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RESEARCH PAPER

Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia

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Abstract

Isoflavonoids are derived from a flavonone intermediate, naringenin, that is ubiquitously present in plants, and play a critical role in plant development and defence response. Isoflavonoids secreted by the legumes also play an important role in promoting the formation of nitrogen-fixing nodules by symbiotic rhizobia. In these plants, the key enzyme that redirects phenylpropanoid pathway intermediates from flavonoids to isoflavonoids is the cytochrome P450 monooxygenase, isoflavone synthase. In an effort to develop a rice variety possessing the ability to induce nodulation (*nod*) genes in rhizobia, the *IFS* gene from soybean was incorporated into rice (*Oryza sativa* L. cv. Murasaki R86) under the control of the 35S promoter. The presence of *IFS* in transgenic rice was confirmed by PCR and Southern blot analysis. Analyses of the *35S-IFS* transgenic lines demonstrated that the expression of the *IFS* gene led to the production of the isoflavone genistein in rice tissues. These results showed that the soybean *IFS* gene-expressed enzyme is active in the R86 rice plant, and that the naringenin intermediate of the anthocyanin pathway is available as a substrate for the introduced foreign enzyme. The genistein produced in rice cells was present in a glycoside form, indicating that endogenous glycosyltransferases were capable of recognizing genistein as a substrate. Studies with rhizobia demonstrated that the expression of isoflavone synthase confers rice plants with the ability to produce flavonoids that are able to induce *nod* gene expression, albeit to varied degrees, in different rhizobia.

Key words: Flavonoids, isoflavone synthase, *nod* gene induction, *Oryza sativa*, rhizobia.

Introduction

Flavonoids are secondary plant metabolites, derived from the phenylpropanoid pathway (Dixon and Steele, 1999; Winkely-Shirley, 2001). Isoflavonoids, which are limited primarily to leguminous plants, are produced by a branch of the phenylpropanoid pathway (Fig. 1). Many studies link the dietary consumption of the isoflavones genestein and daidzein to a range of health benefits (for references see Fader *et al*., 2000; Liu *et al*., 2002). In plants, these compounds are known to be involved in interactions with other organisms and participate in the defence responses of legumes against phytopathogenic micro-organisms (Fader *et al*., 2000). In addition, isoflavonoids also participate in promoting symbiotic relationship between legume plants and rhizobia leading to the formation of nitrogen-fixing root nodules (Broughton *et al*., 2000; Spaink, 2000). Isoflavones act as chemoattractants for the rhizobia and as inducers of nodulation (*nod*) gene expression, leading to the production of Nod factors, which in turn aid in the development of nodules in legume plants.

The substrate for producing the isoflavone genistein is naringenin, an intermediate in the branch of the phenylpropanoid pathway that leads to synthesis of the flavonoids,

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which include flavanones, flavones, flavonols, proanthocyanidins, and anthocyanins. Naringenin is the product of chalcone synthase and chalcone isomerase enzymes that are common to most plants (Fig. 1). Legumes have a unique enzyme that carries out aryl migration of the B-ring to the 3-position of naringenin, paving the way for the production of the isoflavone (Kochs and Grisebach, 1986). This key enzyme that redirects phenylpropanoid pathway-flavonone intermediates to isoflavonoids is the cytochrome P450 mono-oxygenase, isoflavone synthase. In most plants other than legumes, isoflavones are not among the complex variety of secondary metabolites synthesized by the phenylpropanoid pathway. This difference is due to the absence of isoflavone synthase, which converts naringenin into an isoflavone. The identification of the gene encoding isoflavone synthase (Akashi *et al*., 1999; Steele *et al*., 1999; Jung *et al*., 2000) allowed the introduction of an isoflavone synthesizing capacity into *Arabidopsis* and tobacco, and maize BMS cell cultures that do not naturally produce isoflavones (Jung *et al*., 2000; Yu *et al*., 2000; Liu *et al*., 2002). These studies demonstrated that the isoflavone genistein could be synthesized in *Arabidopsis* and tobacco, thus proving that the naringenin substrate present in these anthocyanin-producing plants was available for isoflavone synthase to convert to genistein.

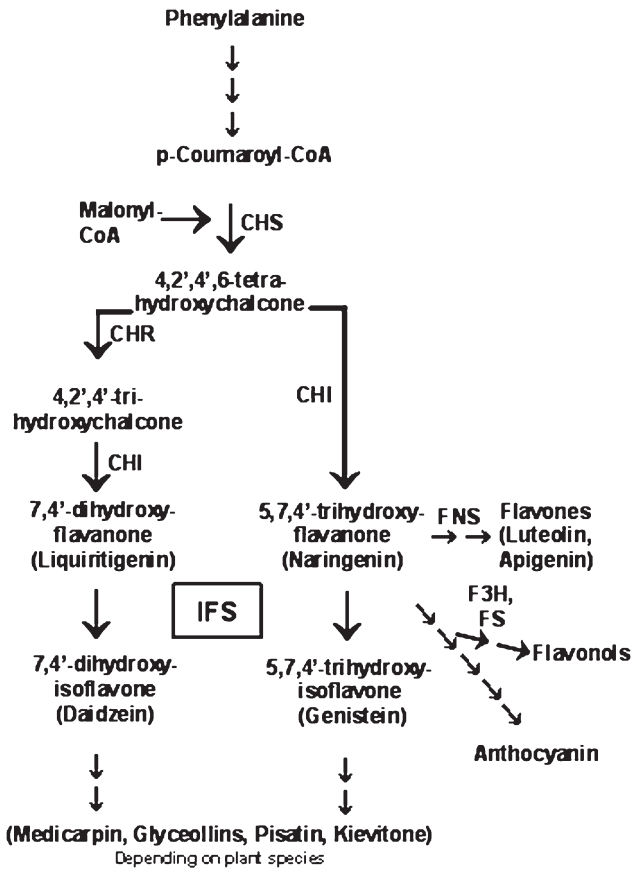


Fig. 1. A partial diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis. CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; FNS, flavone synthase; FS, Flavonol synthase; IFS, isoflavone synthase.

Rice is one of the most important cereal crops of the world, and recently much effort has been directed towards reducing the nitrogen input from chemical fertilizers and providing nitrogen to rice by biological nitrogen fixation (Ladha and Reddy, 2000, 2003). Towards this goal, studies have focused on how to encourage the interaction of rice with rhizobia in order to develop a stable symbiotic association (Reddy *et al*., 1997, 2000*a*, *b*; Sreevidya *et al*., 2005). Rice was shown to harbour at least partial genetic make up in its genome for interacting with rhizobia (Reddy *et al*., 2000*b*, 2002). However, rice varieties have a very low capacity to induce *nod* genes in rhizobia, presumably because of a lack of ability to synthesize *nod* gene-inducing flavonoids (Reddy *et al*., 2000*b*; Rolfe *et al*., 2000). Hence, isoflavone production may pave the way for rice plants to enter into a symbiotic relationship with rhizobia. Because secretion of flavonoids is a first step in the legume-*Rhizobium* interactions, and rice lacks the pathway to produce isoflavones from flavanones such as naringenin, in this study the aim was to transfer the key enzyme, isoflavone synthase for isoflavonoid production in rice, and to determine its ability to synthesize the isoflavonoid genistein, and to induce *nod* genes in rhizobia. In this study, a pigmented rice variety (*Oryza sativa* L. cv. Murasaki R86) was used which produces anthocyanins in leaves, culm, and seed coat (Nakai *et al*., 1998). Any plant that can produce anthocynins must have the ability to generate naringenin, a substrate for isoflavone synthase for the production of genistein (Fig. 1).

Materials and methods

Rhizobial strains

Rhizobial strains, *Rhizobium* NGR234, *A. caulinodans* ORS571, *R. meliloti* 1021, and *B. japonicum* USDA110 harbouring *nod::lacZ* fusions, used in the present study are listed in Table 1, and will be referred to in the text as NGR234, ORS571, *Rm*1021, and USDA110, respectively. The *Rhizobium* and *Bradyrhizobium* strains were routinely grown under aerobic conditions in the dark at 30 °C in yeast mannitol (YM) broth or agar plates (Vincent, 1970), supplemented with the appropriate antibiotics, if needed.

