Available online at www.sciencedirect.com

SCIENCE DIRECT

Plant Science 169 (2005) 726–736

PLANT SCIENCE

www.elsevier.com/locate/plantsci

Expression of the legume symbiotic lectin genes *psl* and *gs52* promotes rhizobial colonization of roots in rice

V.S. Sreevidya [a], Rowena J. Hernandez-Oane [a], Rollando B. So [a], S.B. Sullia [b], Gary Stacey [c], Jagdish K. Ladha [a], Pallavolu M. Reddy [a,*]

[a] *International Rice Research Institute, DAPO Box 7777, Metro Manila, Philippines*
[b] *Department of Microbiology and Biotechnology, Bangalore University, Jnana Bharathi, Bangalore 560056, India*
[c] *National Center for Soybean Biotechnology, Divisions of Plant Science and Biochemistry, University of Missouri, Columbia, MO 65211, USA*

Received 26 March 2005; received in revised form 17 May 2005
Available online 14 June 2005

Abstract

Transgenic rice (*Oryza sativa* L. cv. Murasaki) carrying genes encoding pea (*Pisum sativum*) lectin (PSL) or wild-soybean (*Glycine soja*) lectin-nucleotide phosphohydrolase (GS52) were inoculated with *Rhizobium leguminosarum* bv. v*iciae* or *Bradyrhizobium japonicum* USDA110, respectively, as well as with *Rhizobium* sp. NGR234, and root colonization was assessed in comparison to comparably inoculated control plants. The data showed that expression of PSL and GS52 significantly promoted rhizobial colonization of root epidermal cells including root hairs in rice. In addition, in the case of *R. leguminosarum* bv. v*iciae* and *B. japonicum* USDA110 colonization of the *psl* and *gs52* transgenic rice plants, respectively, the bacterial cells were found to preferentially home towards and aggregate maximally at the root hair tip regions rather than on the root hair "stalks". The above data suggest that the lectins PSL and GS52, which participate in rhizobial recognition by root epidermal cells in pea and soybean, respectively, are also able to facilitate rhizobial attachment and colonization of the epidermal cells in rice roots. Moreover, aggregation of *R. leguminosarum* bv. v*iciae* and *B. japonicum* USDA110 cells preferentially at root hair tip regions suggest that similar to legumes, the PSL and GS52 lectins are targeted to the root hair tips in transgenic rice, enabling higher bacterial attachment/colonization at the tip region. Rhizobial colonization at root hair tips in the *psl* and *gs52* rice plants frequently led to the localized dissolution of the cell wall creating perforations at the tip region. It is likely that the presence of lectins, such as PSL and GS52 leads to structural modifications in cell wall organization of the root hair/epidermal cells, making them prone to localized dissolution by the hydrolytic activity of compatible rhizobia to permit invasion of the root cells.

Keywords: Lectins; *gs52* (*Glycine soja* lectin-nucleotide phosphohydrolase); *psl* (*Pisum sativum* lectin); *Oryza sativa* L.; Rhizobial association; Transformation

1. Introduction

Leguminous plants are capable of forming symbiotic associations with α-proteobacteria belonging to the genera *Rhizobium, Bradyrhizobium, Azorhizobium, Mesorhizobium*, *Sinorhizobium, Allorhizobium* and *Methylobacterium*, as well as with *Burkholderia* and *Ralstonia* that belong to β-proteobacteria group [1]. A complex interaction between the legume host and its bacterial partner leads to the formation of the specialized plant organ, the nodule, in which bacteria carry out nitrogen fixation.

Abbreviations: GUS, β-glucuronidase; EGFP, enhanced green fluorescent protein; PSL, *Pisum sativum* lectin; GS52, *Glycine soja* lectin-nucleotide phosphohydrolase; MSD, Murashige and Skoog medium with 2,4-dichlorophenoxyacetic acid; PCR, polymerase chain reaction

* Corresponding author at: Centro de Ciencias Genómicas, Universidad Naciónal Autónoma de México, Apdo. Postal 565-A, Cuernavaca, Morelos, México. Tel.: +52 777 311 5164; fax: +52 777 317 5581.
E-mail address: pmreddy@ccg.unam.mx (P.M. Reddy).

0168-9452/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2005.05.024

The symbiotic interaction between rhizobia and legume roots is characterized by a high degree of specificity at various levels. Nodulation is regulated by a two-way exchange of plant and bacterial signal molecules [2]. Signal exchange begins in the rhizosphere where rhizobia sense plant-secreted flavonoids, which act as transcriptional activators of the *Rhizobium* nodulation (*nod*) genes [3]. This step is an important determinant of host plant specificity in certain *Rhizobium*-host plant combinations. In the ensuing process, enzymes encoded by the activated *nod* genes aid the synthesis of extracellular lipochitooligosaccharide signal molecules (Nod factors), which act as host-plant specific inducers of nodule organogénesis. Nod factors are considered the main rhizobial inducers for nodulation because the purified molecules elicit many of the plant responses observed during early stages of nodule formation. These responses include changes in free calcium levels and ion balance, alterations in cytoskeletal organization and morphology of root hairs, the initiation of cortical cell divisions, and the formation of nodule primordia, as well as nodules [4]. Nod factors constitute an important determinant of host-plant specificity of nodulation [5].

Nod factors are not the only molecules that influence host determination during the symbiotic interactions. For example, legume lectins, such as PSL (*Pisum sativum* lectin [6]), Le1 (lectin from *Glycine max* [7]), DB46 and GS52 (lectin-nucleotide phosphohydrolases [LNPs] of *Dolichos biflorus* and *Glycine soja*, respectively [8,9]), have also been implicated as playing a role in host-rhizobia recognition. Several lines of evidences derived from studies using mutant rhizobia and transgenic plants, as well as investigations pertaining to anti-lectin serum-mediated inhibition of rhizobia-induced symbiotic responses in legumes have given credence to the lectin recognition hypothesis [6–16].

Legume lectins are sugar-binding proteins, usually harboring at least one noncatalytic sugar-binding site per molecule [17] that interacts with a sugar moiety on the surface of a compatible *Rhizobium*. This interaction is hypothesized to enable the two symbionts to recognize each other, promoting a close association [12,18]. Diaz et al. [6,13] and van Rhijn et al. [16] working with white clover and alfalfa, respectively, demonstrated that transformation with the pea seed lectin (*psl*) gene enabled these plants to enter into symbiotic association and produce nodules with the heterologous, pea-specific microsymbiont, *Rhizobium leguminosarum* bv *viciae*. Similarly, transformation of *Lotus corniculatus* with soybean lectin (*Le1*) gene allowed infection and development of nodule-like structures upon inoculation with the soybean-specific microsymbiont, *Bradyrhizobium japonicum*, which otherwise does not nodulate *L. corniculatus* [7]. These studies not only demonstrated that the host range in *Rhizobium*-legume symbiosis (rhizobial recognition, attachment and infection) is at least partially determined by symbiont-root lectin interactions, but also showed that host range could be extended by transferring the symbiosis-related lectin genes to heterologous legumes.

Rice is the most important staple food for more than 2.5 billion people. The yields of rice will have to increase by an additional 30% in the next 30 years, to meet the demands of the increasing human population [19]. To achieve this yield, the use of fertilizer nitrogen will have to be increased two-fold, which is not sustainable either economically or environmentally [20]. An alternative would be to provide nitrogen to rice through biological nitrogen fixation. Lately, increasing interest has been focused on determining whether legume-like symbiotic nitrogen fixation capability could be transferred to a monocot, such as rice, to render the crop independent of fertilizer nitrogen [20,21]. As part of this effort, we developed transgenic rice expressing legume lectin genes and assessed their ability to form associations with rhizobia. We report here that expression of the legume lectin genes *psl* and *gs52* promoted rhizobial colonization of roots; thus, demonstrating that lectins that participate in the rhizobial recognition in legumes can also facilitate colonization of rice.

