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Short Communication

Enhanced symbiotic nitrogen fixation by *Lotus japonicus* containing an antisense *β*-1,3-glucanase gene

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Abstract We hypothesized that root nodule formation would be promoted by lower expression of a *β*-1,3-glucanase gene (designated as *LjGlu1*), because expression of this gene is increased in transgenic *Lotus japonicus* that shows reduced nodulation. In order to suppress the expression of this gene, we introduced the antisense *LjGlu1* gene into *L. japonicus* via *Agrobacterium rhizogenes*. Although there was no significant difference in shoot and root growth between *L. japonicus* possessing the antisense construct and plants containing an empty vector, the number of root nodules 28 days after inoculation with *Mesorhizobium loti* MAFF303099 increased in the antisense plant compared with the control plant. Moreover, the nitrogen fixation activity of the antisense plant was drastically enhanced. The mechanism of enhanced nitrogen fixation by suppression of the expression of *LjGlu1* is unknown, but this phenomenon should be an important breeding target for leguminous plants. This is the first report of strong enhancement of nitrogen fixation activity by manipulation of a gene of the leguminous host plant.

Key words: Antisense, *β*-1,3-glucanase, *Lotus japonicus*, nitrogen fixation, root nodules.

Nodules form on the roots of legumes under conditions that favor rhizobial entry into the root cells of the host plant. Inside the nodules, the rhizobia differentiate into nitrogen-fixing bacteroids, which convert atmospheric nitrogen into ammonia, a source of fixed nitrogen for the host legume. In return, the host plant provides the rhizobia with photosynthates. Together, the two organisms form a symbiotic relationship enabling the plant to survive in nitrogen-limiting environments. Research has gradually revealed the molecular mechanisms programmed into the host leguminous plants, such as mechanisms for the recognition of the bacterial Nod factors (Radutoiu et al. 2003; Madsen et al. 2003), and of subsequent signal transduction (Tirichine et al. 2006).

Recently, we studied the effect of the phytohormone abscisic acid (ABA) on root nodule formation (Suzuki et al. 2004). We observed that at higher endogenous concentrations of ABA, the number of root nodules decreased whereas at lower endogenous concentrations, it increased. In this research, the endogenous concentration of ABA increased significantly in transgenic *Lotus japonicus* in which *TrEnodDR1* was artificially expressed, driven by a CaMV35S promoter, and the number of root nodules produced by this transformant was drastically reduced; in addition, the expression of a *β*-1,3-glucanase gene that has been designated *LjGlu1* (accession number: AB437902) was clearly up-regulated compared with the control plant. Moreover, when this transformant was treated with abamine, a specific inhibitor of the 9-*cis*-epoxycarotenoid dioxygenase that is required for ABA biosynthesis, expression of *LjGlu1* was restored to some extent (Nakatsukasa-Akune et al. 2005). These results suggest the possibility that *LjGlu1* has an inhibitory function in root nodule formation. Therefore, we hypothesized that if the expression of *LjGlu1* could be suppressed, the number of root nodules would increase. In the present study, we investigated the symbiotic characteristics of *L. japonicus* containing an antisense *LjGlu1* gene and that was inoculated with *Mesorhizobium loti* MAFF303099.

To suppress the expression of *LjGlu1*, we amplified a 550-bp DNA fragment with a cDNA clone (MPD042b01, Asamizu et al. 2000; Sato et al. 2001; Asamizu et al. 2004) obtained from the Kazusa DNA Research Institute as a template. The primer sequences for *LjGlu1* were 5′-GGGGACAAGTTTGTACAAA-