Table 1. *Rhizobial strains carrying* nod::lacZ *fusions*

Rhizobial strain	*nod::lacZ* fusion	Reference
Rhizobium NGR234 (pA27)	*nodSU::lacZ*	Lewin *et al*. (1990)
A. caulinodans ORS571 (pRG290–12::T20)	*nodA::lacZ*	Goethals *et al*. (1989)
R. meliloti 1021 (pRM57)	*nodC::lacZ*	Mulligan and Long (1985)
B. japonicum USDA110 (pZB32, Z977)	*nodY::lacZ*	Banfalvi *et al*. (1988)

Construction of plant transformation vector

The binary transformation vector pMSH2-*cyp93C1* (Fig. 2A) containing the soybean isoflavone synthase gene (*IFS; cyp93C1v1*) under the control of CaMV 35S promoter and the nopaline synthase gene (*nos*) terminator was constructed by ligating the 1.8 kb *SpeI/XbaI* fragment of the *cyp93C1v1* cDNA, derived from pCR2.1/cyp93C1v1 (Siminszky *et al*., 1999), downstream of the CaMV 35S promoter in the *SpeI/XbaI* restricted binary vector pMSH2 (Kawasaki *et al*., 1999), in sense orientation.

Rice transformation and growth conditions

Binary vectors used for rice transformation, pVM200 (a derivative of the pMSH lacking the CaMV 35S promoter at the multiple cloning site; control vector) or pMSH2-*cyp93C1* (*35S-IFS*), were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation (Nagel *et al*., 1990). *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L. cv. Murasaki R86) calli was performed according to Sreevidya *et al*. (2005). Plants were regenerated from transformed calli selected by hygromycin resistance. Fully regenerated plantlets were acclimatized hydroponically in Yoshida nutrient solution (Yoshida *et al*., 1976) for 15 d and then the putative transgenic plants (T_0 generation) were transferred to soil in pots and grown in a transgenic containment greenhouse.

Polymerase chain reaction (PCR), and Southern and northern blot analyses

Genomic DNA was prepared from young rice leaves using the procedure developed by Dellaporta *et al*. (1983) for Southern analysis, and by the CTAB method (Taylor *et al*., 1993) for PCR analysis.

For PCR analysis ~5 ng of genomic DNA was used as a template. The primers, designed to generate the entire coding sequences of *IFS*, were used at 10 μM concentration in a 20 μl reaction mixture containing 0.2 mM of each dNTPs, 2 mM $MgCl_2$, 2 U *Taq* DNA polymerase in PCR buffer, and PCR was performed on Biometra Uno-Thermocycler (Biometra, Gottingen, Germany). The forward and reverse primers were 5′-ATG TTG CTT GAA CTT GCA CT-3′ and 5′-TTA AGA AAG GAG TTT AGA TG-3′, respectively, and the amplification was carried out with an initial cycle of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 45 s at 53 °C, and 1.5 min at 72 °C, and a single final cycle of 10 min at 72 °C. After the completion of PCR, samples were separated on 0.8% (wt/vol) agarose gels, stained with ethidium bromide and visualized on an UV transilluminator.

For Southern analysis aliquots of 10 μg of purified genomic DNA were digested with *Xba*I, size-fractionated on a 0.8% (w/v) agarose gel, alkali transferred onto a Hybond-N^+ nylon membrane (Amersham Biosciences, Buckinghamshire, UK), and hybridized overnight at 42 °C to digoxigenin-11-dUTP labelled PCR fragments of the coding sequence of *IFS*. DNA probe preparation using random primers, hybridization and stringency washing of the membranes, and chemiluminescent detection of the bands with anti-DIG-AP and CSPD were done using the DIG-High Prime DNA Labeling and Detection Kit II as per the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The blots were exposed to X-ray film (Kodak, Rochester, NY, USA) for 1–2 h.

For northern analysis, total RNA was isolated from young rice leaves by guanidine isothiocyanate extraction as per the manufacturer's instructions (TRIzol reagent; GIBCO-BRL, Grand Island, NY). Total RNA (~20 μg) was subjected to denaturing agarose gel electrophoresis and blotted onto the Hybond-N^+ membrane (Amersham) using standard methods (Sambrook *et al*., 1989). The membrane was hybridized with a DIG-labelled *IFS* probe and processed as described above.

Identification of free isoflavones in 35S-IFS *transgenic plants*

Plant tissues were ground in liquid nitrogen, extracted with methanol at 100 mg ml^{-1}, and filtered through 0.2 μm Acrodisc syringe filters (Gelman Sciences, Ann Arbor, MI, USA). To hydrolyse any possible isoflavone conjugates, 3 ml of 1 N HCl were added to 1 ml of the extract and the sample was incubated at 95 °C for 2 h, followed by extraction using 1 ml ethyl acetate. Both unhydrolysed and hydrolysed methanol extracts were separated by HPLC system (Shimadzu SCL10A, Japan) using Luna C18 column (3 μm, 150 mm×4.6 mm; Phenomenex, USA), by isocratic separation with 65% methanol in water at a flow rate of 0.5 ml min^{-1}. The peaks were monitored at 260 nm and 280 nm, and absorbance of the eluents was recorded on UV detector (SPD10A, Shimadzu, Japan). Absorption spectra of the eluents were obtained using a spectrophotometer (DU800, Beckman, USA). The eluent fractions collected at 1 min intervals were evaporated in Speedvac (SC110A Speedvac Plus, Savant, Germany), dissolved in methanol at a concentration of 10 mg ml^{-1} and used for *nod* gene induction studies. Authentic naringenin, genistein (Sigma, St Louis, USA), liquiritigenin (Indofine Chemical Company, Somerville, NJ, USA), and daidzein (Research Biochemicals International, Natick, MA, USA) were dissolved at a concentration of 10 μM in methanol and used as standards in HPLC and spectral analyses.

Isoflavone synthase activity assay

For Isoflavone synthase assays, soluble and membrane protein fractions from control and *35S-IFS* transgenic rice plants were prepared as follows. Root/leaf tissues (1 g fresh weight) of 2 month-old rice plants were frozen with liquid nitrogen, ground to a fine powder, and homogenized in 10 ml of extraction buffer (50 mM TRIS-HCl, pH 8.2, 0.4 M sucrose, 2 mM EDTA, 0.5 mM DTT, 0.3 mM mercaptoethanol, and 10% glycerol). The homogenates were centrifuged at 5000 rpm (5 K) for 10 min at 4 °C. The pellets were discarded, and the supernatants (designated as crude homogenates, containing both soluble and membrane fractions) were collected and stored at –80 °C until further use. A portion of the supernatants were centrifuged again at 15 000 rpm (15 K) for 60 min at 4 °C after adding glycerol to a final concentration of 20%. The resulting pellets (crude microsomal fractions) and supernatants were collected and stored at –80 °C until further use. The protein contents of 5 K and 15 K preparations were determined using the Bradford protein microassay (Bio-Rad, Hercules, CA, USA), and isoflavone synthase activity assays were performed according to Yu *et al*. (2000). Approximately 150 mg of the above protein fractions were incubated for 12 h at room temperature in a reaction mix containing 80 mM K_2HPO_4, 0.5 mM glutathione, 20% sucrose, pH 8.0, with 100 μM naringenin or 100 μM liquiritigenin substrate and 0.4 mM of NADPH. Following incubation, reactions were extracted with methanol. The samples were evaporated and resuspended in 80% methanol, and then were separated on a HPLC system as described above.

