Gene Silencing by Expression of Hairpin RNA in *Lotus japonicus* Roots and Root Nodules

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We investigated the efficacy of self-complementary hairpin RNA (hpRNA) expression to induce RNA silencing in the roots and nodules of model legume *Lotus japonicus*, using hairy root transformation mediated by *Agrobacterium rhizogenes*. Transgenic lines that express β -glucuronidase (GUS) by constitutive or nodule-specific promoters were supertransformed by infection of *A. rhizogenes* harboring constructs for the expression of hpRNAs with sequences complementary to the GUS coding region. GUS activity in more than 60% of the hairy roots was decreased or silenced almost completely. Silencing of the GUS gene was also observed in symbiotic nodules formed on hairy roots in both early and late stages of nodule organogenesis. These results indicate that transient RNA silencing by hairy root transformation provides a powerful tool for loss-of-function analyses of genes that function in roots and root nodules.

Legume plants are able to form symbiotic root nodules by interaction with rhizobia, which provide nitrogen compounds to the host plant by atmospheric nitrogen fixation. *Rhizobium* infection and the subsequent organogenesis of nitrogen-fixing root nodules is accomplished through highly regulated expression of many plant and bacterial genes that are induced by a perpetual interaction between the host plant and the microsymbiont (Spaink 2000; Stougaard 2001). In the past few years, a survey of the molecular mechanisms of legume-*Rhizobium* symbiosis and root nodule development has been made possible by large-scale accumulation of expressed sequence tags (ESTs) for a number of legume species. Expression of a large number of plant genes in nodules has been profiled by in silico analysis of an EST database of *Medicago truncatula* (Fedorova et al. 2002; Gyorgyey et al. 2000) and by a cDNA array of *Lotus japonicus* (Colebatch et al. 2002), allowing us to identify many novel nodule-specific or nodule-enhanced genes. However, the biological functions of most of these genes still remain to be elucidated. In order to discover more about the molecular mechanisms underlying the nodulation process, it is essential to establish efficient systems for the analyses of the functions of these genes.

RNA silencing (RNA interference [RNAi]) is a posttranscriptional gene silencing in which double-stranded RNAs (dsRNAs) in cells cause degradation of RNAs that share the same sequence with the dsRNAs. This phenomenon is proposed to have been developed during evolution as a defense mechanism against foreign genes, including viruses (Voinnet

2001) and transposable elements (Hamilton et al. 2002). RNA silencing provides an efficient technique for gene silencing in living cells by introducing dsRNAs that induce sequence-specific degradation of corresponding mRNAs (Wang and Waterhouse 2002). The first report of gene silencing by such a dsRNA strategy in higher plants was described by Waterhouse and co-workers, who discovered that the constitutive expression of a β -glucuronidase gene (*gusA*) in transgenic calli of *Oryza sativa* was efficiently silenced by additional transformation with a gene that was designed to express the self-complementary 'hairpin' RNA (hpRNA) for *gusA* rather than those with only sense or antisense RNA expression (Waterhouse et al. 1998). This was followed by an improvement of efficiency using an intron sequence as a spacer between target mRNA sequences of inverted directions (Smith et al. 2000). RNA silencing based on artificial hpRNA expression has been also successfully applied to endogenes in *Arabidopsis thaliana*, e.g., *AG*, *CLV3*, *API*, *PAN* (Chuang and Meyerowitz 2000), *CHS*, *FLC1*, *EIN2* (Wesley et al. 2001), and the cystathionine β -lyase gene (Levin et al. 2000). In most of these cases, RNA silencing was shown to cause phenotypes that were more or less the same as those of genomic mutational lines. However, there has been no report on RNA silencing in the roots of plants.

Our aim is to establish an efficient system for loss-of-function analyses of genes that function in roots and root nodules of legume plants. Here, we describe RNA silencing in roots and root nodules of a model legume *Lotus japonicus* by means of transient expression of hpRNAs with hairy root transformation mediated by *Agrobacterium rhizogenes*.

RESULTS

GUS expressing lines of *L. japonicus* and constructions of vectors for hairy root transformation.

