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Transformation of *Medicago truncatula* with *rip1-GUS* Gene

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ABSTRACT : *Medicago truncatula* is a model plant for molecular genetic studies of legumes and plant-microbe interactions. To accelerate finding of genes that play roles in the early stages of nodulation and stress responses, a transgenic plant was developed that contains a promoter-reporter fusion. The promoter of *rip1*, a *Rhizobium*-induced peroxidase gene, was fused to the coding region of β-glucuronidase (GUS) gene and inserted into a modified plant transformation vector, pSLJ525YN, in which the *bar* gene was preserved from the original plasmid but the neomycin phosphotransferase gene was replaced by a polylinker. Transformation of *M. truncatula* was carried out by vacuum infiltration of young seedlings with *Agrobacterium*. Despite low survival rates of infiltrated seedlings, three independent transformants were obtained from repeated experiments. Southern blot analyses revealed that 7 of 8 transgenic plants of the T_1 generation contained the *bar* gene whereas 6 T_1 plants contained the GUS gene. These results indicate that vacuum infiltration is an effective method for transformation of *M. truncatula*. The progeny seeds of the transgenic plants will be useful for mutagenesis and identification of genes that are placed upstream and may influence the expression of *rip1* in cellular signaling processes including nodulation.

Keywords: *Medicago truncatula*, model legume, *rip1-GUS* gene, transformation, vacuum infiltration

Medicago truncatula is a leguminous plant species related closely with the forage crop alfalfa (*M. sativa*). It originates from the Mediterranean region and numerous ecotypes have been collected from there. Unlike alfalfa, however, *M. truncatula* has many features suitable for genetic analyses and research on plant-micorbe interactions. Particularly, this small diploid legume has a tractable genome size (450 Mb/1C) and a short life span (Barker *et al.*, 1990; Blondon *et al.*, 1994), and can be transformed and regenerated readily (Trieu *et al.*, 1996). Furthermore, *M. truncatula* undergoes symbiotic relationship with the nitrogen-fixing soil bacterium *Rhizobium meliloti* (Cook, 1999). Such features have served to place *M. truncatula* as one of the most intensively studied model plants for the past decade (Handberg & Stougaard, 1992; Harrison, 2000). International research programs are actively operated to investigate genome organization and global gene expression profiles of *M. truncatula* (VandenBosch *et al.*, 2000), and results from such concerted efforts are being collected in organized databases (Bell *et al.*, 2001)

Symbiotic nitrogen fixation is established by mutual signal exchange between the plant and microbial partners (Mylona *et al.*, 1995). Flavonoids excreted by legume roots induce *Rhizobium* to produce a Nod factor. Perception of this oligosaccharide signal molecule invokes morphogenetic changes in root hairs that precede the infection of *Rhizobium*. The cellular events taking place in roots following infection are considered to be critical in establishing nodule development and function.

Several plant genes whose expression is activated during early nodulation have been cloned. These genes have been divided into two groups: the early and late nodulin genes. Early nodulin genes are implicated in preinfection process, whereas late nodulin genes are detected around the onset of nitrogen fixation and the functioning of the nodule. Typical early nodulin genes include those involved in the infection processes such as *rip1* (rhizobium-induced peroxidase 1) (Cook *et al.*, 1995), *ENOD12* (Journet *et al.*, 1994) and *ENOD5* (Scheres *et al.*, 1990) and the gene functioning in nodule organogenesis such as *ENOD40* (van de Sande *et al.*, 1996). An example of late nodulin genes is leghemoglobin gene.

The *rip1* gene is one of the earliest known nodulin genes. It is induced in root cells after 3 hours of *Rhizobium* infection (Cook *et al.*, 1995). Its transcript is localized to epidermal cells at the site of infection. Interestingly, its transcript level diminishes at the onset of nodule morphogenesis, when the transcripts of most other early nodulin genes such as *ENOD12*, *ENOD5*, and *ENOD40* increase. Such a distinguishable feature suggests that the expression of *rip1* is governed by an unknown regulatory mechanism.

Mutagenesis of wild type *M. truncatula* generated several mutants that were defective in nodulation and other developmental processes (Penmetsa & Cook, 1997; *ibid*, 2000; Catoira *et al.*, 2000). Positional cloning of such mutant genes are under way using molecular genetic maps (Thoquet *et al.*, 2002) and large-insert genomic DNA libraries (Nam

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et al., 1999). While these efforts are expected to yield valuable results by identifying novel genes that may play roles in nodulation and legume-specific processes, it is worthwhile to produce mutant populations that are more directed at the early stages of nodule development and hence likely to permit rapid identification of genes that regulate early signaling events.