Measurement of the nod gene-inducing activity

Methanol extracts prepared from untransformed or vector transformed control plants, and *35S-IFS* transgenic rice tissues, and the eluents obtained following HPLC separation of the methanol extracts were assessed for their ability to induce *nod* gene expression in rhizobia harbouring *nod::lacZ* fusions (Table 1) as follows. Bacteria were grown overnight to mid-log phase and diluted to an OD_{600} of 0.2 in 2 ml of YM medium containing 50 μl of the rice tissue extracts or the eluents obtained after HPLC separation, together with appropriate antibiotics. All extracts were diluted at 0.01 mg ml^{-1}, in 2 ml of culture. After overnight shaking for 16 h at 30 °C, the cultures were assayed for β-galactosidase activity (expressed by the *nod::lacZ* fusion gene) according to the method of Miller (1972) at a temperature of 37 °C with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) or

chlorophenol red-β-D-galactopyranoside (CRPG) as a substrate. The OD_{420} (ONPG), OD_{574} (CRPG). and OD_{600} (bacterial cells) readings were obtained using a spectrophotometer (DU800, Beckman, USA). All assays with the extracts were conducted at least four times, and mean and standard error of the data points were calculated using the Microsoft Excel program, with appropriate controls in each experiment. Data obtained with *nod* gene induction assays were subjected to Student's *t* test to delineate significance of any differences in β-galactosidase activities elicited in rhizobia in response to the plant extracts of *35S-IFS* plants versus vector-transformed control rice plants. Threshold levels of significance were adjusted for multiple comparisons by Bonferroni's correction. Corrected *P*-values were then calculated according to sample size. Bonferroni correction for multiple comparisons was applied to enhance stringency level while determining the significance of any differences between the compared groups by the *t* test. Thus, the significance test performed was at a highly stringent two-tailed alpha level (significance level) of 0.01.

β-galactosidase assays of rhizobia performed with appropriately diluted methanol (solvent used for the dilution of extracts) served as negative controls, and the assays with the authentic flavonoids (100 μM) apigenin, 7,4-dihydroxyflavone, daidzein, genistein, liquiritigenin, luteolin, and naringenin served as positive controls.

Results

Generation of transgenic rice carrying the soybean IFS *gene*

Transgenic rice plants carrying the *IFS* gene fused to the cauliflower mosaic virus 35S promoter were generated using *A. tumefaciens*-mediated transformation. The plantlets regenerated from the hygromycin-resistant transformed rice calli were analysed for the presence of *IFS* using gene-specific primers in the polymerase chain reaction. PCR analysis showed that 11% of the putative transgenic plants of rice contained the *IFS* gene (Table 2), and these were designated as *35S-IFS* plants. Southern analyses of the *35S-IFS* plants revealed that all of them had a single copy of the *IFS* gene, and were derived from five independent transformation events (Fig. 2B). Northern analysis of the selected representative *35S-IFS* plants revealed that the *IFS* gene was expressed in the transgenic lines (Fig. 2C). During the generation of the primary transgenic plants, most of the *35S-IFS* plants, excepting D121, showed normal growth and development similar to untransformed or vector control plants (Fig. 3A). Representative *35S-IFS* plants (T_0) derived from each independent line (D90, D93, D95, D114, and D121) were used in *nod* gene induction studies. Untransformed and/or vector-transformed rice plants served as controls.

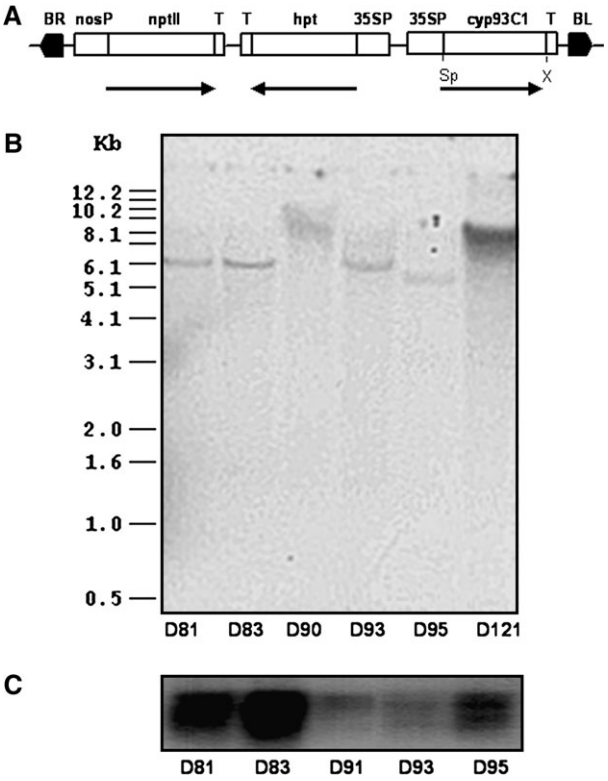


Fig. 2. (A) Binary vector carrying the *35S-IFS* chimeric gene, and (B) Southern and (C) northern blot analysis of the representative primary transgenic rice plants harbouring *35S-IFS* generated from independent transformation events. For Southern blot analysis, rice genomic DNA from the representative primary *35S-IFS* transgenic plants was digested with *Xba*I and hybridized with the DIG-labelled *IFS* cDNA inserts. For northern analysis, total RNA from young leaves of the representative transgenic rice plants harbouring *35S-IFS* was isolated by guanidium thiocyanate extraction, separated on formaldehyde-agarose gels, blotted, and hybridized with a DIG-labelled *IFS* probe. Numbers below each lane in Southern or northern blots denote the selected transformation lines of *35S-IFS* rice plants (D81, D83, D90, D91, D93, D95 and D121). BR: right border; BL: left border; *nos* P: nopalene synthase promoter; *35S* P: CaMV 35S promoter; *npt*II: kanamycin resistance gene; *hpt*: hygromycin resistance gene; *cyp93c1*: soybean isoflavone synthase cDNA; T: Nos terminator; S: *Spe*I; X: *Xba*I.

Table 2. *Characteristics of* 35S-IFS *and vector control transgenic plants of rice*

Vector	Putative transgenic plants regenerated	PCR positive plants for *IFS* or *hpt*	Number of independent lines based on Southerns	Copy number of the transgenes	Phenotype of the transgenic plants
pMSH2-*cyp93C1* (*35S-IFS*)	65	7 (11%)[a]	5	1	Normal[c]
pVM200 (control vector)	70	34 (49%)[b]	3	1–3	Normal

[a] *IFS*-positive.
[b] *hpt*-positive.
[c] All *35S-IFS* plants showed normal growth and development, excepting the transgenic plant D121 which exhibited a retarded growth phenotype. Values in parentheses depict percentage of the PCR-positive transgenic plants.

Evaluation of nod *gene expression in rhizobia in response to standard flavonoids*

Most of the known plant substances that act as signals to rhizobia are flavonoids. Flavonoids selectively activate the expression of the nodulation (*nod*) genes of symbiotic rhizobia (Broughton *et al*., 2000). Flavonoids interact with a class of transcriptional activators (NodD) of the LysR family that bind to highly conserved 49 bp DNA motifs (*nod* boxes) found in the promoter regions of many nodulation loci, thus aiding in the expression of *nod* genes. Despite the fact that *nodD* genes are present in all rhizobia, their symbiotic characteristics vary from one species to another, and *Rhizobium* species respond in different ways to flavonoids. NodD homologues from the same or different strains may have different flavonoid preferences. In the present study, authentic flavonoids such as apigenin, daidzein, 7,4-dihydroxyflavone, genistein, liquiritigenin, luteolin, and naringenin have been tested in order to ascertain flavonoid preferences for *nod* gene activation in the rhizobial strains used in this study (Table 3). Quantitative measurements of the expression of the *nod::lacZ* fusions harboured by various rhizobia were made by assaying β-galactosidase activity in bacteria. The rhizobia tested in the present study exhibited differential responses towards various flavonoids. A comparison of data presented in Table 3 gives a clear picture of potent and weak inducers of *nod* gene expression in NGR234, ORS571, *Rm*1021, and USDA110. The most potent inducers that showed half-maximal *nod* gene induction were 7,4-dihydroxyflavone, apigenin, naringenin, liquiritigenin, daidzein, and genistein in the case of NGR234, naringenin and liquiritigenin in ORS571, luteolin and 7,4-dihydroxyflavone in *Rm*1021, and genistein and daidzein in USDA110. These results agree with the findings reported earlier for the same strains, reiterating differential flavonoid preferences for the induction of *nod* gene expression in different rhizobia (Mulligan and Long, 1985; Peters *et al*., 1986; Banfalvi *et al*., 1988; Bassam *et al*., 1988; Goethals *et al*., 1989; Le Strange *et al*., 1990; Lewin *et al*., 1990; Kobayashi *et al*., 2004).