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AAAGCAGGCTGGCCCCCCTCGACATG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTGAT-CTGGCCTTGTCAATTGG-3′. At the beginning of this process, we intended to create an RNAi construct for *LjGlu1* using Invitrogen's Gateway Technology (Tokyo, Japan). The amplified fragment was inserted into pDONR221 by BP clonase. Resultant plasmid DNA was reacted with pHKNGWRNAi, which was generated by inserting a CaMV35S promoter-RNAi cassette-OCS terminator fragment of a plasmid pHELLSGATE8 into multi-cloning site of a plasmid pHKN29 that was made from pCAMBIA1300 by replacing the hygromycin resistant gene with sGFP (Kumagai and Kouchi 2003). However, sequencing analysis to confirm the construct showed that we obtained a CaMV35S promoter+ antisense *LjGlu1*+OCS terminator cassette (antisense) instead of the desired RNAi cassette. We then designated the result as pHKN29-antiLjGlu1. Both pHKN29 (an empty vector) and pHKN29-antiLjGlu1 were introduced into *Agrobacterium rhizogenes* strain LBA1334 by means of electroporation. The induction of hairy roots in *L. japonicus* Miyakojima MG-20 was performed according to the method of Kumagai and Kouchi (2003). Plants in which hairy roots had been induced for 3 weeks were transferred into an artificial soil mix (vermiculite/perlite, 5 : 1 v/v) containing B&D medium (Broughton and Dilworth 1971) and inoculated with *M. loti* MAFF303099. The bacteria were cultivated in liquid YM medium (Keele et al. 1969) at 28°C on a rotary shaker for 3 days. Cells were harvested by centrifugation and resuspended in sterile water to a concentration of 1.0×10^7 cells/ml. For inoculation, 1 ml of this bacterial suspension was used.

Figure 1A (bright field) and 1B (fluorescence field) show hairy root induction and a root nodule of *L. japonicus* (antisense) 28 days after inoculation (DAI) with *M. loti*. We assessed that transformation efficiency as very high, because about 70% of emerged roots showed GFP fluorescence both with the empty vector and with the antisense construct (data not shown). Quantitative real-time RT-PCR for analysis of the expression of *LjGlu1* in transformed *L. japonicus* was carried out according to the method of Shimoda et al. (2005). We used the following primer sequences: for *LjGlu1*, 5′-GGGGACAAGTTTGTACAAAAAAGCAG-GCTGGCCCCCCTCGACATG-3′ and 5′-GGCTAAG-GAGACCTTG-3′, and for eIF4A (the internal control), 5′-AGAGGGTTTAAAGATCAAAT-3′ and 5′-ATGTC-AATTCATCACGTTTT-3′. Hairy roots of *L. japonicus* showing GFP fluorescence (2 weeks after hairy root induction followed by inoculation with the rhizobia) at 28 DAI with *M. loti* were used for the RNA extraction. As shown in Figure 1C, the expression of *LjGlu1* in the antisense transformant was significantly reduced compared with that in plants with the empty vector.

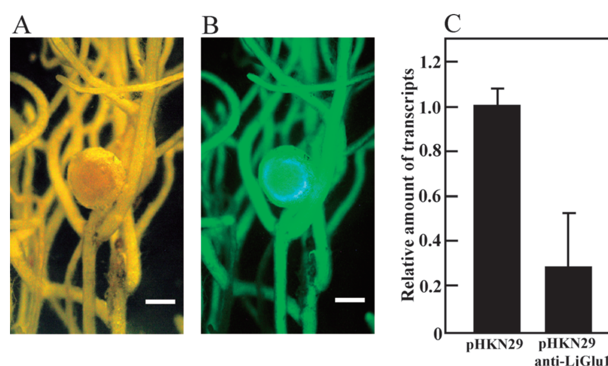

Figure 1. Expression analysis of the transformed hairy roots of *L. japonicus*. Microscopic observations of the nodules that formed on the transgenic hairy roots 28 DAI with *M. loti*. Hairy roots induced by *A. rhizogenes* were observed using (A) bright-field and (B) fluorescence-field microscopy. The hairy roots showing GFP fluorescence possess the T-DNA region of antisense *LjGlu1* (antiLjGlu1). Scale bars represent 1 mm in both (A) and (B). (C) Expression of *LjGlu1* in transformed hairy roots 28 DAI. The relative amount of transcripts were rectified against LjeIF-4A (internal control) transcripts and were normalized relative to the mean value in control plants, which was set to 1. The mean values indicate the average of five (pHKN29) and seven (pHKN29-antiLjGlu1) independent experiments, and range bars represent standard deviations.

Therefore, these antisense hairy roots could be used for our experimental purposes.