2. Methods

2.1. Chemicals and enzymes

All chemicals including antibiotics, plant culture media salts, vitamin solutions and phytohormones used in the present study were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless specified otherwise, while enzymes and PCR reagents were from New England Biolabs (Beverly, MA, USA).

2.2. Rhizobial strains and plasmids

Rhizobial strains used in this study were *Bradyrhizobium japonicum* USDA110 [22], *Rhizobium leguminosarum* bv. *viciae* 248 [23] and *Rhizobium* NGR234 [24]. These strains henceforth will be referred to in the text as USDA110, *Rlv* 248 and NGR234, respectively.

Rlv 248 was marked with enhanced green fluorescent protein (EGFP) by triparental mating using *Escherichia coli* DH5α containing the plasmid pMP2464 [25], and a helper plasmid pRK2013 [26]. The *Rlv* 248 transconjugants were selected on minimal medium (M9 [27]) plates containing 10 mM glucose, 10 mM KNO_3 and 25 μg ml^{-1} gentamycin. The transconjugants of *Rlv* 248 expressing the EGFP was confirmed by fluorescence microscopy. *E. coli* S17.1 containing transposon based GUS marker pCAM120 (Tn5ssgusA20), which has the *gusA* gene under the control of a constitutive kanamycin resistance gene promoter [28], was used to tag NGR234. pCAM120 was transferred to NGR234 by conjugation using a filter mating technique [29]. Because the *E. coli* strain used in conjugation is an auxotroph, the transconjugants were selected on minimal medium, as

described above, supplemented with 200 μg ml^{-1} spectinomycin. The presence of *gusA* in the NGR234 transconjugants was confirmed by the appearance of blue color in the bacterial colony growing on medium with 1 mM x-gluc (5-bromo-4-chloro-3-indolyl glucuronide, sodium salt; Biosynth AG, Staad, Switzerland). Representative transconjugants of *Rlv* 248::EGFP and NGR234::GUS, showing no differences in growth as compared to their respective wild-type strains, were chosen for the inoculation experiments. The *Rhizobium* and *Bradyrhizobium* strains were routinely grown under aerobic conditions in dark at 30 °C in yeast mannitol (YM) broth or agar plates [30], supplemented with the appropriate antibiotics, if needed.

2.3. Construction of plant transformation vectors

Plasmids were constructed using standard recombinant techniques [27]. The binary transformation vector pMSH1-2809, which includes the *35S* CaMV promoter transcriptionally fused to the *psl* coding sequence and the *nos* terminator, was constructed as follows. A 0.9-kb *Hind*III fragment of the *psl* cDNA (wild-type *psl* from pMP2809) was excised from the pAGS-wt PSL vector [14,31] and ligated into the multiple cloning site of *Hind*III-digested pBluescript SK$^+$ (Stratagene, La Jolla, CA, USA), and the orientation determined by restriction analysis. The resultant recombinant plasmid pBS-*psl* was then digested with *Xba*I/*Kpn*I, and the 0.9-kb *psl* fragment was isolated and introduced downstream of the 35S CaMV promoter in *Xba*I/*Kpn*I-digested binary vector pMSH1 [32], yielding pMSH1-2809 (Fig. 1a).

The binary vector pVM220 contains the *gh3* promoter [33] transcriptionally coupled to the *gs52* coding sequence and the CaMV polyA signal sequence. The plasmid was constructed as follows: A 3-kb *Eco*RI fragment containing the *gh3-gusA* cassette was isolated from pJJ430 [34] and introduced into *Eco*RI digested-pBluescript SK$^+$, yielding pBS-*gh3-gusA*. The resultant plasmid (pBS-*gh3-gusA*) was digested with *Eco*RI/*Nco*I to release the 0.75-kb *gh3* promoter, which was subsequently ligated into *Sac*I/*Xba*I digested-pRT101 expression vector [35] in the presence of *Sac*I/*Eco*RI and *Nco*I/*Xba*I adaptors to generate pRT110. In the following step, the 1.5-kb *gs52* cDNA derived as an *Xba*I fragment from pTOPO52 (pDAY52new [9]) was introduced into pRT110 at the *Xba*I site, in the sense orientation between *gh3* promoter and CaMV polyA signal sequence, to yield pRT120S. The pRT120S was then digested with *Sph*I, and a 3-kb fragment containing the entire cassette of *gh3-gs52*-polyA signal together with flanking sequences was moved into *Sph*I digested-pUC19, yielding pUC120. The resultant pUC120 was digested with *Sac*I, and the 2.5-kb expression cassette containing only the *gh3-gs52*-polyA signal sequence was isolated and cloned into the *Sac*I site of the binary vector pVM200 to generate pVM220 (Fig. 1b). The pVM 200 is a derivative of the pMSH1 lacking the 35S CaMV promoter at the multiple cloning site.

2.4. Genetic transformation, regeneration of transgenic plants and growth conditions

Binary vectors used for rice transformation, pVM200 (control vector), pVM220 (*gh3-gs52*) or pMS1-2809 (*35S-psl*), were introduced into *Agrobacterium tumefaciens* EHA105 by electroporation [36]. *A. tumefaciens* strains harboring the transformation vectors were grown at 30 °C in Luria-Bertani (LB) broth supplemented with 50 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin to an OD_{600} of 1.0. The bacterial cells from overnight cultures were harvested by centrifugation at 5000 × *g* for 10 min, washed and resuspended in MSD medium (see below) containing 51 μM acetosyringone (Aldrich, Milwaukee, WI, USA) to a final OD_{600} of 0.04–0.06, and used for the transformation of rice calli as described below.

Mature rice seeds (*Oryza sativa* L. cv. Murasaki) were dehusked and surface sterilized with 70% (v/v) ethanol for 1 min followed by treatment with 20% sodium hypochlorite for 1 h. Seeds were then washed extensively with sterile distilled water. Callus was induced from these surface-sterilized seeds by plating them on MSD medium {composition: Murashige and Skoog [37] basal salts (MS salts), Kao and Michayluk [38] vitamins (1X), 58.4 mM sucrose, 18.1 μM 2,4-dichlorophenoxyacetic acid, 0.8% (wt/vol) agarose, pH 5.8} and grown at 28 °C under continuous light. Three weeks after callus induction, the embryogenic calli were immersed in the *A. tumefaciens* suspension (OD_{600} = 0.04–0.06) in liquid MSD medium having 51 μM acetosyringone for 15 min, blotted dry with sterilized filter paper to remove excess liquid, and cocultivated on MSD agarose medium containing 51 μM acetosyringone in the dark at 28 °C. After 3 days of cocultivation, calli were washed with sterile distilled water five-times and twice with sterile water containing 500 μg ml^{-1} carbenicillin, placed on selection medium [MSD medium containing 500 μg ml^{-1} carbenicillin and 50 μg ml^{-1} hygromycin, 0.8% (wt/vol) agarose] and incubated in continuous light for 2 weeks at 25 °C. The microcalli that proliferated after this initial selection were further subcultured on the same selection medium except that hygromycin concentration was increased to 100 μg ml^{-1}. After two rounds of selection, the actively dividing hygromycin-resistant calli were plated on plant regeneration medium containing MS basal salts, MS vitamins (1X [37]), 5.4 μM *N*-naphthalene acetic acid, 23.2 μM kinetin and 83.3 mM maltose, 0.6% (wt/vol) agarose, pH 5.8, and grown for 3–4 weeks under 10 h light/14 h dark photoperiod at 25 °C. The regenerated shoots were then transferred to the rooting medium containing 39.6 mM KNO_3, 2.6 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4{\cdot}7H_2O$, 1 mM $CaCl_2{\cdot}2H_2O$, 1.7 mM $NaH_2PO_4{\cdot}2H_2O$, 7.2 μM $MnSO_4{\cdot}4H_2O$, 7.7 μM $ZnSO_4{\cdot}7H_2O$, 0.5 μM $CuSO_4{\cdot}5H_2O$, 48.5 μM H_3BO_3, 0.5 μM $NaMoO_4{\cdot}2H_2O$, FeEDTA (20.2 μM Na_2EDTA, 19.8 μM $FeSO_4{\cdot}7H_2O$), MS vitamins, 87.6 mM sucrose, 164.7 mM sorbitol, 0.6% (wt/vol) agarose, pH 5.8 for 3–4 weeks under 12 h light/12 h dark photoperiod at 25 °C. Fully regenerated