Transgenic *L. japonicus* (B129 'Gifu' line) plants harboring a chimeric *gusA* gene were used as model plants for RNA silencing. Two different transgenic lines with *gusA* were generated: line 16E contains *gusA* driven by the cauliflower mosaic virus (CaMV) 35S promoter and line 9Y contains *gusA* driven by the *lj27* promoter that is specific for nodule infected cells and is the *L. japonicus* ortholog of soybean *GmN479* (Kouchi and Hata 1993). Southern blot analysis of genomic DNA from T₂ plants of these lines indicated that they both had a single copy of T-DNA insert in their genome (data not shown). They were both homozygous for the transgenes. Histochemical β -glucuronidase (GUS) staining demonstrated that line 16E exhibits strong GUS expression in all parts of the plant whereas, in the line 9Y, GUS activity is specific to nodule infected cells (Fig. 1).

A binary vector, pHKN29, for hairy root transformation was constructed from pCambia1300 by replacing hygromycin

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phosphotransferase (HPT) with the green fluorescent protein (GFP) (Niwa et al. 1999), thus enabling GFP fluorescence under UV illumination as a marker of transgenic hairy roots. Two constructs for RNA silencing were made in pKANNIBAL (Wesley et al. 2001) using a sequence of 582 nt at the 3' end and a 325-nt sequence at the middle part of the GUS coding region, followed by ligation into the cloning sites of pHKN29 to generate plasmids pHKN30 and pHKN31, respectively (Fig. 2).

RNA silencing in hairy roots.

Transgenic *L. japonicus* plants, line 16E, were transformed with hpRNA constructs pHKN30 and pHKN31, with pHKN29 as a control, by hairy root transformation with *A. rhizogenes* LBA1334. All the hairy roots formed on the edge of sectioned hypocotyls were tested for GFP fluorescence and then stained with 5-bromo-4-chloro-3-indoryl- β -D-glucuronide (X-glucuronide). The results are shown in Figure 3. All the transgenic roots transformed with pHKN29 were stained blue by X-glucuronide, indicating that expression of *gusA* was not influenced by additional insertion of GFP in the plant genome (Fig. 3A and D). In contrast, transgenic hairy roots transformed with pHKN30 (Fig. 3B and E) and pHKN31 (Fig. 3C and F) had no apparent GUS activity, and detailed observations demonstrated that GUS activity was not detected in any parts of the roots, including root hairs and the root apical meristem (data not shown).

Fluorometric assays of GUS enzyme activity of the extracts from transgenic hairy roots are shown in Figure 4. Extracts from eight of ten independent hairy roots transformed with pHKN30 and from eight of nine hairy roots transformed with pHKN31 showed very low or nondetectable GUS activity, whereas extracts from the roots transformed with pHKN29

showed perceptible GUS activities of between 0.1 and 0.225 pmol 4-methyl umbelliferone/h per μ g of protein.

These results show that RNA silencing of preintroduced *gusA* was induced uniformly in hairy roots transformed with constructs for expression of hpRNA.

Spread of silencing from roots to upper parts of plants.

In higher plants, it is known that RNA silencing triggered in one part of the plant can spread systemically. Previous reports indicate that a nonsilenced scion grafted onto a silenced stock becomes silent, but the reverse combination of grafting did not lead to transmission of silencing (Palauqui et al. 1997). These observations suggest strongly that the spread of silencing is unidirectional from the lower part of the plant stem to the upper. We tested the transmission of RNA silencing from silence-induced transgenic roots to the untransformed upper part of the plants. Extracts of leaves from plants with hairy roots that were transformed with either pHKN30, pHKN31, or pHKN29 were quantitatively assayed for GUS activity (Fig. 5). Although the GUS activity in the leaves varied considerably between individual plants, the averages were 25 and 33% for the plants with pHKN30- and pHKN31-transformed roots, respectively, of that for the leaves from the control plants with pHKN29-transformed roots. These results suggest that the silencing of the GUS reporter gene in transgenic hairy roots is transmitted to nontransformed shoots, even though the extent of the silencing in the shoots is more limited and more varied than that in the transgenic hairy roots.

RNA silencing in nodules on transgenic hairy roots.