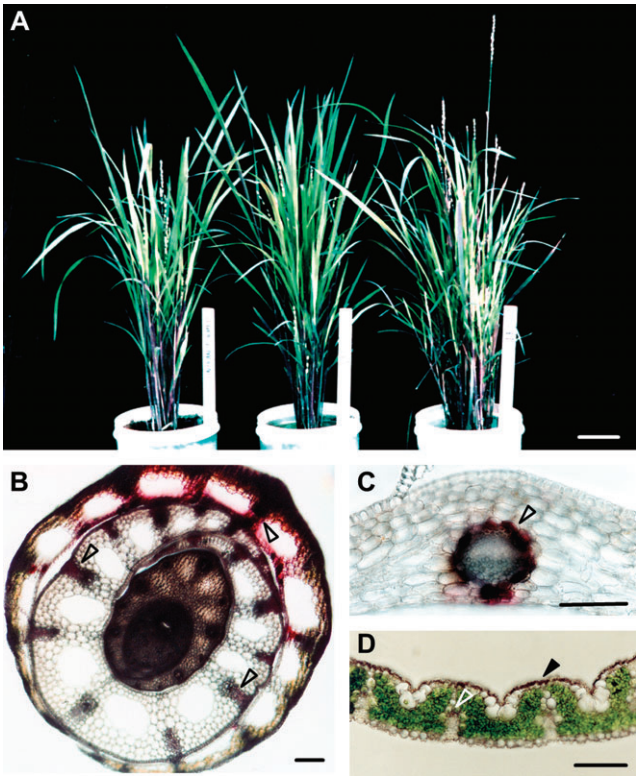


Fig. 3. (A) Comparison of growth of transgenic rice plants transformed with vector (centre) and a *35S-IFS* chimeric gene (right) with an untransformed control plant (left). The *35S-IFS* transgenic plant shown in this figure is that of D93. Note normal growth and development of the *35S-IFS* transgenic plant. (B–D) Photomicrographs of transverse sections of stem (B), sheath (C), and leaf (D) showing the production of anthocyanin pigment in cells in the vicinity of the vascular bundles (open arrowheads) and leaf epidermis (closed arrowhead).

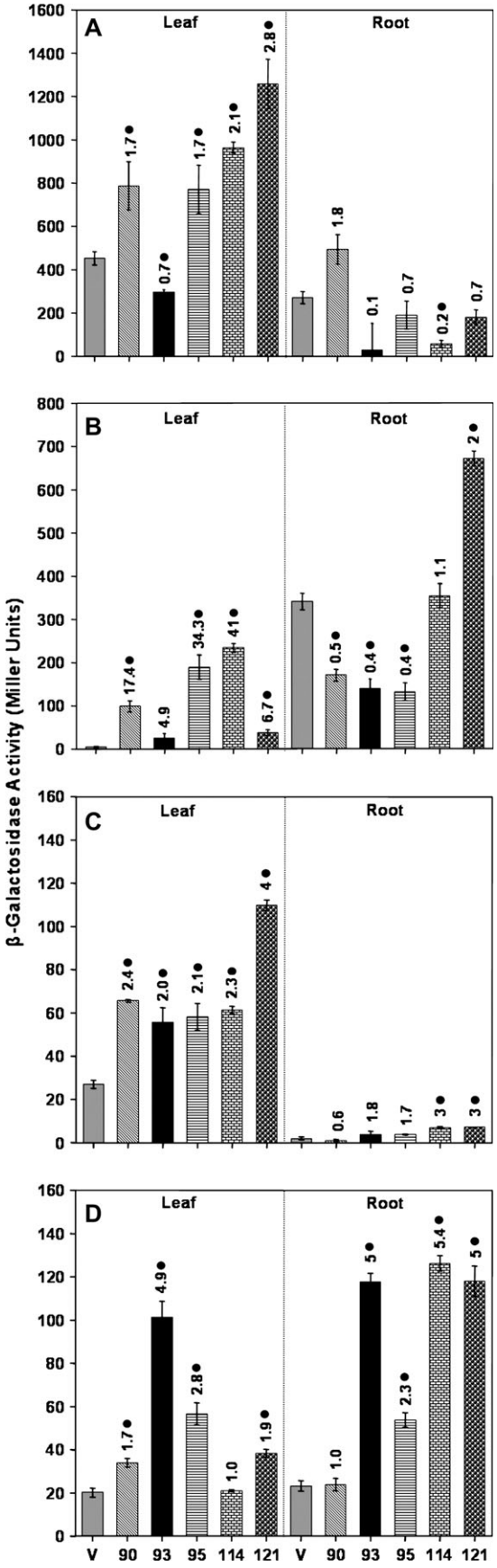
Assessment of the ability of the wild-type R86 and vector-transformed control rice plants to induce nod *gene expression in rhizobia*

Earlier studies showed that root exudates or plant extracts from various indica and japonica rice varieties have only a limited capacity to induce the transcription of *nod* genes in rhizobia (Reddy *et al*., 2000*b*; Rolfe *et al*., 2000).

Table 3. *β-galactosidase expressed by* Rhizobium *NGR234* (nodSU::lacZ), A. caulinodans *ORS571* (nodA::lacZ), R. meliloti *1021* (nodC::lacZ), *and* B. japonicum *USDA110* (nodY::lacZ) *in response to the authentic flavonoids*

Half-maximal β-galactosidase activities (Miller units): NGR234, 5145; ORS571, 2340; Rm1021, 155; USDA110, 792. SE: Standard error.

Flavonoid	β-galactosidase activity (Miller units) ±SE			
	Rhizobium NGR234 (*nodSU::lacZ*)	*A. caulinodans* ORS571 (*nodA::lacZ*)	*R. meliloti* 1021 (*nodC::lacZ*)	*B. japonicum* USDA110 (*nodY::lacZ*)
Naringenin	7041±533	4679±273	13±1	149±16
Liquiritigenin	6691±129	4271±72	80±8	98±5
Genistein	5359±170	322±39	6±1	1584±142
Daidzein	6845±486	178±52	18±2	824±39
Apigenin	8803±702	214±54	94±7	270±19
Luteolin	539±81	515±29	310±4	154±8
7,4-Dihydroxy flavone	10289±234	941±85	230±25	638±30



In order to ascertain the extent of the *nod* gene-inducing abilities of the R86 rice variety used in the present study, root and leaf extracts prepared from the vector transformed (for example, see Fig. 4) and untransformed wild-type control plants were examined for their ability to induce β-galactosidase activities in NGR234, ORS571, *Rm*1021, and USDA110. The study showed that extracts of both vector-transformed and the untransformed control behaved very similarly in inducing *nod* gene expression in rhizobia (data not shown). Extracts derived from the control rice plants elicited modest levels of *nod* gene expression in NGR234 and ORS571, while they produced very low levels of induction in *Rm*1021 and USDA110.