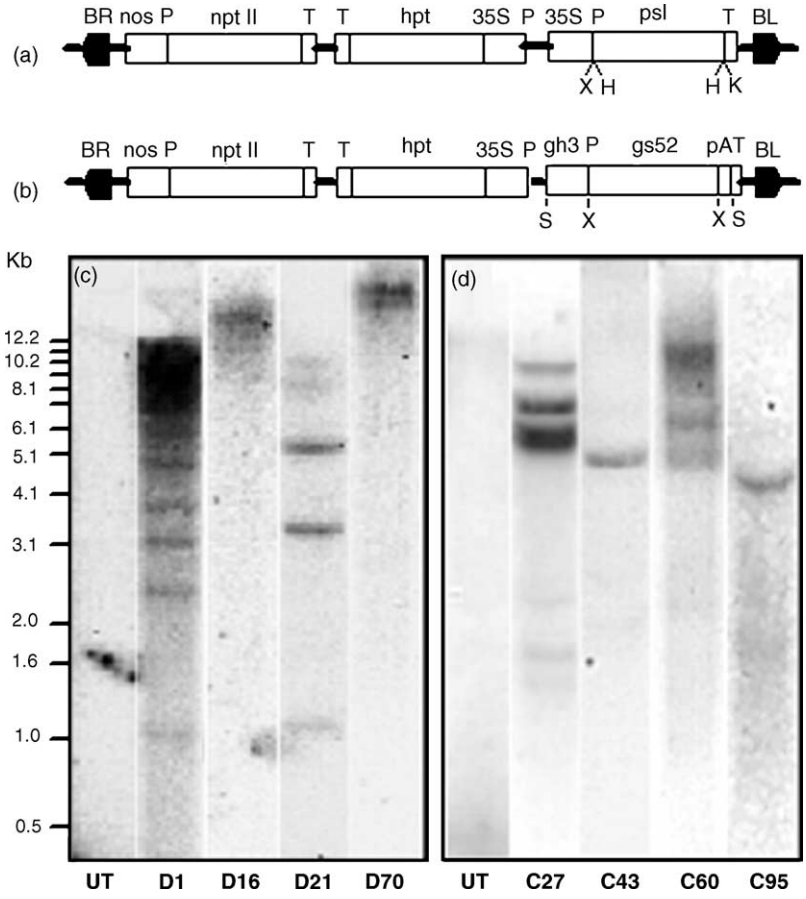

Fig. 1. Binary vectors carrying (a) *35S-psl* and (b) *gh3-gs52* chimeric genes, and (c) and (d) Southern blot analysis of the representative primary transgenic rice plants harboring *35S-psl* (c) and *gh3-gs52* (d) generated from independent transformation events. Rice genomic DNA from the *35S-psl* or *gh3-gs52* transgenic plants was digested with *Kpn*I or *Spe*I, and hybridized with the DIG-labeled *psl* or *gs52* cDNA inserts, respectively. Lanes represent Southern blot analysis of the untransformed plants (UT) and the independent transformation lines of *35S-psl* (D1, D16, D21 and D70) and *gh3-gs52* (C27, C43, C60 and C95). BR: right border; BL: left border; *nos* P: nopalene synthase promoter; *35S* P: CaMV 35S promoter; *gh3* P: soybean auxin-responsive promoter; *npt*II: kanamycin resistance gene; *hpt*: hygromycin resistance gene; *psl*: pea lectin gene; *gs52*: soybean lectin-nucleotide phosphohydrolase; T: nos terminator; pA: CaMV polyA signal sequence; H: *Hind*III; K: *Kpn*I; S: *Sac*I; X: *Xba*I.

plantlets were acclimatized hydroponically in Yoshida nutrient solution [39] for 15 days and then the putative transgenic plants (T_0 generation) were transferred to soil in pots and grown in a transgenic containment greenhouse.

2.5. Polymerase chain reaction (PCR) and Southern blot analyses

Genomic DNA was prepared from young rice leaves using the procedure developed by Dellaporta et al. [40] for Southern analysis, and by the CTAB method [41] 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 *psl* and *gs52*, 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). For *psl*, forward and reverse primers were 5′-TGC ATG CAA TTA TAA CCA AT-3′ and 5′-CTA TGC ATC TGC AGC TTG CT-3′, respectively, and the amplification was carried out with an initial cycle of 3 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 1 min at 55 °C, and 1.5 min at 72 °C, and a single final cycle of 10 min at 72 °C. For *gs52*, forward and reverse primers were 5′-ATG GTA CTT GTG CTA TGG TC-3′ and 5′-CCC AGC ATA AGC TTT AAA TA-3′, respectively, and the amplification was performed with an initial cycle at 94 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 3 min and 72 °C for 1 min, and a single final cycle at 72 °C for 10 min. 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 appropriate restriction endonucleases, size-fractionated on a 0.8% (wt/vol) agarose gel, alkali transferred onto a Hybond-N nylon membrane (Amersham Biosciences, Buckinghamshire, UK), and hybridized overnight at 42 °C to digoxigenin-11-dUTP

labeled PCR fragments of the full length coding sequences of *psl* and *gs52*. 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.

2.6. Rice culture and rhizobial inoculations

Rhizobial inocula were essentially prepared as described by Reddy et al. [42] excepting that the bacterial cells were finally washed and resuspended in N-free Yoshida rice culture solution [39].

Rice seeds were surface sterilized as described above, and germinated on LB agar plates in dark at 37 °C. After 3 days, the germinated seedlings free of any visual microbial contamination were transferred to 80 ml glass tubes containing 20 ml of N-free Yoshida rice culture solution (pH 5.0) and incubated for an additional 3 days in the plant growth room (maintained at a 14 h light/10 h dark cycle, at temperatures of 27 °C/25 °C, respectively). One millilitre of mid-log rhizobial suspension containing approximately 10^6 cells (see above) was inoculated on rice seedlings and the culture tubes were returned to the growth room for incubation. For the assessment of bacterial colonization, roots were sampled on the 4th day after inoculation.