To examine the efficacy of RNA silencing in root nodules, transgenic hairy roots formed on the line 16E by *A. rhizogenes* harboring pHKN29, pHKN30, and pHKN31 were inoculated with *Mesorhizobium loti*. In our growth conditions, nodulation on the transgenic hairy roots appeared to proceed at a rate similar to that on the nontransformed roots. Figure 6 shows nodule primordia just emerging on the transgenic roots after GUS staining. There was no staining of nodule primordia on the hairy roots transformed with pHKN30 (Fig. 6B and E) or pHKN31 (Fig. 6C and F), whereas nodule primordia on the roots transformed with pHKN29 showed strong GUS activity (Fig. 6A and D).

We further examined RNA silencing at the inner symbiotic zone of nodules using another transgenic *L. japonicus* line 9Y, in which *gusA* was driven by the *lj27* promoter that is specific to nodule infected cells (Fig. 1C) at the late stage of nodule formation. Transgenic hairy roots formed on the plants of line 9Y with either pHKN29, pHKN30, or pHKN31 were inoculated with *M. loti*, and the nodules were harvested 14 days after inoculation, followed by fluorometric assay of GUS activities. The results are shown in Figure 7. Nodules from seven of ten

Fig. 1. β -glucuronidase (GUS) staining of transgenic *Lotus japonicus*. Seedlings of **A**, the transgenic line 16E that has a *gusA* gene driven by the CaMV35S promoter, and **B**, wild-type Gifu. The seedlings were stained by X-glucuronide and photographed after bleaching in 100% ethanol. Micrographs of root nodule sections (110 μ m thick) of **C**, transgenic line 9Y that has a *gusA* gene driven by *lj27* promoter and **D**, of Gifu after GUS staining.

Fig. 2. Schematic diagrams of T-DNA insertions of binary vector for hairy root transformation.

independent hairy roots transformed with pHKN30 or pHKN31 showed very low or nondetectable GUS activity, whereas nodules from all the hairy roots with pHKN29 showed strong GUS activity.

To verify silencing of the *gusA* gene in this system, we examined accumulation of *gusA* mRNA in nodules of 9Y plants (Fig. 8). The results of the reverse transcription-polymerase chain reaction (RT-PCR) assay indicated that the levels of *gusA* mRNA in the nodules formed on pHKN30- and pHKN31-transformed roots were substantially reduced compared with that in the nodules on control (pHKN29-transformed) roots.

DISCUSSION

The introduction of a gene expressing hpRNAs into roots by *A. rhizogenes*-mediated hairy root transformation induces efficient silencing of the target gene in the roots and nodules of *L. japonicus*. To our knowledge this is the first report of RNA silencing by hpRNAs in a root system of higher plants. The expression of *gusA* that was previously introduced into the plant genome was reduced or almost completely silenced in 60

to 70% of the roots supertransformed by the hpRNAs (Figs. 4 and 7).

Hairy root transformation is a “partial” transformation, because the transgene is not present in all plant cells, but only in the hairy roots induced by infection of *A. rhizogenes*. Our data indicate that the silencing occurs uniformly in the transgenic hairy root tissues, including nodules (Figs. 3, 6, and 8). However, spreading of the silencing state to nontransformed roots did not occur (Fig. 3), even though systemic spread of the silencing state is one of the intriguing features of RNA silencing (Hamilton et al. 2002). This may be explained by the fact that gene silencing by hpRNA expression transmits unidirectionally only from the lower to the upper parts of the stems (Palauqui et al. 1997). Indeed, our results strongly suggest that the silencing state in hairy roots is transmitted to untransformed leaves, though the silencing in the leaves was not complete (Fig. 5). Incomplete or partial silencing of the target gene in the untransformed shoots might be due to a nonuniform distribution of the silencing state in these tissues (Voinnet and Baulcombe 1997). In our experiments, GUS activities in the shoots of line 16E plants varied considerably between individ-

Fig. 3. Silencing of *gusA* gene in transgenic hairy roots. Hairy roots from line 16E induced by inoculation of *Agrobacterium rhizogenes* harboring **A** and **D**, pHKN29, **B** and **E**, pHKN30, and **C** and **F**, pHKN31 are shown. Upper panels (**A**, **B**, and **C**) show GUS staining. Lower panels (**D**, **E**, and **F**) show GFP fluorescence.

ual plants. Thus, further examinations including more lines are required to obtain conclusive data on systemic spread of gene silencing to the shoots from transgenic roots.