Induction of rhizobial nod *genes by transgenic rice expressing* IFS

To ascertain if the presence of the soybean *IFS* gene in transgenic rice confers the competence to produce *nod* gene-activating flavonoids, the methanol extracts generated from leaves and roots of representative *35S-IFS* rice plants (D90, D93, D95, D114, and D121) from each independent transformation line were analysed for their ability to induce *nod* gene expression in NGR234, ORS571, *Rm*1021, and USDA110 harbouring *nod::lacZ* fusions. When NGR234 carrying *nodSU::lacZ* fusion was tested for *nod* gene induction, the leaf extracts derived from D90, D95, D114, and D121 transgenic lines induced significantly higher β-galactosidase activity compared with the leaf extract from vector-transformed control plants (Fig. 4A). By contrast, compared with control plants, none of the root extracts generated from various transgenic plants were able to enhance *nod* gene induction in NGR234. In the case of ORS571, compared with vector control plants, even though leaf extracts derived from all *35S-IFS* transgenic plants, in general, were able to promote *nod* gene expression, the leaf extracts particularly from the D90, D95, D114, and D121 lines produced a significant enhancement in *nod* gene

Fig. 4. β-galactosidase expressed by (A) *Rhizobium* NGR234 (*nodSU::lacZ*), (B) *A. caulinodans* ORS571 (*nodA::lacZ*), (C) *R. meliloti* 1021 (*nodC::lacZ*), and (D) *B. japonicum* USDA110 (*nodY::lacZ*) in response to the extracts prepared from leaves and roots of rice plants transformed with *35S-IFS* or vector. Plant extracts derived from: V, vector transformed control plant; D90, D93, D95, D114, and D121, representative *35S-IFS* plants from five independent transformation lines. β-galactosidase activity of various rhizobia was determined after 16 h exposure to plant extracts. The level of induction is expressed after subtracting the background level of β-galactosidase activity that was about 715 units in *Rhizobium* NGR234, 236 units in *A. caulinodans* ORS571, 4 units in *R. meliloti* 1021, and 9 units in *B. japonicum* USDA110. The bars represent means of five (four in the case of *A. caulinodans*) β-galactosidase assays, and vertical lines represent standard error values. Numbers on the top of each bar represent the relative increase/decrease in β-galactosidase activity induced by the *35S-IFS* plant extracts compared with vector control plants. Black dots above the bars depict significant differences (*t* test, $P \leqslant 0.01$ after Bonferroni correction) in β-galactosidase activities induced by the extracts from *35S-IFS* plants versus that from vector control plants.

induction (Fig. 4B). By contrast, only the root extract obtained from D121 could promote a significant increase in β-galactosidase activity in ORS571. Analysis with *Rm*1021 revealed that the leaf extracts derived from the *35S-IFS* plants of all transgenic lines were able significantly to enhance *nod* gene expression in this bacterium, compared with the leaf extracts from control transgenic plants transformed with the vector alone (Fig. 4C). However, when the ability of root extracts from the *35S-IFS* transgenic plants was assessed, it was found that they were able to induce β-galactosidase activity in *Rm*1021 at much lower levels than the leaf extracts from the respective transgenic plants. In the case of USDA110, almost all leaf and root extracts derived from the *35S-IFS* plants (excepting the leaf extract from D114 and the root extract from D90, which showed no difference in *nod* gene induction levels compared with the vector control) stimulated *nod* gene expression significantly in this bacterium (Fig. 4D). The above results suggest that manipulation by incorporating the *IFS* gene bestowed rice plants with the ability to produce flavonoids that are able to regulate *nod* gene expression, albeit to varied degrees, in NGR234, ORS571, *Rm*1021, and USDA110. Further, it is interesting to note that both leaf and root extracts derived from most of the *35S-IFS* transgenic lines were able to stimulate *nod* gene expression in USDA110, compared with other rhizobia, where leaf extracts were more effective than root extracts (excepting the root extract derived from D121 on *nod* gene induction in ORS571) in promoting *nod* activity. This observation is intriguing since *nod* gene expression in USDA110 is maximally induced by isoflavones, such as genistein (Table 3; Banfalvi *et al*., 1988), and the present study showed that *IFS*-transformed rice plants are indeed able to produce genistein (see below).

nod *gene induction in* B. japonicum *USDA110 by the HPLC-separated fractions of methanol extracts derived from transgenic rice expressing* IFS

Investigations with the crude methanol extracts revealed that the leaf and root extracts derived from *35S-IFS* transgenic plants were generally able to induce greater *nod* gene expression in USDA110 (see above). In particular, both root and leaf extracts from D93 elicited very high levels of *nod* gene expression. To investigate the ability of the D93 plant extracts to induce *nod* gene expression in USDA110 further, the methanol extracts prepared from leaves and roots of D93 were fractionated by the HPLC system equipped with a reverse-phase Luna C18 column, and the fractions collected at 1 min intervals were assessed for their ability to induce *nodY::lacZ* fusion in USDA110 as against the fractions collected from the extracts derived from untransformed plants. Analysis of various HPLC fractions of leaf and root extracts produced conspicuous differences in their abilities to induce *nod* gene expression in USDA110 (Fig. 5A, B). The 'leaf fractions' of D93 obtained at 6, 8, 13, 14, and 21 min significantly stimulated *nod* gene induction in USDA110 compared with the 'leaf fractions' derived from control plants at similar retention times (Fig. 5A). It is interesting to note that a number of fractions from the wild-type R86 leaf extract produced inhibitory effect on *nod* gene expression, in particular those with 13, 14, and 21 min retention times exerted a significant inhibition compared with comparable fractions obtained from D93 plants. In the case of D93 transgenic plants, however, only one 'leaf fraction' that was eluted at the 10 min retention time elicited reduced *nod* gene expression compared with a comparable fraction from control plants. Overall, the above results indicated that R86 leaves, besides *nod* gene-inducing compounds, also produce compounds that are inhibitory to *nod* gene expression in USDA110, and these inhibitory effects, at least partially, could be alleviated by transforming R86 plants with the *IFS* gene, which perhaps enables the plants to produce new flavonoid compounds that are conducive to *nod* gene induction.

In contrast to the 'leaf fractions', none of the HPLC fractions derived from the roots of wild-type R86 plants exerted any inhibitory effect on *nod* gene expression in USDA110 (Fig. 5B). Nonetheless, expression of *IFS* in R86 roots, by and large, substantially enhanced their ability to promote *nod* induction. The 'root fractions' of D93 obtained at 5, 6, 7, 8, 14, 16, 21, 22, and 24 min retention times significantly stimulated *nod* gene expression in USDA110 compared with the 'root fractions' derived from control plants at similar retention times (Fig. 5B). The only 'root fraction' of D93 that produced a marginal, but significant reduction in *nod* gene expression (compared with the comparable fraction from control plants) was eluted at a retention time of 12 min. All other 'root fractions' of D93 collected at the retention times of 9, 10, 11, 13, 17, and 21 min promoted *nod* gene induction similar to the corresponding fractions derived from the roots of control plants. Taken together these results suggest that R86 roots inherently produce compounds that are modestly stimulatory to *nod* gene expression in USDA110, and this ability could be improved by transforming R86 plants with *IFS*.

Identification of genistein in acid hydrolysed methanol extracts of transgenic rice roots expressing IFS*, and determination of the ability of the acid hydrolysed-fractions to induce* nod *gene expression in* B. japonicum

A comparison of the HPLC chromatograms obtained with the leaf and root extracts of IFS-transformed plants with those acquired with R86 control rice plants revealed no new peak with a retention time (R_F) 7.3–7.4 min corresponding to the genistein standard, in *35S-IFS* plants (for example, see HPLC profiles of D93 extracts in Fig. 5, insets). In legumes, free isoflavones rarely accumulate to high levels in plant tissues. Instead they are usually conjugated to carbohydrates (Graham, 1991). This prompted the hypothesis that the