To enrich the pool of mutagenizable legumes and increase the possibility for screening early nodulation genes, reporter gene fusions were constructed and introduced into *M. truncatula* in this study. The transgenic plants prepared by infiltration of *Agrobacterium* provide resources for isolating genes involved in legume-specific processes.

MATERIALS AND METHODS

Plant growth conditions

M. truncatula Gaertn Jemalong (A17) was used. Plants were grown in 7 cm diameter pots, one plant per pot. The light level was 0.15 mmol $m^{-2}s^{-1}$ with 16 h light / 24°C and 8 h dark / 22°C.

Treatment of *M. truncatula* seeds

M. truncatula seeds were obtained by breaking open the pods. The seeds were soaked in concentrated H_2SO_4 for 10 min, washed 3 times with distilled water and then sterilized in 30 % bleach, 0.1 % Tween 20 for 5 min. The seeds were then washed 3 times in sterile distilled water and spread on filter paper in petri plates (10 × 100 mm^2), approximately 20 seeds per plate. The plates were wrapped with parafilm and aluminum foil. The seeds were vernalized by incubation at 4°C for 12 - 18 days.

Preparation of vectors and gene fusions

The plant transformation vector pSLJ525 (Jones *et al.*, 1992) (Fig. 1A) was digested with *Hin*dIII and treated with the Klenow fragment at 37°C for 30 min. The blunt-ended vector was self-ligated and digested with *Bam*HI The Klenow fragment was used again to fill in the staggered restriction ends. The resulting vector lacking two unique restriction sites was digested with *Cla*I and *Sal*I, of which *Sal*I was treated only for 15 min for partial digestion. From the digested fragments separated by gel electrophoresis, a 25.2-kb *Cla*I/*Sal*I fragment was isolated and ligated with the oligonucleotide polylinker (Fig. 1B) The polylinker was prepared by annealing two separately synthesized complementary strands. The resulting vector was used to carry the reporter fusions. The two plasmids pRG-FR and pRL-rN