Hairy root transformation is a short-cut strategy to test the function or regulation, or both, of genes in roots and nodules (Hansen et al. 1989). It should be noted, however, that *Agrobacterium rol* genes cotransformed with the transgene of interest may disturb the hormonal balance of the plants (Schmulling et al. 1988). Nevertheless, hairy root transformation with hpRNA is a powerful approach for investigating functions of nodule-specific genes. Only six weeks are needed to test the nodulation phenotypes, compared with conventional transformation by *A. tumefaciens* that needs at least eight months to obtain seeds of the transformants. Furthermore, regeneration of the shoots from hairy roots to obtain transgenic seed is very easy in *L. japonicus* (Stiller et al. 1997). When transformed hairy roots show unusual phenotypes, the plant clones can be regenerated from exactly the same roots, allowing analysis of the phenotypes in greater detail, as well as eliminating *rol* genes through segregation in progenies of the regenerated plants.

Taken together, our results indicate that transient RNA silencing through a hairy root transformation procedure provides a powerful tool for loss-of-function analysis of genes that function in roots and root nodules. Primary analyses of functions of a number of nodule-specific or nodule-enhanced genes identified by a cDNA array analysis (H. Kouchi, unpublished data) are currently underway.

MATERIALS AND METHODS

Construction of binary vectors.

Plasmid DNA recombination was done with *Escherichia coli* DH5 α according to standard procedures (Sambrook and Russel 2001). PCR products were subcloned in pBluescript II SK+ and were verified for correct amplification by DNA sequencing. For stable transformation of *L. japonicus* to make transgenic lines harboring *gusA*, a binary vector pCambia1301 (Cambia, Canberra, Australia) was used. A binary vector, pHKN29, for hairy root transformation was made from pCambia1300 by replacing the hygromycin resistant gene with sGFP (S65T) (Niwa et al. 1999).

Constructs for RNA silencing.

The 3' end (582 bp) and a middle region (325 bp) of GUS coding sequence were amplified by PCR from pCambia1301, using a primer set of 5'-TTGGATCCTCGA-GCATCAGGGCGGCTATAC-3' and 5'-TTATCGATGGTAC-CTGCCAGTCAACAGA-3' and a set of 5'-TTGGATCCTCG-AGTATTGCCAACGAACC-3' and 5'-TTATCGATGGTACC-GAAGTTCATGCCAG-3', respectively. These fragments were

ligated in pKANNIBAL (Wesley et al. 2001) at *Kpn* I/*Xho* I and *Clal*/*Bam* HI sites so that the sense and antisense fragments were situated at both sides of a pyruvate decarboxylase kinase (Pdk) intron. The constructed cassettes were excised by digestion with *Nor* I and were cloned into pART27 (Gleave 1992), followed by transfer into pHKN29 to make pHKN30 and pHKN31, respectively. These binary vector constructs were transformed into appropriate *Agrobacterium* strains by direct transfer.

Plant transformation.

Transgenic *L. japonicus* B129 'Gifu' lines with CaMV35S::*gusA* or *lj27* promoter::*gusA* were made by a conventional transformation procedure with *A. tumefaciens* AGL1, as described by Stiller and associates (1997). Homozygous T₃ progeny with a single transgene were used for hairy root transformation.

Hairy root transformation using *A. rhizogenes* LBA1334 (Offringa et al. 1986) was performed according to a procedure for white clover (Diaz et al. 1989), with some modifications for optimization with *L. japonicus* (C. L. Diaz, personal communication). In brief, *L. japonicus* 'Gifu' seedlings (5 to 6 days old) grown on a pack of filter paper were placed in the *A. rhizogenes* suspension in a petri dish and then cut at the base of the hypocotyl. The seedlings with cotyledons were transferred onto agar plates containing half-strength B&D medium (Imaizumi-Anraku et al. 1997), were placed vertically in a growth cabinet, and were grown for 5 days for cocultivation. Then, the plants were transferred onto fresh agar medium containing 300 μ g of cefotaxime per ml and were grown for ten more days. Hairy roots that emerged from the base of the hypocotyls were tested for GFP fluorescence, and the plants with transgenic hairy roots were transplanted to sterilized vermiculite pots supplied with half-strength B&D medium containing 1 mM ammonium nitrate. After a week, the plants were inoculated with *Mesorhizobium loti* Tono (Kawaguchi et al. 2002) and continued to grow with the nitrogen-free medium.

