mannitol stresses, the highest level of flavones was present in leaves, but the lowest level was found in roots. GmFNSII-2 induction by stresses was more rapid compared with GmFNSII-1, whereas the latter had a higher transcript level than GmFNSII-2. We also showed that RNAi-mediated suppression of two GmFNSII genes resulted in a reduction in the levels of flavones, led to the accumulation of MDA and H2O2 in roots and greatly inhibited the growth of plant seedlings under salt stress. However, the detailed molecular mechanism underlying flavone accumulation induced by glucose and jasmonates and the flavone function in stress relief in soybean still remain elusive, and future investigations are needed to address these questions.

Materials and Methods

MeJA, glucose, mannitol and NaCl treatments

Soybean (cultivar ‘Hefeng47’) seeds were germinated in pots containing sterilized vermiculite and well-watered with sterile...
1/4 strength Murashige and Skoog (MS) basal salts solution. The seedlings were grown in greenhouse conditions (28/24°C day/night temperature, photoperiod of 16/8 h, 400 μmol m⁻² s⁻¹ light intensity and 60% humidity). In the gene expression and aglycone content experiments, V1 stage plants (first trifoliate vegetative stage, approximately 15 days after sowing) were carefully pulled out and washed to remove excess vermiculite, then transferred to either sterile 1/4 MS solution (control treatment) or to 1/4 MS solution containing appropriate concentrations of glucose (0.4 M), mannitol (0.4 M), methyl jasmonate (100 M) or NaCl (100, 150 and 200 mM) for different treatments. The samples from leaves, stems and roots were collected separately in three biological replicates for all treatments at each time point, immediately frozen in liquid nitrogen and stored at −70°C for RNA and flavone extraction.

Real-time RT–PCR analysis of GmFNSII-1 and GmFNSII-2 expression

Total RNA was isolated from soybean tissues using the TRIzol reagent (Sangon Biotech (Shanghai) Co., Ltd.) for CDNA synthesis according to the manufacturer’s instructions. The quality and integrity of RNA were determined using a NanoDrop ND-1000 spectrophotometer and agarose gel electrophoresis, respectively. First-strand cDNAs were synthesized with the PrimeScript® RT reagent Kit with gDNA Eraser (TAKARA BIO). The real-time PCRs were performed employing a Mastercycler ep realplex Sequence Detection System and software (Eppendorf). All the PCRs were performed under the following conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 20 s. The specificity of amplicons was verified by melting curve analysis (60–95°C) after 40 cycles and agarose gel electrophoresis. Three biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate. Primers used for GmFNSII-1 transcript analysis were 5'-AGGTCGCAATGAGAATGAGGC-3' and 5'-CATCCGCAAGAGGTGTTAC-3'. For GmFNSII-2 transcript analysis, primers 5'-AGGTCGCAATGAGAATGAGGC-3' and 5'-CATCCGCAAGAGGTGTTAC-3' were used. Soybean ACTIN (GenBank: V00450.1) was used as a reference gene for data normalization and to calculate the relative transcript levels.

HPLC analysis

HPLC and flavone quantification methods were modified as described previously (Boué et al. 2003, Jiang et al. 2010). Briefly, fresh plant tissue samples (100 mg) were ground in liquid nitrogen, extracted with 1 ml of 80% methanol at room temperature with rigorous shaking for 12 h, and centrifugated at 12,000 × g for 10 min, and then the supernatant was filtered through a 0.2 μm membrane prior to HPLC analysis. The products were monitored at 260 nm on an Agilent 1100 series HPLC system using a Venusil MP-C18 column (250 mm × 4.6 mm). Elution was carried out at a flow rate of 1.0 ml min⁻¹ with the following solvents: A = HPLC grade water (pH 3.0 adjusted with acetic acid), B = acetonitrile; 5% B to 65% B in 60 min, then 65% B to 100% B in 10 min followed by holding at 100% B for 5 min. Peak identification was confirmed by retention time against flavone aglycone standards and by UV absorption spectra. Apigenin and luteolin were purchased from Sigma-Aldrich, and DHF was obtained from the National Institutes for Food and Drug Control, China. The flavone aglycone content includes the total content of apigenin, luteolin and DHF.

Isolation of the promoter of GmFNSII-1 and GmFNSII-2 sequences and construction of deletion and mutation promoter—GUS vectors