2.7. Histochemical localization of GUS activity and microscopy methods

GUS staining of roots inoculated with *gusA* tagged NGR234 was performed at 37 °C for 12–24 h according to Jefferson et al. [43]. After staining, roots were rinsed in 100 mM potassium phosphate buffer, pH 7.0, and fixed for 4 h in a solution containing 2.5% (v/v) glutaraldehyde buffered with 200 mM sodium cacodylate, pH 7.2. Subsequently, the roots were rinsed in 100 mM potassium buffer and examined for bacterial colonization in whole specimens or in sections (30 μm thick sections cut with Microcut H1250, Energy Beam Sciences Inc., USA) with a Zeiss Axiophot II optical microscope using bright field optics (Carl Zeiss, Jena, Germany).

For the plants inoculated with USDA110, the roots were excised, fixed with glutaraldehyde solution (as above), washed, and viewed for bacterial colonization by brightfield using a Zeiss Axiophot II microscope. For seedlings inoculated with *Rlv* 248::EGFP, the excised roots were washed with sterile distilled water and examined directly using the Zeiss Axiophot II microscope under bright field optics, as well as using epifluorescence mode with appropriate filter sets for visualizing the EGFP bacteria. For laser scanning confocal microscopy (LSCM), washed root segments or sections were directly examined using Zeiss LSM510 microscope in the epifluorescence confocal mode. Image scanning was carried out with the 488-and 543-nm laser lines. GFP fluorescence was observed with a 488-nm Ar/Kr laser, and light of the desired wavelengths was collected by using a 510-to 525-nm bandpass emission filter. Images were obtained with 40× and 100× NA Plan-Neofluar lenses.

3. Results

3.1. Generation of transgenic rice carrying the legume lectin genes psl and gs52

Transgenic rice carrying *psl* and *gs52* genes fused to the cauliflower mosaic virus *35S* promoter and the soybean auxin-responsive promoter *gh3*, respectively, were generated using *A. tumefaciens*-mediated transformation. The plantlets regenerated from the hygromycin resistant transformed rice calli were analyzed for the presence of *psl* and *gs52* by PCR using gene specific primers. This analysis showed that 27% and 41% of putative transgenic plants contained the *psl* and *gs52* genes, respectively (Table 1). These plants were designated as *35S-psl* and *gh3-gs52* plants. Southern analysis of the *35S-psl* and *gh3-gs52* plants revealed that they were generated from four independent transformation events in each case, and the copy number of the transgenes ranged from 1 to 6 in *35S-psl* and 1–4 in *gh3-gs52* plants (Fig. 1c, d). The transgenic plants (T_0 generation) were transferred to soil in pots and grown in a transgenic containment greenhouse. During the generation of the primary transgenic plants, no deleterious effects on growth and development could be detected in vector control plants versus those harboring *psl* or *gs52*. On the contrary, the expression of GS52 appeared to promote plant growth (see below). Primary transformants were self-pollinated and the resulting seeds (T_1) were collected, germinated, and grown in Yoshida rice culture solution under sterile

Table 1
Characteristics of transgenic plants of rice

Vector	Putative transgenic plants regenerated	PCR-positive plants for *psl*, *gs52* or *hpt*	Independent lines based on Southerns	Copy number of the transgenes	Phenotype of the transgenic plants
pMSH1-2809 (*35S-psl*)	71	19 (27%)	4	1–6	Normal
pVM220 (*gh3-gs52*)	116	48 (41%)	4	1–4	Enhanced growth
pVM200 (control vector)	70	34 (49%)	3	1–3	Normal

Values in parentheses depict percentage of the PCR-positive transgenic plants.