GUS staining and enzyme assay.

Whole plants or detached roots were immersed in a staining solution (1.9 mM X-glucuronide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% TritonX-100, 20% methanol, and 100 mM sodium phosphate, pH 7.0) and were put under vacuum for a few minutes, followed by incubation at 37°C in the dark. Whole plant samples were bleached in 100% ethanol after staining.

Quantitative assay of GUS activity was performed by a method using 4-methyl umbelliferyl glucuronide (4-MUG)

Fig. 4. Fluorometric assays of β -glucuronidase activities of transgenic hairy roots of the line 16E. Only GFP-positive roots were selected.

Fig. 5. β -glucuronidase activities in the leaves of the plants (line 16E) with hairy roots transformed with hpRNAs. The averages are shown in the right panel. Error bars indicate $\pm$ standard deviation.

(Jefferson et al. 1987). Harvested plant materials were homogenized with a plastic rod in 100 μ l of extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% TritonX-100, 0.1% sarcosyl, and 10 mM 2-mercaptoethanol) in a 1.5-ml microtube. The homogenate was frozen in liquid nitrogen and then thawed at room temperature. After centrifugation to remove debris, the supernatant was diluted by 4 volumes of extraction buffer. Substrate solution (2.5 mM 4-MUG in 50% methanol) and the extract were mixed at 1:4 (vol/vol) and then were incubated in the dark at 37°C for 20 min, followed by terminating reactions by adding 1.5 volumes of 200 mM sodium carbonate. Amounts of 4-methyl umbelliferone formed were determined fluorospectrometrically with excitation at 355 nm and emission at 460 nm.

RT-PCR.

Total RNA was isolated from a transgenic root with nodules (14 days after inoculation), using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to manufacturer's instructions. Reverse transcription was performed by Super-ScriptII (Invitrogen, San Diego, CA, U.S.A.) with DNase (RQ1, Promega, Madison, WI, U.S.A.) -treated total RNA and oligo-dT₁₂₋₁₈ as primers. Synthesized cDNA (equivalent to 12 ng of total RNA) was used for PCR (45 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s) with the sets of primers specific to GUS (5'-TATCACCGTGGTGACGCATGT-3' and 5'-TTACATTGACGCAGGTGATCG-3') and Lj27 (5'-AGAT-CAGATGATTTGGGTGGTC-3' and 5'-ACGGCAAACATCATGCTGACA-3') as an internal control. Real-time detection

Fig. 6. β -Glucuronidase staining of nodule primordia formed on transgenic hairy roots of line 16E. Nodule primordia 7 days after *Mesorhizobium loti* inoculation are shown for **A** and **D**, pHKN29, **B** and **E**, pHKN30, and **C** and **F**, pHKN31. Upper panels (**A**, **B** and **C**) show X-glucuronide staining. Lower panels (**D**, **E** and **F**) show GFP fluorescence.

Fig. 7. Fluorometric assays of the β -glucuronidase (GUS) activities of nodules formed on transgenic hairy roots of line 9Y harboring *gusA* driven by nodule-specific *lj27* promoter. Six to ten nodules were harvested from each independent hairy root, and the extracts were subjected to fluorometric GUS assay.

Fig. 8. Accumulation of *gusA* transcripts in nodules on transgenic hairy roots of line 9Y. Total RNA prepared from three to six independent transgenic roots with nodules were subjected to reverse transcription followed by real-time polymerase chain reaction. The data are normalized using the amounts of *Lj27* transcripts estimated by the same reaction.

of PCR products was performed using LightCycler 350S (Roche, Branchburg, NJ, U.S.A.).

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