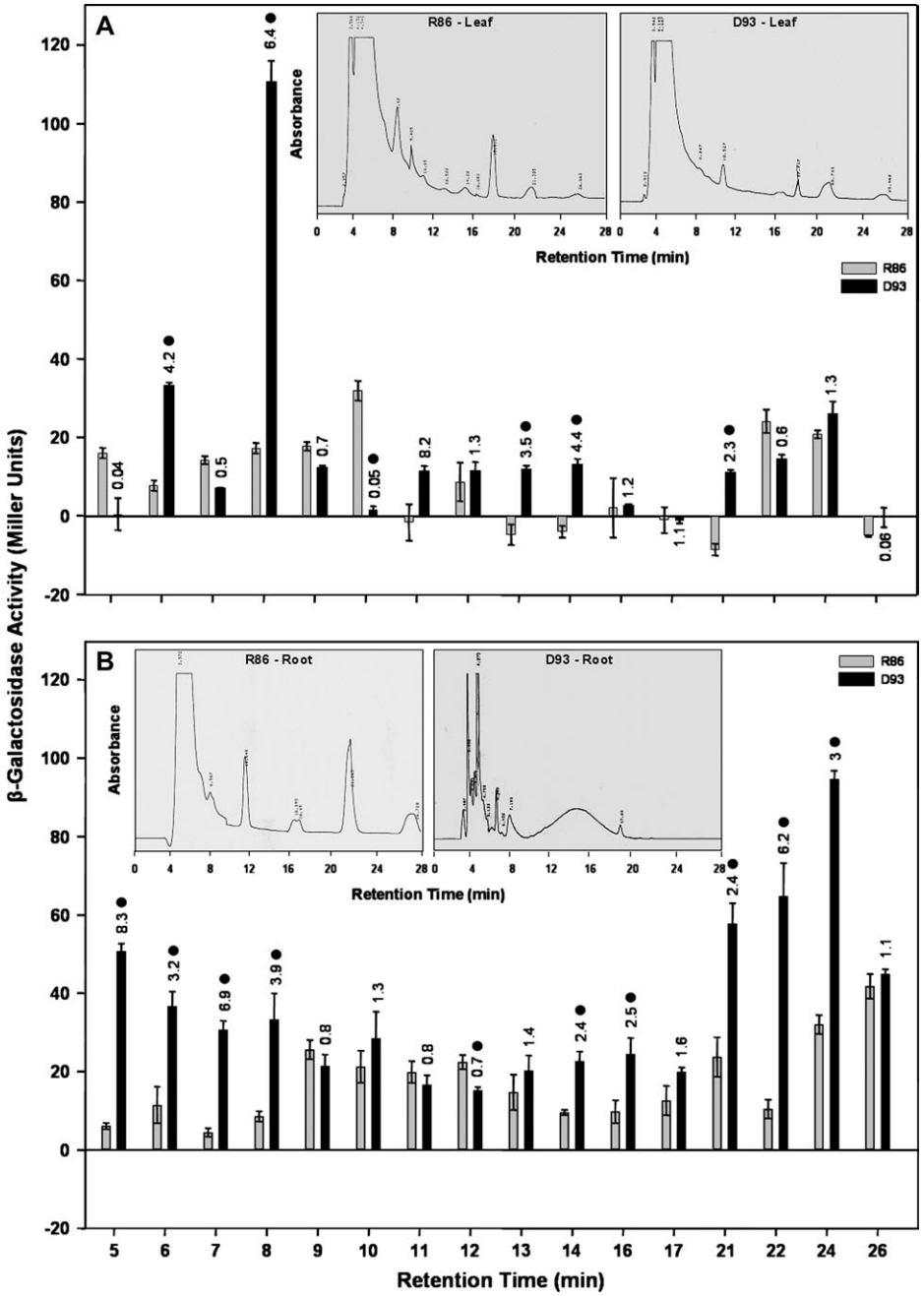


Fig. 5. β-galactosidase expressed by *nodY::lacZ* gene fusion in *B. japonicum* USDA110 in response to the HPLC fractions derived from the methanol extracts of (A) leaves and (B) roots of untransformed (R86) and *IFS*-transformed (D93) rice plants. β-galactosidase activity of *B. japonicum* was determined after 16 h exposure to HPLC fractions derived from the leaf extracts. The level of induction is expressed after subtracting the background level of β-galactosidase activity that was about 9 units in *B. japonicum* USDA110. The bars represent means of four β-galactosidase assays, and vertical lines represent standard error values. Numbers on the top of each bar represent the relative increase/decrease in β-galactosidase activity induced by the HPLC fractions of D93 leaf and root extracts compared with the fractions obtained from the leaves and roots of untransformed control plants at various retention time points. Black dots above the bars depict significant differences ($P \leq 0.05$, t test) in β-galactosidase activities induced by the fractions collected from the leaf and root extracts of D93 transgenic plant versus that from untransformed control plants. Insets in (A) and (B): HPLC profiles of methanol extracts prepared from the leaves and roots of untransformed R86 and *IFS*-transformed D93 rice plants. Each panel in the insets presents HPLC chromatograms at 260 nm.

genistein produced in *35S-IFS* plants probably converted to a glycosylated form, thus evading elution at an expected retention time (similar to the genistein standard) during HPLC fractionation. Therefore, in order to liberate genistein as an aglycone free-form from any possible glycoconjugates, methanol extracts of the roots of D93 and D121 *35S-IFS* transgenic rice plants were subjected to acid hydrolysis, and analysed for the presence of genistein by HPLC. Upon acid

hydrolysis, a peak corresponding to the R_F value of the genistein standard could be detected in the samples of the root extracts from D93 and D121 (Fig. 6B, C, arrows). The UV spectra of the eluents of these peaks (in methanol) showed a λ_{max} of 262 nm (Fig. 6B, C, boxed), which coincided with the published absorption maximum of the aglycone genistein standard (Kosslak *et al*., 1987), thus further showing that the peaks are that of genistein. In contrast to D93 and D121, samples of the acid hydrolysed root extracts from other *35S-IFS* transgenic plants failed to produce such a genistein peak. Untransformed control plants never showed any peak corresponding to the peak of the genistein standard (Fig. 6A). The finding obtained with D93 and D121 suggests that rice is able to synthesize genistein upon transformation with the *IFS* gene, and that the naringenin intermediate of the anthocyanin pathway is available for the introduced foreign enzyme for the production of genistein. Non-detection of the genistein peak in the samples from the rest of the transgenic rice plants may be due to low levels of its synthesis, or rapid conversion of the generated genistein to other forms of flavonoids, leaving no detectable levels of accumulation of this isoflavonoid in the plant tissues. Indeed drastic variations in the production of genistein were also commonly encountered among independent transgenic *Arabidopsis* lines expressing soybean IFS (Liu *et al*., 2002).