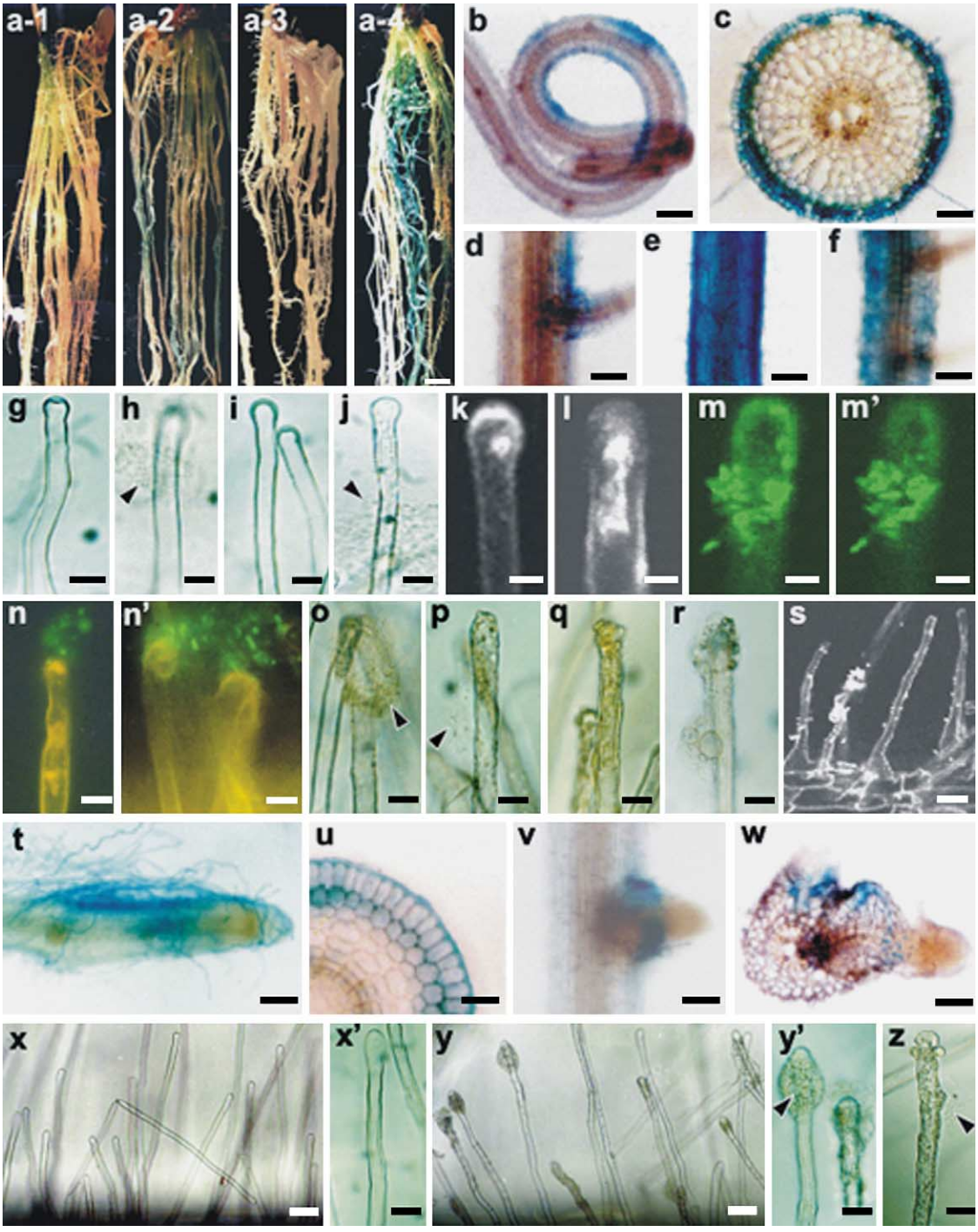

Fig. 2. Light and laser scanning confocal micrographs showing the colonization patterns and responses of transgenic rice roots expressing the legume symbiosis-related lectin genes upon inoculation with rhizobia. (a–f) Colonization of rice roots by *gusA*-tagged *Rhizobium* NGR234. (a) The roots of the untransformed, as well as the *psl*-carrying rice seedlings, showing GUS staining upon inoculation with NGR234::GUS (a-2) and (a-4). The GUS staining was much stronger in roots of transgenic seedlings containing *psl* (a-4), as compared to that in untransformed seedlings (a-2) suggesting more intense rhizobial colonization in transgenic plants. No GUS staining was observed in roots of uninoculated seedlings of both wild type (a-1) and *psl* harboring transgenic rice (a-3). (b) In young roots, GUS activity was mostly concentrated in the elongation zone in *psl* harboring transgenic plants suggesting that this is the preferred zone of initial colonization by NGR234. (c) A transverse 30-μm section from the elongation zone of a transgenic root showing intense GUS staining in the epidermal and hypodermal cell layers revealing heavy colonization by NGR234. (d) GUS activity at the sites of lateral root emergence in the root of a *psl*-plant depicting the rhizobial colonization within the fissures created by lateral root emergence. (e) GUS staining in the matured portion of a root showing the extended zone of colonization by NGR234 upon prolonged incubation with the bacteria. (f) Surface of a *psl* transgenic root showing dissolution of epidermal cells, perhaps due to cellulolytic activity of the colonizing NGR234 cells. (g–s) Characterization of the colonization patterns by *EGFP*-tagged *R. leguminosarum* bv. *viciae* 248 in untransformed, vector transformed and transgenic rice roots transformed with *psl*. (g) and (h) Photomicrographs of the root hairs of the uninoculated (g) and *Rlv* 248-inoculated (h) roots of untransformed rice. Rhizobial cells were only found as loosely associated aggregates (arrowhead in h) around root hairs upon

conditions. PCR analysis was again performed on these T_1 segregating populations of the plants using *psl* and *gs52* specific primers. Only the PCR-positive T_1 *35S-psl* and *gh3-gs52* plants were used in various inoculation experiments. Untransformed and vector transformed rice plants inoculated with various rhizobia served as controls.

3.2. Transgenic rice expressing the legume lectin genes exhibit enhanced colonization of roots by rhizobia

The broad host-range NGR234, as well as narrow host-range *Rlv* 248 and USDA110 strains, were used to study rhizobial interactions with transgenic rice harboring the legume lectin genes. The broad host-range NGR234 was specifically chosen because of its exceptional ability to recognize and nodulate a wide variety of legumes as well as the non-legume *Parasponia* [44], and presumably possesses an ability to interact with a range of legume lectins. NGR234 also served as a control rhizobial strain in the present study. On the other hand, from among the narrow-host range strains, *Rlv* 248 and USDA110 were selected to investigate rice-rhizobial interactions, respectively, in *35S-psl* and *gh3-gs52* transgenic plants since these rhizobia, in a host-specific manner, interact with and nodulate *P. sativum* and *G. soja* from which the *psl* and *gs52* genes were derived [6,9]. The *35S-psl* and *gh3-gs52* plants derived from various independent transgenic rice lines as well as control plants were inoculated with NGR234::GUS, *Rlv* 248::EGFP or USDA110 and the level of root colonization was visualized using brightfield, fluorescence and/or laser scanning confocal microscopy.

The roots of *35S-psl* plants inoculated with NGR234::GUS stained more intensely for GUS than roots of vector transformed or untransformed control plants (Fig. 2a1–4). Similar results were also obtained with the *gh3-gs52* roots, although the GUS staining was less intensive than with *35S-psl* plants (data not shown). The intense blue staining of the roots of *35S-psl* and the *gh3-gs52* plants indicated greater NGR234 attachment and colonization in the roots of *psl* or *gs52* transgenic plants as compared to vector transformed and untransformed control plants. A closer examination of the GUS stained *35S-psl* and *gh3-gs52* roots revealed that in the young roots, GUS activity was mostly concentrated in the elongation zone identifying this as the preferred zone of initial colonization (Fig. 2b, t). In the fully developed *35S-psl* roots, however, GUS staining was observed throughout the root surface indicating a gradual spread of rhizobial colonization as the root matures (Fig. 2e, f). In addition, GUS staining of the transverse sections of the *35S-psl* roots showed extensive intra- and intercellular colonization of the epidermal and hypodermal cells by NGR234 (Figs. 2c and 3e). At times the colonization of the outer cell layers in the *35S-psl* roots by NGR234 was so extensive that it led to the dissolution of epidermal cell layer perhaps due to cellulolytic activity of the bacterial cells (Fig. 2f). In contrast, the distribution of NGR234 cells on the roots of *gh3-gs52* plants was mostly confined to the elongation zone regardless of the degree of maturity of root (Fig. 2t). Moreover, in these transgenic plants, colonization was confined to the root surface (Fig. 2u), although intracellular colonization of the epidermal/hypodermal cells was occasionally observed (Fig. 3b). Nevertheless, in both types of transgenic plants, NGR234 cells were readily able to invade the root cortex through injured epidermal cells and more frequently through natural wounds caused by the emergence of young lateral roots (Fig. 2d, v, w, Fig. 3c, d, f). The bacteria colonized intracellularly (Fig. 3c), as well as intercellular spaces in the cortical region.

Similar to that observed with NGR234, the *35S-psl* plants inoculated with *Rlv* 248::EGFP also showed extensive colonization of the roots, including widespread intracellular colonization of the epidermal cells (Fig. 3h–k). In some cases, extensive colonization led to the dissolution of the epidermis (Fig. 3k), as compared to roots of vector transformed (Fig. 3g) or untransformed control plants. The USDA110 inoculated *gh3-gs52* plants also exhibited greater root colonization (but not to the extent of *35S-psl* plants) when compared to control plants (data not shown). The intensity of colonization was comparable to that seen

inoculation in untransformed rice. (i) and (j) Root hairs from the uninoculated (i) and inoculated (j) roots of transgenic rice transformed with vector only. Similar to that in untransformed rice, upon inoculation, *Rlv* 248 cells only developed into loose aggregates (arrowhead in j) around root hairs in vector-transformed rice. (k–s) Light and laser scanning confocal micrographs of the root hairs of *psl* transgenic rice uninoculated (k) or inoculated (l–s) with *Rlv* 248::EGFP. (l) A root hair from transgenic *psl*-rice plant showing the cytoplasm reorganization, upon inoculation with *Rlv* 248. (m–o) Homing of the *Rlv* 248 cells towards sub-apical (m, m′) and apical (n, n′, o-arrowhead) regions of root hairs in *psl* transformed rice plants. Green fluorescence-emitting *Rlv* 248 cells (m–n′) can be clearly seen at the homing zones in the root hairs. (p–r) Photomicrographs of the root hairs of *psl* transformed plants exhibiting cellulolytic activity upon colonization by *Rlv* 248 (arrowhead points to bacterial colonization). (s) Laser scanning confocal micrograph of root hairs of a *psl* transformed rice depicting typical polar attachment of *Rlv* 248 cells to the root hair surface, and congregation of the bacterial cells at the root hair apex. (t–w) Colonization of roots of transgenic rice carrying the soybean *gs52* gene by *gusA*-marked NGR234. (t) Photomicrograph showing the colonization by NGR234::GUS in the root elongation zone in transgenic rice transformed with *gs52*. (u) A transverse 30-μm section with GUS staining at the root surface showing that colonization of NGR234 is largely confined to root surface in transgenic rice harboring *gs52*. (v–w) Intact root (v) and a 30 μm transverse section of a root (w) showing GUS staining at the sites of lateral root emergence in roots of *gs52* transformed plants depicting the colonization by NGR234 within the fissures created by the lateral root emergence. (x–z) Characteristics of root colonization by *B. japonicum* USDA110 in transgenic rice transformed with vector only, or with *gs52*. (x) and (x′) Photomicrographs showing no noticeable colonization of root hairs in the USDA110-inoculated transgenic rice plants transformed with vector alone. (y) and (y′) Photomicrographs illustrating heavy colonization of the apical zones of root hairs by USDA110 in transgenic rice carrying *gs52*. Note the initiation of USDA110-induced lysis at the root hair tip (y′, z-arrowheads) upon colonization. Droplets of cytoplasm (arrowhead) oozing out at heavily colonized root hair tip can be clearly seen in (y′). Bar = (a) 10 mm; (b) 500 μm; (c) 100 μm; (d–f, v) 1 mm; (k–m, m′) 10 μm; (g–j, n, n′, o–r, x′, y′, z) 20 μm; (s, u) 40 μm; (t) 300 μm; (w) 80 μm; (x, y) 50 μm.