Lotus japonicus in which hairy roots were induced for 5 days were transferred to B&D agar plates and inoculated with *M. loti*. Then, we analyzed the growth of shoots and hairy roots showing GFP fluorescence in the treated plants 28 DAI with the rhizobia. As shown in Figure 2A (shoot length) and 2B (root length), no significant difference in shoot or root length was observed between plants with the empty vector and the antisense transformants. For the analysis of root nodule formation, we transferred *L. japonicus* in which hairy roots has been induced for 3 weeks into an artificial soil mix (vermiculite/perlite, 5 : 1 v/v) containing B&D medium and inoculated with the rhizobia. The number of root nodules on hairy roots showing GFP fluorescence in the antisense transformant 28 DAI with *M. loti* increased compared to that in the control, but the difference was not significant (Figure 2C). To assess nitrogen fixation activity, we measured acetylene reduction activity as follows: We placed plants in glass tubes containing wet filter paper, and replaced the gas phase with an acetylene–air mixture (C_2H_2 : air=1 : 4 v/v). After 2 h of incubation at 25°C, we analyzed the gas phase using a GC-3A gas chromatograph (Shimadzu, Kyoto, Japan) to determine the amount of ethylene. Surprisingly, the nitrogen fixation activity per plant of the antisense transformant was dramatically and significantly enhanced compared with that of the control (Figure 2D). The nitrogen fixation activity per root nodule was also increased about twofold. This is the first report of strong enhancement of nitrogen fixation activity resulting from

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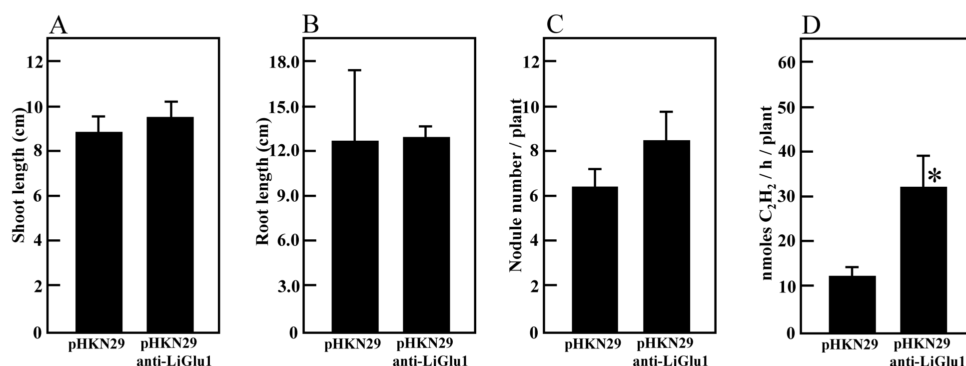

Figure 2. Phenotypic analysis of transformed hairy roots 28 DAI with *M. loti*. pHKN29, plants treated with *A. rhizogenes* containing plasmid pHKN29; pHKN29-antiLjGlu1, plants treated with *A. rhizogenes* containing plasmid pHKN29-antiLjGlu1. (A, B) At least 14 plants were used for the analysis of (A) shoot and (B) root growth. Error bars indicate standard deviations. (C) Average numbers of root nodules per plant, and (D) average acetylene reduction activity per plant. Range bars represent standard errors. At least 25 plants were used for each analysis. Asterisks indicate statistically significant differences (*, $P<0.01$) between the control plants and the antisense transformants.

the manipulation of a gene of the leguminous host plant.

A DNA database search using the *LjGlu1* sequence as a query revealed at least ten genes that resemble β-1,3-glucanase in *L. japonicus*, and the highest homology between *LjGlu1* and these genes was around 60%. It is well known that β-1,3-glucanase produces β-glucan fragments from the cell wall of a pathogenic fungus, and induces phytoalexin biosynthesis in the host cells. In *L. japonicus*, it has been reported that expression of two β-1,3-glucanase genes (which are not identical to *LjGlu1*) that may be involved in the plant's defense response was induced transiently during the infection or nodule initiation stage (Kouchi et al. 2004). In *Nicotiana tabacum*, pathogenesis-related β-1,3-glucanase genes were down-regulated by treatment with ABA (Rezzonico et al. 1998). However, the expression of *LjGlu1* was suppressed during infection with rhizobia and the initiation stages of nodule organogenesis (Kouchi et al. 2004) and was up-regulated by treatment with exogenous 0.5 μM ABA (data not shown). Thus, *LjGlu1* may not be a pathogenesis-related β-1,3-glucanase and may have a negative function in root nodule formation. If so, the tendency to increase the number of root nodules in the antisense transformants is reasonable.