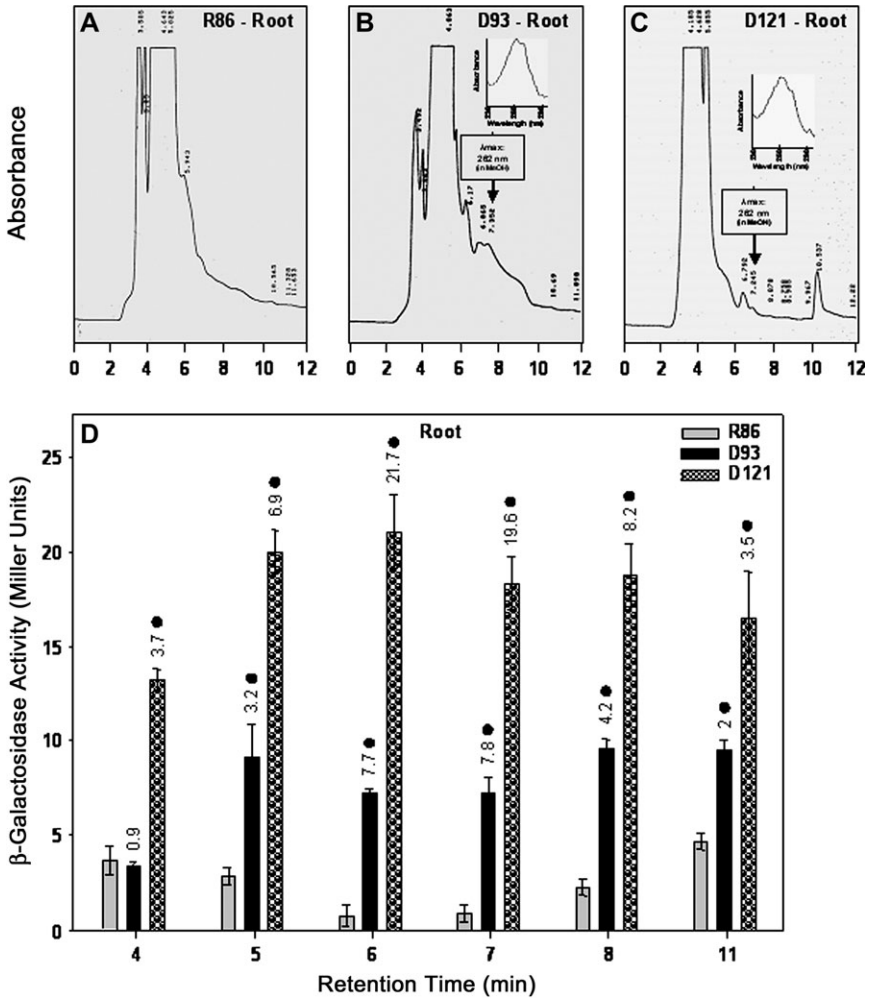


Fig. 6. (A–C) HPLC profiles of acid-hydrolysed methanol extracts of the roots of untransformed R86 (A), and *IFS*-transformed D93 (B), and D121 (C) rice plants. A new peak is depicted by an arrow, and its UV spectrum and λ_{max} are presented in boxes. Each panel presents HPLC chromatograms at 260 nm. (D) β-galactosidase expressed by *nodY::lacZ* gene fusion in *B. japonicum* USDA110 in response to the HPLC fractions derived from the acid-hydrolysed methanol extracts of roots of R86, D93, and D121 rice plants. β-galactosidase activity of *B. japonicum* was determined after 16 h exposure to HPLC fractions obtained from the acid-hydrolysed root extracts. The level of induction is expressed after subtracting the background level of β-galactosidase activity that was about 9 units in *B. japonicum* USDA110. The bars represent means of four β-galactosidase assays, and vertical lines represent standard error values. Numbers on top of each bar represent the relative increase/decrease in β-galactosidase activity induced by the HPLC fractions derived from the acid-hydrolysed root extracts of D93 and D121 plants compared with the fractions obtained from the roots of untransformed control plants at various retention time points. Black dots above the bars depict significant differences ($P \leq 0.05$, *t* test) in β-galactosidase activities induced by the fractions collected from the acid-hydrolysed root extracts of D93 or D121 transgenic plants versus that from untransformed control plants.

To determine the *nod* gene-inducing abilities of the fractions derived from the acid-hydrolysed methanol extracts, the individual HPLC eluents belonging to D93 and D121 plants were tested on USDA110 (*nodY::lacZ*). Most of the HPLC fractions obtained from the acid-hydrolysed methanol extracts of roots of D93 and D121 transgenic plants significantly stimulated *nod* gene induction in USDA110, compared with the comparable root fractions derived from control plants at similar retention times (Fig. 6D). It is noteworthy that the fractions from the D93 and D121 root extracts that eluted around the retention time of 7 min produced about an 8–20-fold increase in β-galactosidase activity in the test bacterium. Coincidentally, the retention time span covering elution of the above fractions extended over the genistein peak-retention time observed with the acid-hydrolysed D93 and D121 root extracts (Fig. 6B, C).

Isoflavone synthase activity in leaves and roots of the 35S-IFS *transgenic rice*

Results of the above study showed that the plant extracts derived from both D93 and D121 were able to elicit high *nod* gene induction in USDA110 (Fig. 4D), and the HPLC profiles of the acid-hydrolysed root extracts from these plants revealed a new peak corresponding to that of the isoflavone genistein (Fig. 6B, C), an expected product of isoflavone synthase activity in plant tissues. In order to verify the ability of *35S-IFS* plants to produce isoflavones, an *in vitro* enzyme activity assay was performed utilizing the protein fractions derived from both leaves and roots of D93 plants to evaluate the isoflavone synthase enzyme functioning in transgenic tissues. Isoflavone synthase activities of soluble and membrane protein fractions were assayed for their ability to convert either naringenin to genistein or liquiritigenin to daidzein, in the presence of the NADPH cofactor, and the methanol extracts of the reaction mixtures were analysed for any genistein or daidzein formation by HPLC. Figures 7 and 8 show the HPLC profiles of the products derived after *in vitro* isoflavone synthase assay reactions with the leaf and root protein fractions of D93 transgenic plants compared with untransformed control plants. The enzyme assays with supernatant protein fractions from both roots and leaves of D93 plants did not produce any new compounds (data not shown) indicating the absence of isoflavone synthase activity in these protein fractions. This result was anticipated as isoflavone synthase is a membrane bound protein (Yu *et al*., 2000). By contrast, the crude homogenate protein fraction (which contained both soluble and membrane components) from leaves and the partially purified microsomal protein fraction (pellet) from roots of D93 plant produced new compounds (as revealed by new peaks in the HPLC profiles) upon incubation in a reaction mix containing naringenin or liquiritigenin together with NADPH (Figs 7E, F, 8B, arrows). R_F values of the peaks of the new products corresponded to the R_F values of the genistein (7.3 min; upon incubation with the naringenin substrate, see Fig. 7E, arrow) and daidzein (6.3 min; upon incubation with the liquiritigenin substrate; see Figs 7F, 8B, arrows) standards. Untransformed control plants never showed any peaks corresponding to the peaks of the genistein and daidzein standards (Figs 7B, C, 8A). Results obtained with this study showed that D93 plants are able to produce active isoflavone synthase that is able to convert naringenin to genistein (Fig. 7E, arrow) and liquiritigenin to daidzein (Figs 7F, 8B, arrows).

Discussion

In legumes, the molecular dialogue between symbiotic partners is triggered when root-secreted plant flavonoids act as transcriptional activators of the *Rhizobium* nodulation (*nod*) genes. The *nodD* gene product of rhizobia physically binds to flavonoids, and initiates the induction of nodulation genes leading to the formation of nitrogen-fixing nodules (Broughton *et al*., 2000; Spaink, 2000). Isoflavones, a class of flavonoids, are derived from the flavonone intermediate naringenin that is universally present in plants. This reaction is carried out by the enzyme, isoflavone synthase (Kochs and Grisebach, 1986) (Fig. 1). cDNAs encoding isoflavone synthase had been cloned from soybean and other legume species (Akashi *et al*., 1999; Steele *et al*., 1999; Jung *et al*., 2000). Soybean isoflavone synthase has been expressed in non-legumes such as *Arabidopsis thaliana* and tobacco (*Nicotiana tobaccum*), and the maize BMC cell line, leading to the formation of the isoflavonoid genistein (Jung *et al*., 2000; Yu *et al*., 2000; Liu *et al*., 2002).

Cereal crops such as rice do not naturally produce isoflavones. Synthesis of isoflavones in this crop may enhance its nutritional value. In addition, isoflavone production may pave the way for rice plants to enter into a symbiotic relationship with rhizobia. Indeed, there has recently been much interest in elucidating the potential to transfer the symbiotic nitrogen-fixing ability to rice in an effort to reduce chemical nitrogen fertilizer input in rice farming (Reddy *et al*., 2002; Ladha and Reddy, 2003). Because the secretion of flavonoids is a first step in the legume–*Rhizobium* interactions, and rice lacks the pathway to form isoflavones from flavanones such as naringenin (Fig. 1), transgenic R86 rice plants that express the soybean isoflavone synthase gene under the control of the 35S promoter were generated using *Agrobacterium*-mediated transformation, and tested for their ability to produce the isoflavone genistein, and to induce *nod* gene expression in rhizobia. Isoflavone synthase activity assays and HPLC analysis of the acid-hydrolysed tissue extracts performed with D93 and D121 *35S-IFS* transgenic rice plants showed that the soybean *IFS* gene-expressed enzyme is active in R86 and enables the synthesis of the isoflavone genistein in plant tissues. These results suggested that, in rice cells, the