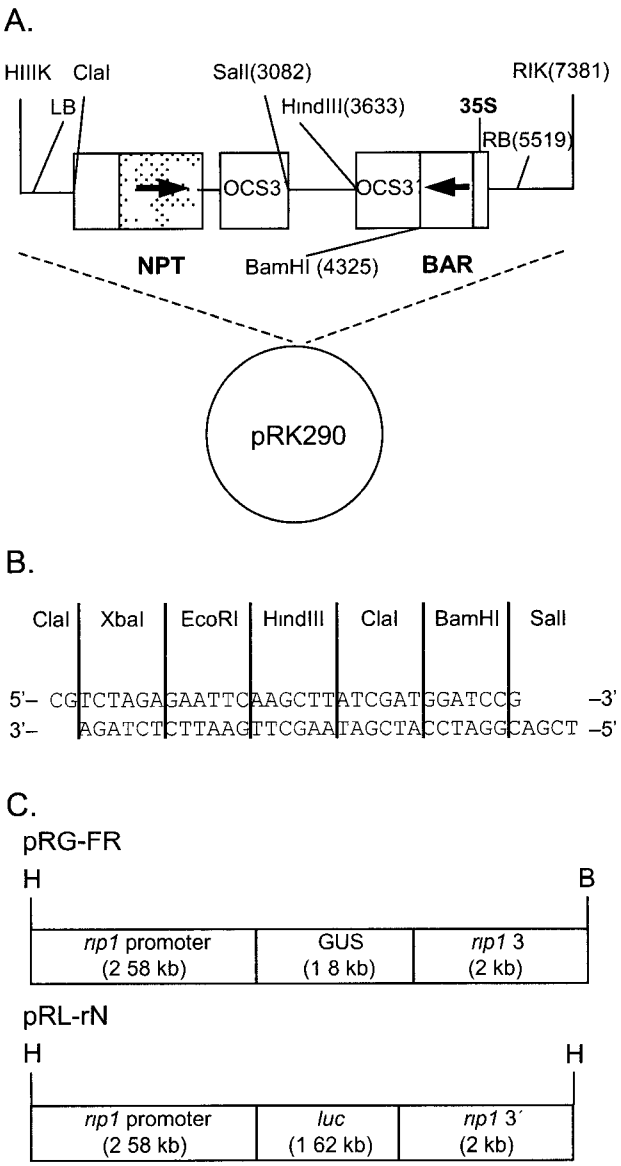

Fig. 1. Structure of plant transformation vectors and gene fusions. A. pSLJ525 (Jones *et al.*, 1982), the plant transformation vector based on the binary plasmid pRK290, contains the phosphinothricin acetyl transferase (BAR) coding region driven by the cauliflower mosaic 35S promoter (35S) and the nptII (neomycin phosphotransferase II) cassette. Both the *bar* and *nptII* genes are terminated by the 3' untranslated sequences of octopine synthase gene (OCS3'). LB and RB designate left and right borders of T-DNA, respectively The relative nucleotide positions of restriction sites are shown in parentheses B Nucleotide sequences of the polylinker used to replace the *nptII* cassette To nullify the *Cla*I site at the 5' end after ligation, an adenosine residue following the staggered cut (CG) was omitted. **C.** Structures of the pRG-FR and pRL-rN plasmids The fusions between *rip1* promoter / 3' flanking regions (*rip1* 3') and the *uidA* gene (GUS) or the luciferase gene (*luc*) were placed in the pBluescript SK+ vector (Peng and Cook, unpublished) B· *Bam*HI, H· *Hin*dIII.

(Peng & Cook, unpublished) (Fig. 1C) were used to provide the *rip1-GUS* and *rip1-luc* fusions, respectively. The fusion fragments were isolated by digestion with *Hin*dIII/*Bam*HI (pRG-FR) and *Hin*dIII (pRL-rN) and ligated to the restriction digested polylinker-containing vector.

Transient expression assay

M. truncatula seedlings were used for particle bombardment experiments. A helium driven Biolistic PDS-1000 Particle Delivery System (BioRad) was used by following the manufacturer's instructions. Luciferase activity was measured 20 h after bombardment. Detection of luciferase activity was carried out according to the methods described by Luehrsen *et al.* (1992). Colorimetric GUS assay was carried out according to the procedures described by Gallagher (1992). Values obtained from the luciferase and GUS activity measurements were normalized and plotted as a function of input DNA concentration.

Transformation of *M. truncatula* via seedling infiltration

Transformation of *M. truncatula* with *Agrobacterium* was carried out via seedling infiltration as described by Trieu *et al.* (2000) with minor modifications. *Agrobacterium tumefaciens* strain EHA105 (Dr. M. J. Harrison) carrying the pSLJ525 derivatives was grown in TY medium at 28°C for 2 days up to OD_{600} of 1.8. The cells were pelleted by centrifugation and resuspended in seedling vacuum infiltration medium (Trieu *et al.*, 2000). Petri plates (10×100 mm^2) containing vernalized seedlings were covered with vacuum infiltration medium and placed in a vacuum chamber of the PDS-1000 Particle Bombardment System (BioRad). Vacuum was applied to 25 inches Hg for 1 min two times, and the seedlings were blotted on sterile 3 MM paper (Whatman) and spread onto seedling co-cultivation medium, approximately 20 seedlings per petri plate (10×100 mm^2). The plates were incubated in a growth chamber at 20°C, 16 h daylength for 2 days, and the seedlings were planted into pots containing soil with 3 seedlings per pot and kept under dark conditions for 6 days.

Southern blotting

Plant genomic DNA was extracted following the methods of Chen & Ronald (1999). The electrophoresed genomic DNA in the agarose gel was irradiated with UV light (50 mJ) in a Gene Linker chamber (BioRad). The gel was then blotted onto a Hybond N(+) nylon filter (Amersham) with 0.4 N NaOH, 1.5 M NaCl as the capillary solution. The filter was dried, crosslinked, and used for hybridization as described (Nam *et al.*, 1999).

The *bar* gene probe was prepared from pSLJ525 by digesting the plasmid with *Sal*I and *Xho*I. The resulting 1.9-kb fragment was isolated and used. The GUS gene probe was prepared by digesting pSLJ525YN-GUS with *Nco*I and *Pst*I and isolating a 1.8-kb fragment. The probes were labeled with α-^{32}P-dCTP (3000 Ci/mmole, Amersham) using a Ready-to-Go DNA labeling kit (Pharmacia).

RESULTS AND DISCUSSION

Preparation of plant transformation vectors containing gene fusions

The model legume *M. truncatula* can be transformed via infiltration of flowers and seedlings with *Agrobacterium* (Trieu *et al.*, 2000). Transformants are often selected for the resistance to phosphinothricin, a capability provided by the *bar* (phosphinothricin acetyl transferase) gene of the plant transformation vector. To introduce the *rip1-GUS* fusion into *M. truncatula* by using the same strategy, an appropriate plant transformation vector was prepared. The plasmid pSLJ525 (Jones *et al.*, 1992) was developed using the pRK290 backbone and contains the *bar* gene and neomycin phosphotransferase type II (*nptII*) gene. However, because the *nptII* gene is not required for the present study, pSLJ525 was modified by replacing the *nptII* cassette with a synthetic polylinker.