Two pairs of primers, 1R/1F and 2R/2F (Supplementary Table S2), were designed based on the Glycine max genomic sequences (http://www.plantgdb.org/GmGDB/) to amplify the GmFNSII-1 and GmFNSII-2 promoter sequences using soybean cultivar ‘Hefeng47’ genomic DNA as template. The PCR products were sequenced by Sangon Biotech Co., Ltd. Regulatory elements in these regions were analyzed using the online programs PLACE (http://www.dna.affrc.go.jp/PLACE/) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Three DNA fragments containing different deletions of the GmFNSII-1 promoter region were amplified with the appropriate forward primers (1F, 1DF1 and 1DF2) and reverse primer (1R). The forward primers contained a HindIII restriction site, and the reverse primer included a BamHI restriction site (Supplementary Table S2, underlined sequences). The PCRs were performed according to the manufacturer’s instructions using LA Taq DNA Polymerase (TAKARA BIO). The amplified products were sequenced by Sangon Biotech Co. Ltd. The confirmed fragments were digested with HindIII and BamHI restriction endonucleases and directly inserted into the HindIII–BamHI site located upstream of the GUS reporter gene in the vector pBI121. The resulting plasmids were named P1, P1D1 and P1D2, respectively. Using the same method, plasmids P2, P2D1 and P2D2 were constructed for 5′-deletion of the GmFNSII-2 promoter. The corresponding fragments were amplified from genomic DNA with the three forward primers 2F, 2DF1 and 2DF2 and the reverse primer 2R.

GmFNSII-1 mutant promoter (P1m) was constructed by PCR, in which P1 plasmid was used as a template together with two primer pairs, 1F/1mR and 1mF/1R; the PCR products (P1-1 and P1-2) were digested by Sall and then ligated into the fragment (P1m fragment) containing P1-1 and P1-2, the P1m fragment was then double digested by BamHI/HindIII, and the double-digested fragment was directly inserted into vector pBI121 as the promoter of the GUS reporter gene. All of the primers are detailed in Supplementary Table S2.

The construction of GmFNSII-RNAi vectors and transformation by Agrobacterium rhizogenes were as described by Jiang et al. (2010).
Ex vitro composite plant production

According to a previously described method (Taylor et al. 2006), A. rhizogenes strain K599 harboring the described vectors (all deletion promoter–GUS vectors, RNAi vectors and control vectors) were cultured in LB broth and resuspended in sterile 1/4 strength MS salts (OD$_{600}$ ~ 0.3). Apical stem sections, excised from greenhouse-grown V3 stage soybean seedlings, were inserted into the inoculated Agricultural Rockwool saturated with the resuspended cells, placed in open Petri dishes within plant growth trays covered with clear plastic domes and incubated at room temperature overnight, then the trays were opened until the plant materials were fully wilted, the Rockwool was saturated with deionized water and the box was closed. Trays were checked weekly to make sure the cubes were wet during the induction period. Incubation conditions were 22°C with 100 μmol m$^{-2}$ s$^{-1}$ light intensity and a 16 h photoperiod. After about 2 weeks, transgenic roots were observed emerging from the cut site. The Rockwool was removed using forceps, and the roots of the composite plants were stained for GUS to determine whether they were transgenic.

Histochemical and fluorimetric analysis of GUS expression

For histochemical staining, transgenic hairy roots were immersed in GUS staining solution [1 mM X-gluc in 100 mM sodium phosphate buffer, pH 7.0, containing 10 mM EDTA, 0.1% Triton and 0.5 mM K$_4$Fe(CN)$_6$H$_2$O] at 37°C for 16 h (Jefferson et al. 1987). After staining, tissues were incubated in 70% (v/v) ethanol for 24 h to reduce background.

GUS activity was determined using the fluorimetric GUS assay (Jefferson et al. 1987). Fluorescence output was measured in a spectrofluorophotometer (LS50B, Perkin Elmer, Inc.) with excitation at 365 nm and emission at 455 nm. Protein concentration was determined according to the method described by Bradford (1976). GUS activity was expressed as picomoles of 4-methylumbelliferone (MU) (Sigma 1508) per minute per milligram of protein.

Lipid peroxidation and H$_2$O$_2$ detection

Lipid peroxidation was determined by the thiobarbituric reaction to measure the amount of MDA according to the protocols described by Zhang et al. (2009). Briefly, soybean roots were weighed and homogenized in 4 ml of 10% trichloroacetic acid (TCA) solution. After the sample was centrifuged at 10,000 g for 30 min, an equal volume of 0.6% thiobarbituric acid in 10% TCA was added to the supernatant. Then the mixture was incubated in boiling water for 15 min and the reaction was stopped in an ice bath. The mixture was centrifuged at 10,000 g for 20 min and the absorbance of the supernatant was measured at 450, 532 and 600 nm.

H$_2$O$_2$ was detected by an endogenous peroxidase-dependent in situ histochemical staining procedure using DAB as described previously (Watanabe and Lam 2008). Plant tissues were placed in a solution containing 1 mg ml$^{-1}$ DAB (pH 5.5, Sigma-Aldrich) for 2 h at room temperature. The plant tissues were washed with water and cleared in boiling ethanol (96%) for 10 min, and rinsed in 50% ethanol followed by distilled water. The treatment decolorized the seedlings, except for the deep brown polymerization product produced by the reaction of DAB with H$_2$O$_2$. After cooling, the seedlings were washed with fresh ethanol and preserved at room temperature in ethanol and photographed. H$_2$O$_2$ level was visualized as a reddish-brown coloration (Thordal-Christensen et al. 1997).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


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