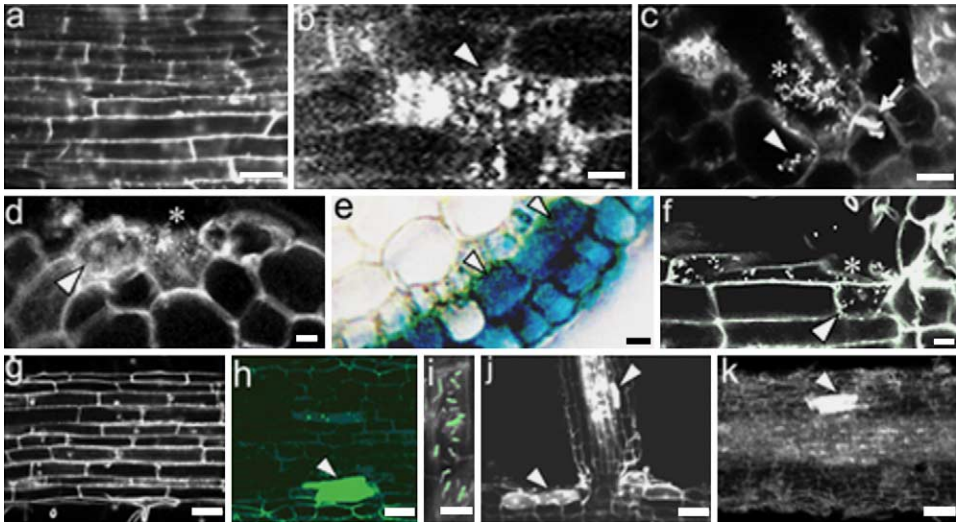

Fig. 3. Inter- and intracellular colonization by NGR234::GUS (b–f) and *Rlv* 248::EGFP (h–k) in the roots of transgenic rice expressing GS52 (b, c) and PSL (d–f, h–k) as compared to control plants (a, g). (a) A laser scanning confocal micrograph of a root of untransformed rice plant inoculated with NGR234. (b) A lightmicrograph showing intracellular colonization of an epidermal cell by NGR234 in *gs52* expressing rice root. (c) A laser scanning confocal micrograph of a 30 μm transverse section of a root showing inter- (asterisk) and intracellular (arrowhead) colonization including an invading pocket (arrow) of NGR234 cells in the area of a fissure created by lateral root emergence in transgenic rice transformed with *gs52*. (d–f) Laser scanning confocal (d and f) and light (e) micrographs of 30 μm sections of roots showing the inter- (asterisks) and intracellular (arrowheads) colonization by NGR234 in epidermal (d) and hypodermal (e) cell layers, and at the site of lateral root emergence (f) in transgenic rice expressing *psl*. The micrograph (e) was obtained from a GUS stained root section. (g) A laser scanning confocal micrograph illustrating the normal root surface in vector transformed rice plant inoculated with *Rlv* 248. (h–k) Laser scanning confocal micrographs showing intracellular colonization (arrowheads) of the epidermal cells in main (h) and lateral (j) roots in the *psl*-transformed rice, inoculated with *Rlv* 248. (i) An enlarged view of an epidermal cell with intracellular colonization by *Rlv* 248 in a main root. (k) A laser scanning confocal micrograph of the root surface of *psl* transformed plants colonized by *Rlv* 248::EGFP cells showing dissolution of epidermal cell. Note a small group of the intact root epidermal cells with the intracellular colonizing bacteria (arrowhead). Bar = (a, c, d–f, i) 10 μm; (b) 2 μm; (g, h, j, k) 40 μm.

with NGR234 with these transgenic plants. In spite of the similarities in the degrees of root colonization by NGR234 *vis-à-vis Rlv* 248 in *35S-psl* plants, or by NGR234 *vis-à-vis* USDA110 in *gh3-gs52* plants, striking differences were seen in colonization of root hairs in the root elongation zone. In the case of both the *35S-psl* and *gh3-gs52* plants, NGR234::GUS cells were found uniformly distributed throughout the surface of the root hairs (note uniform GUS staining of the root hairs in Fig. 2c, t). In contrast, colonization by *Rlv* 248 and USDA110 in *35S-psl* and *gh3-gs52* plants, respectively, was found preferentially in the root hair tip regions, relative to the root hair ''stalks'' (Fig. 2m–o, s, y, y′). In some instances, up to 95% of the root hairs present in the elongation zone of *gh3-gs52* roots were colonized at the tip regions by USDA110. Colonization by *Rlv* 248 at the root hair apical region of *35S-psl* plants appeared to induce changes in cytoplasmic streaming inside the root hairs (compare the distribution of cytoplasm in uninoculated and inoculated *psl*-transgenic root hairs in Fig. 2k and l). In both *35S-psl* and *gh3-gs52* plants, colonization by *Rlv* 248 and USDA110, respectively, frequently led to dissolution of the cell wall resulting in the formation of perforations at the root hair tips (Fig. 2p–r, y′, z), and the occasional release of cytoplasm from the perforated sites (Fig. 2r, y′, z). This may be due to localized cellulolytic activity of the bacterial cells. Dissolution of the cell wall specifically at the root hair tip was not observed in the roots colonized by NGR234. Rhizobial association with root hairs of vector and untransformed control plants was sparse and random, and no morphological changes were observed in the root hairs in contact with rhizobia (Fig. 2h, j, x, x′) and they appeared similar to uninoculated controls (Fig. 2g, i, k).

3.3. Expression of gs52 promotes plant growth, and enables enhanced lateral root proliferation upon rhizobial inoculation in rice

Transgenic rice plants carrying *gs52* showed enhanced growth under greenhouse conditions and appeared more robust when compared to untransformed and vector transformed control plants (Fig. 4a–c). In addition, the transgenic *gh3-gs52* plants inoculated with NGR234 showed enhanced proliferation of lateral roots in comparison to control plants. The number of laterals per cm root length on plants expressing GS52 increased from an average of 16 in uninoculated plants to 22 when inoculated with NGR234 (Table 2).