Fig. 1 shows the structure of pSLJ525 and the inserted polylinker. Two unique restriction sites of pSLJ525 (*Hin*dIII and *Bam*HI sites at 3633 and 4325 nucleotide positions, respectively) (Fig. 1A) were removed by Klenow filling-in reactions to ensure the usage of *Hin*dIII and *Bam*HI sites introduced in the polylinker (Fig. 1B). The modified plasmid was digested with *Cla*I and *Sal*I, of which *Sal*I was treated only partially. A fragment of 25.2 kb in size was isolated from the digest and ligated with the polylinker. These procedures gave rise to a new plasmid named as pSLJ525YN, which maintained the *bar* gene, yet had a polylinker in place of the *nptII* cassette.

The pSLJ525YN vector served as a vehicle to carry the *rip1* fusions. Restriction fragments of pRG-FR and pRL-rN, which contained GUS and luciferase genes, respectively (Fig. 1C), were inserted into the vector. The resulting fusion constructs, pSLJ525YN-GUS and pSLJ525YN-luc, were subsequently used for plant transformation.

Transient expression of the reporter genes

To test whether the two fusion constructs would express their reporter genes adequately in plant cells, transient expres-

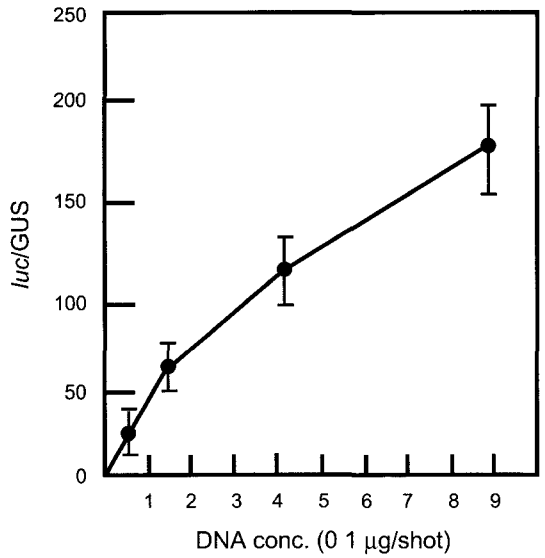

Fig. 2. Transient expression of the reporter genes in *M. truncatula* root tissue. Activities of luciferase (*luc*; pRL-rN) and β-glucuronidase (GUS; pRG-FR) were measured quantitatively and their ratios were plotted as a function of test plasmid concentration. Three to five replicates were used per measurement. Bars indicate standard errors

sion assay was carried out The two plasmids, pSLJ525YN-GUS and pSLJ525YN-luc, were introduced into young root cells by microprojectile bombardment and the activities of β-glucuronidase and luciferase were monitored by separate colorimetric measurements. To quantify the activities of β-glucuronidase and luciferase, the luciferase plasmid was titrated against a constant amount of the GUS plasmid. Fig. 2 shows the ratios of the two enzyme activities (*luc*/GUS) as a function of the DNA concentration used for each shot. The ratio remained linear up to 0.15 µg of input test DNA. These results indicated that both the GUS and luciferase plasmids directed transient expression of their reporter genes in the plant cell and further supported the feasibility of plant transformation.

Transformation of *M. truncatula* with *rip1-GUS* fusion

The *rip1-GUS* fusion was introduced into *M. truncatula* with *Agrobacterim* by vacuum infiltration of seedlings. The procedure developed by Trieu *et al.* (2000) was followed to carry out infiltration. Three separate experiments were conducted, of which two gave transformants. As summarized in Table 1, the survival rates of infiltrated plants were fairly low, ranging from 4 % to 24 %. Except for the first experiment, however, the survived seedlings produced 200 - 300 seeds in total, of which 50 - 60 seeds germinated. The resulting seedlings in the T_1 generation were subjected to phosphinothricin (PPT) treatment. As determined by the resistance to PPT, 5 and 12 transformants were obtained from the second and third experiments, respectively. An average transformation frequency was 14.6 % for the two experiments. These results are largely consistent with the previous results (Trieu *et al.*, 2000), although continuing experiments may improve the survival and transformation frequencies.