In *Glycine max*, it was reported that β-1,3-glucanase activity exhibited a transient pattern in root nodules, with the maximum activity occurring following a decline in the nitrogen fixation rate at 28 DAI (Mohammadi and Karr 2002). The fact that nitrogen fixation was reduced by higher expression of β-1,3-glucanase is common to both *L. japonicus* and *G. max*. Although the mechanism for the enhancement of nitrogen fixation activity is unknown, this phenomenon should be very important for the molecular breeding of leguminous plants.

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References

Asamizu E, Nakamura Y, Sato S, Tabata S (2000) Generation of 7137 non-redundant expressed sequence tags from a legume, *Lotus japonicus*. *DNA Res* 7: 127–130

Asamizu E, Nakamura Y, Sato S, Tabata S (2004) Characteristics of the *Lotus japonicus* gene repertoire deduced from large-scale expressed sequence tag (EST) analysis. *Plant Mol Biol* 54: 405–414

Broughton WJ, Dilworth MY (1971) Control of leghemoglobin synthesis in snake beans. *Biochem J* 125: 1075–1080

Keele BB, Hamilton PB, Elkan GH (1969) Glucose catabolism in *Rhizobium japonicum*. *J Bacteriol*: 971184–1191

Kouchi H, Shimomura K, Hata S, Hirota A, Wu GJ, Kumagai H, Tajima S, Suganuma N, Suzuki A, Aoki T, Hayashi M, Yokoyama T, Ohyama T, Asamizu E, Kuwata C, Shibata D, Tabata S (2004) Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. *DNA Res* 11: 263–274

Kumagai H, Kouchi H (2003) Gene silencing by expression of hairpin RNA in *Lotus japonicus* roots and root nodules. *Mol Plant Microbe Interact* 16: 663–668

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. *Nature* 425: 637–640

Mohammadi M, Karr AL (2002) β-1,3-glucanase and chitinase activities in soybean root nodules. *J Plant Physiol* 159: 245–256

Nakatsukasa-Akune M, Yamashita K, Shimoda Y, Uchiumi T, Abe M, Aoki T, Kamizawa A, Ayabe S, Higashi S, Suzuki A (2005) Suppression of root nodule formation by artificial expression of the *TrEnodDR1* (coat protein of *white clover cryptic virus 1*)

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gene in *Lotus japonicus*. *Mol Plant Microbe Interact* 18: 1069–1080

Radutoiu S, Madsen LH, Madsen EB, Felle HH, Umehara Y, Grønlund M, Sato S, Nakamura Y, Tabata S, Sandal N, Stougaard J (2003) Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425: 585–592

Rezzonico E, Flury N, Meins Jr F, Beffa R (1998) Transcriptional down-regulation by abscisic acid of pathogenesis-related β-1,3-glucanase genes in tobacco cell cultures. *Plant Physiol* 117: 585–592

Sato S, Kaneko T, Nakamura Y, Asamizu E, Kato T, Tabata S (2001) Structural analysis of a *Lotus japonicus* genome. I. sequence features and mapping of fifty-six TAC clones which cover the 5.4 Mb regions of the genome. *DNA Res* 8: 311–318

Shimoda Y, Nagata M, Suzuki A, Abe M, Sato S, Kato T, Tabata S, Higashi S, Uchiumi T (2005) Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in *Lotus japonicus*. *Plant Cell Physiol* 46: 99–107

Suzuki A, Akune M, Kogiso M, Imagama Y, Osuki K, Uchiumi T, Higashi S, Han SY, Yoshida S, Asami T, Abe M (2004) Control of nodule number by the phytohormone abscisic acid in the roots of two leguminous species. *Plant Cell Physiol* 45: 914–922

Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandal N, Albrektsen AS, Kawaguchi M, Downie A, Sato S, Tabata S, Kouchi H, Parniske M, Kawasaki S, Stougaard J (2006) Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. *Nature* 441: 1153–1156

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