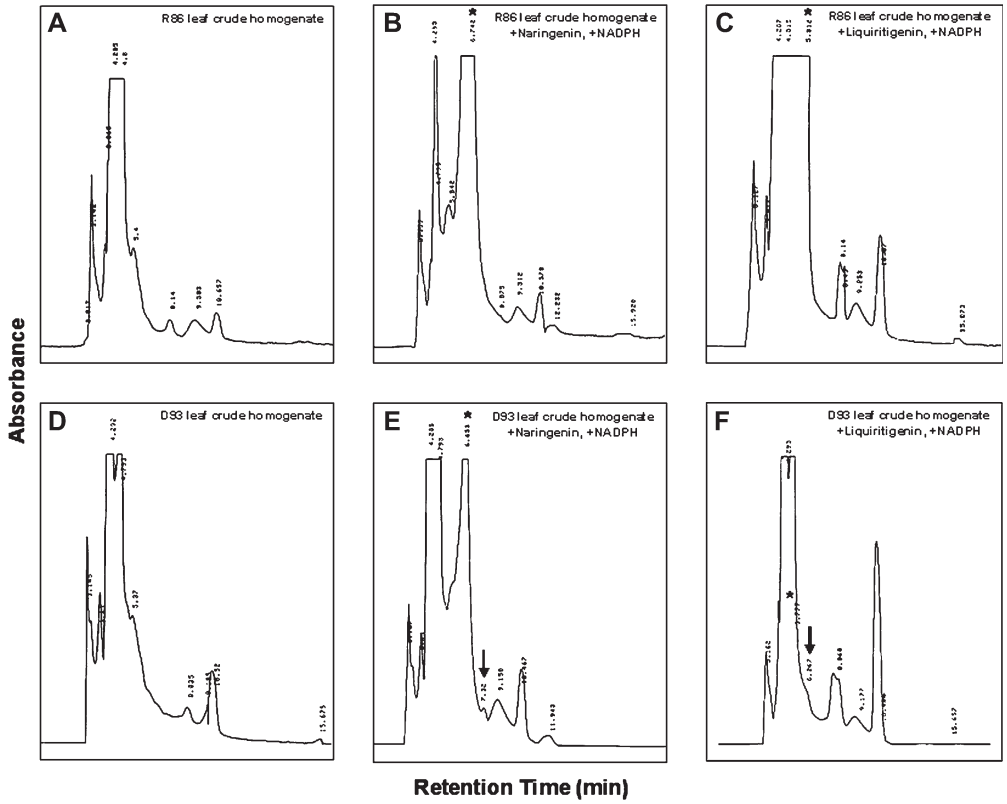


Fig. 7. Isoflavone synthase assays of crude homogenates comprising both soluble and membrane fractions of protein prepared from rice leaves. Each panel presents HPLC chromatograms at 260 nm. The assays include leaf protein from (A–C) untransformed R86 and (C–F) *IFS*-transformed D93 rice plants incubated for 12 h in the absence (A, D) or the presence of naringenin+NADPH (B, E) and liquiritigenin+NADPH (C, F). Twenty min of isocratic separation using a C18 column and 65% methanol as the mobile phase were employed. Naringenin and liquiritigenin peaks are denoted by asterisks, and new peaks are arrowed.

naringenin intermediate in anthocyanin synthesis could be captured by isoflavone synthase for genistein synthesis.

Except in D93 and D121 plants, HPLC analyses of the acid-hydrolysed extracts prepared from root tissues of other transgenic lines did not reveal any peak corresponding to the genistein standard. Failure to detect genistein with acid-hydrolysed extracts from some transgenic lines may be due to its low level of production, or due to preferential channelling of naringenin to flavonol biosynthesis, leaving no detectable levels of accumulation of this isoflavonoid in the plant tissues. In an analogous study with *IFS*-transformed tobacco, the isoflavone genistein was also not detected in acid-hydrolysed extracts, and the more sensitive GC-MS assay was necessary to detect the small amount of genistein produced (Yu *et al*., 2000). Based on these findings, Yu *et al*. (2000) speculated that the low level of genistein in tobacco tissues could be due to the low expression of the transgene, or to the unavailability of the naringenin substrate due to inactivity of the flavonoid pathway.

In soybean, isoflavones usually exist as glucosyl and malonyl-glucosyl conjugates (Graham, 1991). In the case of the *35S-IFS* transgenic rice plants, the genistein peak could be detected by HPLC analysis only when the isoflavone was liberated as a free aglycone by acid hydrolysis, indicating that genistein produced in rice tissues is converted to a glycosylated form. The presence of genistein in a conjugated form in rice tissues that do not naturally synthesize isoflavones indicates that endogenous conjugation enzymes are able to use this novel compound as a substrate. In the *IFS*-transformed non-legume dicot plants such as *Arabidopsis* and tobacco and in the monocot maize BMS cell line, genistein was always found to be present in conjugated forms (Yu *et al*., 2000; Liu *et al*., 2002). Since malonyl conjugates of anthocyanins are commonly found in many non-legume dicots and malonyltransferase activities have been studied in flowers of several plants (Yamaguchi *et al*., 1999), Yu *et al*. (2000) suggested that these types of activities may be responsible for producing the conjugates in tissues that do not naturally encounter genistein.

Analysis of the *35S-IFS* plants for their ability to induce *nod* gene expression revealed that the expression of isoflavone synthase confers rice plants with the ability to produce flavonoids that are able to modulate *nod* gene expression, albeit to varied degrees, in different rhizobia. An interesting observation of the study is that both leaf and root extracts derived from almost all *35S-IFS* transgenic lines were able to stimulate *nod* gene expression in

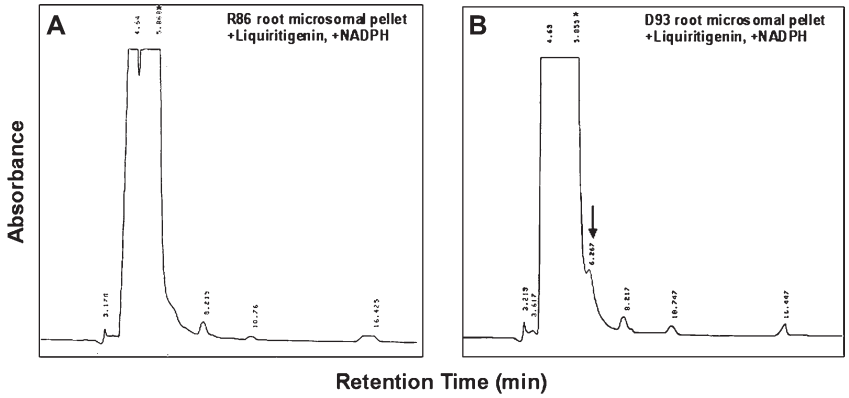


Fig. 8. Isoflavone synthase assays of crude microsomal pellet fraction from rice roots. Each panel presents HPLC chromatograms at 260 nm. The assays include root protein from (A) untransformed R86 and (B) *IFS*-transformed D93 rice plants incubated for 12 h in the presence of liquiritigenin+NADPH. Twenty minutes of isocratic separation using a C18 column and 65% methanol as the mobile phase were employed. The liquiritigenin peak is denoted by an asterisk, and a new peak is arrowed.

USDA110 (Fig. 4D). This observation is significant because *nod* gene expression in USDA110 is maximally induced by isoflavones, such as genistein (Table 3), which *IFS*-transformed rice is able to synthesize due to isoflavone synthase activity (see above; also Fig. 6B, C). These studies also showed that leaf extracts from most of the *35S-IFS* rice plants could elicit enhanced *nod* gene expression in other rhizobia such as NGR234, ORS571, and *Rm*1021. These results indicated that the expression of isoflavone synthase enables rice to synthesize new compounds that are able to promote *nod* gene induction in rhizobia. It will be interesting to ascertain the identity of the newly synthesized compounds, and to incorporate appropriate genetic amendments in rice in order to encourage the synthesis of these *nod* gene-inducing compounds in roots, a suitable site for rhizobial interaction. It is known that the expression of genes encoding certain transcription factors such as C1 myb-type transcription factor of maize and the AmMyb305 of *Antirrhinum majus* can regulate the induction of various genes that encode enzymes of the phenylpropanoid pathway (Grotewold *et al*., 1998; Sablowski *et al*., 1994). Transcription factors such as these may be expressed in host plant cells to activate the expression of genes in the phenylpropanoid pathway, thereby increasing the encoded enzyme activities and the flux of compounds in the desired plant tissues (Jung *et al*., 2003).

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