4. Discussion

Early during symbiotic interaction rhizobia attach themselves to the epidermal cell surface and root hair tip

Fig. 4. Growth of (a) wild-type versus transgenic rice plants transformed with (b) vector and (c) *gh3-gs52* chimeric gene.

region prior to initiating infection thread formation. Legume lectins have been implicated as mediating, at least in part, this attachment process [18,45]. Consistent with this hypothesis, expression of PSL and GS52-LNP significantly promoted the colonization of rice roots by rhizobia. These findings indicated that the legume lectins PSL [6] and GS52 [9], which participate in the rhizobial recognition in pea (*P. sativum*) and wild-soybean (*G. soja*), respectively, are also able to perform a similar function in rice. In addition, transgenic rice expressing PSL and GS52 showed preferential attachment/colonization of *Rlv* 248 and USDA110, respectively, at root hair tips. In legumes, it was suggested that targeting of pea (PSL) and soybean (Le1 and DB46-LNP) lectins to the root hair tips likely mediates bacterial aggregation and attachment at these root hair zones [7,13,46]. Our results suggest that PSL and GS52 lectins are correctly processed and targeted to the root hair tips in rice prompting higher bacterial attachment/colonization at that site. The apparent targeting of the expressed heterologous PSL and GS52 lectins (even though they were expressed with the constitutive 35S CaMV and the auxin-responsive promoters, respectively) to the root surface in rice is likely due to the activity of signal sequences present in these proteins [9,31]. Indeed, targeting of Le1 lectin to root hair tips of *L. corniculatus* was shown to occur even when expressed with 35S promoter [7]. In the *35S-psl* and *gh3-gs52* rice plants, the dissolution of the cell wall leading to perforations at the root hair tip seen upon rhizobial attachment suggest that expression of PSL and GS52 lectins may make the cell wall more susceptible to invasion by rhizobia. It is likely that PSL and GS52 modify cell wall architecture making root hair/epidermal cells more susceptible to the hydrolytic activity of compatible rhizobia to permit bacterial invasion. Resolution of lectin-mediated structural modifications that facilitate rhizobial interaction with cell wall is one of the challenges for the future.

Expression of GS52 in rice, in addition to enhancing rhizobial colonization, also promoted plant growth and lateral root proliferation (about 55% higher than in control plants) upon rhizobial inoculation (Table 2). Earlier studies showed that inoculation of rice with aquatic rhizobia promoted differentiation of thick short lateral roots, probably due to the activity of auxins produced by the root colonizing bacteria [42]. In the present study, however, inoculation only promoted higher lateral root differentiation in the *gh3-gs52* plants and not in the control plants suggesting a specific role for GS52 in this process. Our data do not provide a mechanism for the enhanced growth and lateral root formation in GS52 expressing plants. However, Thomas et al. [47] reported that transgenic expression of the pea apyrase in *Arabidopsis thaliana* produced plants with increased growth rates and phosphate transport when supplied with either inorganic phosphate or ATP. Therefore, the apyrase activity of GS52 (which is a lectin-nucleotide phosphohydrolase) could be enhancing rice growth through this effect on phosphate metabolism.

In conclusion, this study showed that expression of PSL and GS52 promoted rhizobial colonization in rice. These data are consistent with the notion that lectins are important for rhizobial recognition/attachment in legumes. Legume lectins were also implicated in facilitating infection thread formation by rhizobia [7,45]. Perhaps consistent with this suggestion, expression of GS52 or PSL in rice appeared to enable localized dissolution of the cell wall at the root hair tip in response to rhizobial inoculation. However, there was no evidence of infection thread formation in the rice root hairs. It is likely that the genetic mechanisms enabling rhizobia-triggered infection thread formation are lacking in rice.

Table 2
Effect of *Rhizobium* NGR234 inoculation on lateral root differentiation in wild-type and *gh3-gs52* transgenic rice plants

Rice	Uninoculated		Inoculated	
	LR (cm^{-1})	TSLR (cm^{-1})	LR (cm^{-1})	TSLR (cm^{-1})
Wild-type	15.9 ± 1.7	0.06 ± 0.09	14.09 ± 1.67	0.39 ± 0.31
gh3-gs52 transgenic	15.96 ± 1.91	0.03 ± 0.07	21.9 ± 1.85	0.49 ± 0.35

Data represent the mean values (± standard deviation) of the number of lateral roots (LR) and thick short lateral roots (TSLR)/cm root length.

Acknowledgements

We gratefully acknowledge the following scientists for kindly providing us the seeds, cDNAs, plasmids, and bacterial strains: K. Shimamoto for Murasaki rice seeds, C.L. Diaz and J.W. Kijne for pAGS-wt PSL, B.G. Rolfe for pJJ430, H.P. Spaink for pMP2464, Kate Wilson for *E. coli* S17.1 harboring pCAM121, H. Kouchi for pMSH1 and *A. tumefaciens* EHA105, and W.J. Broughton for *Rhizobium* NGR234. Our thanks are also extended to John Bennett for permitting us to use transgenic containment greenhouse. This research was partially supported by BMZ/GTZ, Germany. Research on the GS52 apyrase in the laboratory of GS was supported by a Grant (DE FG02-02ER15309) from the US Department of Energy, Basic Energy Bioscience Program.