Southern blot analysis of transformants

To verify the presence of the T-DNA in the genomes of the transformants, Southern blot analyses were carried out. Eight transformants obtained from the second infiltration experiment (Table 1) were used to extract genomic DNA. The DNA was digested with *Bam*HI, blotted and probed with the *bar* gene for a right border analysis (Fig. 3A). Seven of eight transformants contained a copy of the *bar* gene. Six of them were apparently the same size of approximately 12 kb. These results suggested that the six transformants could be siblings.

The blot was also probed with a *uidA* (GUS) gene fragment for the left border analysis (Fig. 3B). When the identical transformants were used, three different fragment sizes (approximately 6.5 kb, 7.5 kb and 9.0 kb) were found to hybridize to the left border probe. Interestingly, a transformant (#4) that had hybridized with the *bar* probe lacked the sequences complementary to the GUS probe, indicating that the integration of the T-DNA sequence was incomplete or prematurely terminated. These results indicate that six of eight transformants (75 %) contained both the right and left border sequences. Moreover, at least three independent transformation events occurred in a single infiltration experiment. Although further experiments are necessary to dem-

Table 1. Summary of the transformation experiments.

Experiment	No. of plants	No. of plants	Percentage	No. of seedlings	Percentage
	Infiltrated	survived	germination	resistant to PPT	transformation
1	25	1	0	0	0
2	25	4	25.7	5	9.6
3	25	6	18.3	12	19.6

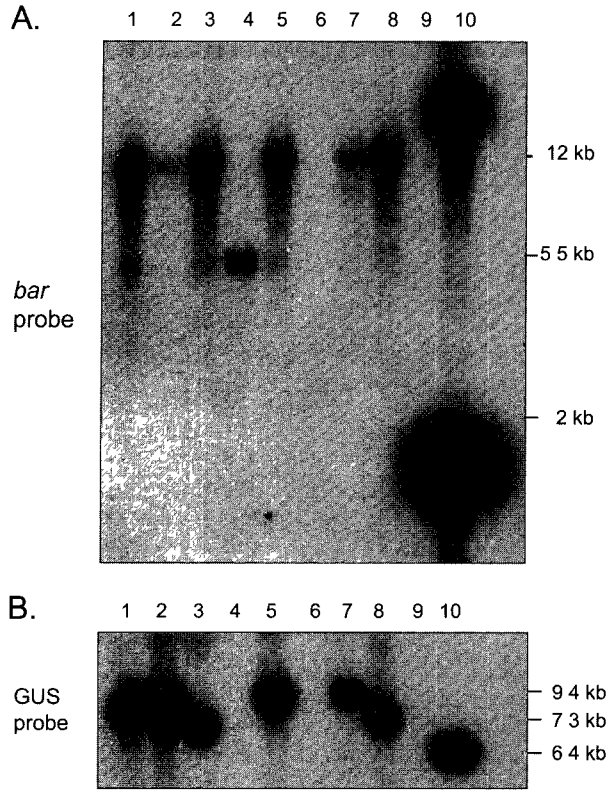

Fig. 3. Southern blot analysis of transformants prepared from *rip1-GUS* fusion Genomic DNAs isolated from eight transformants were digested with *Bam*HI The DNA fragments were separated by agarose gel electrophoresis, blotted to nylon membranes and hybridized with the *bar* probe (**A**) or the GUS probe (**B**) In both A and B, lanes 1-8 were loaded with DNAs from individual transformatns, whereas lane 9 was loaded with untransformed wild type *M truncatula* DNA digested with *Bam*HI. Lane 10 was loaded with the pSLJ525 vector as a control

onstrate the precise number of unique transformation events, it is cautiously concluded that the *rip1-GUS* gene fusion construct was introduced into *M. truncatula.*

Usefulness of transgenic plants for mutagenesis

In this study a transgenic line of *M. truncatula* containing *rip1-GUS* fusion was developed. *M. truncatula* is a model legume used for a variety of research objectives. They include plant-microbe interactions such as symbiotic nitrogen fixation, molecular genome analyses at the structural and functional levels, and map-based cloning of genes that are associated with diverse physiological processes. One of the genetic approaches that have been successful with model plants such as *Arabidopsis* is to mutagenize transgenic plants containing reporter genes. These strategies often resulted in the isolation of novel genes that are placed upstream and regulate expression of the downstream promoter of the transgene (Li *et al.*, 1999). As the putative product of *rip1* has been predicted to function in cell wall crosslinking and fortification during early stages of nodulation, mutageneis of the *rip1-GUS* transgenic plant provides a unique opportunity for isolating upstream components of the nodulation pathway. Discovery of genes that are involved in early signaling processes is important in understanding the cellular events that define legume-specific processes. Eventually, such valuable genes will be useful for improving leguminous crop plants.

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