References

[1] W.-M. Chen, L. Moulin, C. Bontemps, P. Vandamme, G. Béna, C. Boivin-Masson, Legume symbiotic nitrogen fixation by β-proteobacteria is widespread in nature, J. Bacteriol. 185 (2003) 7266–7272.
[2] R. Geurts, T. Bisseling, *Rhizobium* Nod factor perception and signalling, Plant Cell 14 (2002) S239–S249.
[3] W.J. Broughton, S. Jabbouri, X. Perret, Keys to symbiotic harmony, J. Bacteriol. 182 (2000) 5641–5652.
[4] W. D'Haeze, M. Holsters, Nod factor structures, responses, and perception during initiation of nodule development, Glycobiol. 12 (2002) 79R–105R.
[5] H.P. Spaink, Root nodulation and infection factors produced by rhizobial bacteria, Annu. Rev. Microbiol. 54 (2000) 257–288.
[6] C.L. Diaz, L.S. Melchers, P.J.J. Hooykaas, B.J.J. Lugtenberg, J.W. Kijne, Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis, Nature 338 (1989) 579–581.
[7] P. van Rhijn, R.B. Goldberg, A.M. Hirsch, *Lotus corniculatus* nodulation specificity is changed by the presence of soybean lectin gene, Plant Cell 10 (1998) 1233–1249.
[8] M.E. Etzler, G. Kalsi, N.N. Ewing, N.J. Roberts, R.B. Day, J.B. Murphy, A nod factor binding lectin with apyrase activity from legume roots, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 5856–5861.
[9] B.R. Day, C.B. McAlvin, J.T. Loh, R.L. Denny, T.C. Wood, N.D. Young, G. Stacey, Differential expression of two soybean apyrases, one of which is an early nodulin, Mol. Plant-Microbe Interact. 13 (2000) 1053–1070.
[10] L.J. Halverson, G. Stacey, Host recognition in the *Rhizobium*–soybean symbiosis: evidence for the involvement of lectin in nodulation, Plant Physiol. 77 (1985) 621–625.
[11] L.J. Halverson, G. Stacey, Effect of lectin on nodulation of wild type *Bradyrhizobium japonicum* and a nodulation defective mutant, Appl. Environ. Microbiol. 51 (1986) 753–760.
[12] J.W. Kijne, The Rhizobium infection process, in: G. Stacey, R. Burris, H. Evans (Eds.), Biological Nitrogen Fixation, Chapman & Hall, New York, 1992, pp. 349–398.
[13] C.L. Díaz, T.J.J. Logman, H.C. Stam, J.W. Kijne, Sugar-binding activity of pea lectin expressed in white clover hairy roots, Plant Physiol. 109 (1995) 1177–1197.
[14] R.R. van Eijsden, C.L. Díaz, B.S. de Pater, J.W. Kijne, Sugar-binding activity of pea (*Pisum sativum*) lectin is essential for heterologous infection of transgenic white clover hairy roots by *Rhizobium leguminosarum* biovar *viciae*, Plant Mol. Biol. 29 (1995) 431–439.
[15] C.L. Díaz, H.P. Spaink, J.W. Kijne, Heterologous rhizobial lipochitin oligosaccharides and chitin oligomers induce cortical cell divisions in red clover roots, transformed with the pea lectin gene, Mol. Plant-Microbe Interact. 13 (2000) 268–276.
[16] P. van Rhijn, N.A. Fujishige, P.O. Lim, A.M. Hirsch, Sugar-binding activity of pea lectin enhances heterologous infection of transgenic alfalfa plants by *Rhizobium leguminosarum* biovar *viciae*, Plant Physiol. 126 (2001) 133–144.
[17] N. Sharon, H. Lis, Lectins, Springer-Verlag, 2004, pp. 1–470.
[18] A.M. Hirsch, Role of lectins (and rhizobial exopolysaccharides) in legume nodulation, Curr. Opin. Plant Biol. 2 (1999) 320–326.
[19] M.V. Rosegrant, M.A. Sombilla, N. Perez, Global food projections to 2020: implications for investment, food, agriculture and the environment, discussion paper no.5. IFPRI, Washingon, DC, USA, 1995, pp. 1–54.
[20] J.K. Ladha, P.M. Reddy, Nitrogen fixation in rice systems: state of knowledge and future prospects, Plant Soil 252 (2003) 151–167.
[21] J.K. Ladha, P.M. Reddy (Eds.), The quest for nitrogen fixation in rice, International Rice Research Institute, Makati City, Philippines, 2000, pp. 1–354.
[22] A.J. Nieuwkoop, Z. Banfalvi, N. Deshmane, D. Gerhold, M.G. Schell, K.M. Sirotkin, G. Stacey, A locus encoding host range is linked to the common nodulation genes of *Bradyrhizobium japonicum*, J. Bacteriol. 169 (1987) 2631–2638.
[23] D.P. Josey, J.L. Beynon, A.W.B. Johnston, J.E. Beringer, Strain identification in *Rhizobium* using intrinsic antibiotic resistance, J. Appl. Microbiol. 46 (1979) 343–350.
[24] A. Lewin, E. Cervants, W. Chee-Hoong, W.J. Broughton, *nodSU*, two new nod genes of the broad host range *Rhizobium* strain NGR234 encodes host-specific nodulation of the tropical tree *Leucaena leucocephla*, Mol. Plant-Microbe Interact. 3 (1990) 317–326.
[25] N. Stuurman, C.P. Bras, H.R.M. Schlaman, A.H.M. Wijfjes, G. Bloemberg, H.P. Spaink, Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants, Mol. Plant-Microbe Interact. 13 (2000) 1163–1169.
[26] D.H. Figurski, D.R. Helinski, Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans, Proc. Natl. Acad. Sci. U.S.A. 76 (1979) 1648–1652.
[27] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Second ed., Cold Spring Harbor Laboratory Press, New York, 1989.
[28] K.J. Wilson, A. Sessitch, J.C. Carbo, K.E. Giller, A.D.L. Akkermans, R.A. Jefferson, ß-Glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram negative bacteria, Microbiology 141 (1995) 1691–1705.
[29] R. Simon, U. Priefer, A. Puhler, A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis of Gram negative bacteria, Bio/Technology 1 (1983) 784–791.
[30] J.M. Vincent, IBP Handbook 15, in: A Manual for the Practical Study of Root Nodule Bacteria, Blackwell Scientific Publications, London, 1970, , pp. 1–164.
[31] R.R. van Eijsden, P.J. Hoedemaeker, C.L. Díaz, B.J.J. Lugtenberg, B.S. de Pater, J.W. Kijne, Mutational analysis of pea lectin. Replacement of the Asn125 by Asp in the monosaccharide-binding site eliminates mannose/glucose-binding activity, Plant Mol. Biol. 20 (1992) 1049–1058.
[32] T. Kawasaki, K. Henmi, E. Ono, S. Hatakeyama, M. Iwano, H. Satoh, K. Shimamoto, The small GTP-binding protein Rac is a regulator of cell death in plants, Proc. Natl. Acad. Sci. U.S.A. 96 (1999) 10922–10926.
[33] G. Hagen, G. Martin, Y. Li, T. Guilfoyle T, Auxin-induced expression on the soybean GH3 promoter in transgenic tobacco plants, Plant Mol. Biol. 17 (1991) 567–579.
[34] P.J. Larkin, J.M. Gibson, U. Mathesius, J. Weinman, E. Gartner, E. Hall, G.J. Tanner, B.G. Rolfe, M.A. Djordjevic, Transgenic white clover. Studies with the auxin-responsive promoter, GH3, in root gravitropism and lateral root development, Transgenic Res. 5 (1996) 325–335.

[35] R. Töpfer, V. Matzeit, B. Gronenborn, J. Schell, H.H. Steinbiss, A set of plant expression vectors for transcriptional and translational fusions, Nucleic Acids Res. 15 (1987) 5890–5896.
[36] R. Nagel, A. Elliot, A. Masel, R.G. Birch, J.M. Manners, Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, FEMS Microbiol. Lett. 67 (1990) 325–328.
[37] T. Murashige, F. Skoog, A revised medium for rapid growth and bio-essays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.
[38] K.N. Kao, M.R. Michayluk, Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media, Planta 126 (1975) 105–110.
[39] S. Yoshida, D.A. Forno, J.H. Cock, K.A. Gomez, Laboratory manual for physiological studies of rice, Third edition, International Rice Research Institute, Los Banos, Philippines, 1976, , pp. 61–64.
[40] S.L. Dellaporta, J. Wood, J.B. Hick, A plant DNA minipreparation: version II, Plant Mol. Biol. Rep. 1 (1983) 19–21.
[41] B.H. Taylor, J.R. Manhart, R.M. Amasino, Isolation and characterization of plant DNAs, in: B.R. Glick, J.E. Thompson (Eds.), Methods in Plant Molecular Biology and Biotechnology, CRC Press Inc., Boca Raton, Florida, USA, 1993, pp. 37–47.
[42] P.M. Reddy, J.K. Ladha, R.B. So, R.J. Hernandez, M.C. Ramos, O.R. Angeles, F.B. Dazzo, F.J. de Bruijn, Rhizobial communication with rice roots: induction of phenotypic changes, mode of invasion and extent of colonization in roots, Plant Soil 194 (1997) 81–98.
[43] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J. 6 (1987) 3901–3907.
[44] S.G. Pueppke, W.J. Broughton, *Rhizobium* sp. strain NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host ranges, Mol. Plant-Microbe Interact. 12 (1999) 293–318.
[45] J.W. Kijne, M.A. Bauchrowitz, C.L. Diaz, Root lectins and rhizobia, Plant Physiol. 115 (1997) 869–873.
[46] G. Kalsi, M.E. Etzler, Localization of a Nod factor-binding protein in legume roots and factors influencing its distribution and expression, Plant Physiol. 124 (2000) 1039–1048.
[47] C. Thomas, Y. Sun, K. Naus, A. Lloyd, S. Roux, Apyrase functions in plant phosphate nutrition and mobilizes phosphate from extracellular ATP, Plant Physiol. 119 (1999) 543–551.