Leaf growth of *Lotus japonicus* hypernodulation mutant *har1-4*

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Summary

In soybean (*Glycine max*. (L) Merr.), hypernodulation mutant lines produced small-size leaves due to the smaller number of leaf cells, compared to wild type. So the autoregulation of nodulation is related to the control system of leaf cell proliferation in soybean. In this study, leaf growth of hypernodulation mutant *har1-4* in *Lotus japonicus* was compared with wild type. Leaf area of hypernodulation mutant *har1-4* is similar with the wild type. Cell number of leaf of *har1-4* tended to be lower than that of wild type, while cell area of *har1-4* tended to be larger than that of wild type. These results indicate that a part of the autoregulation of nodulation might be related to cell proliferation in leaves also in *L. japonicus* as shown in soybean. The system of autoregulation of nodulation in legume should be a part of general plant mechanisms rather than specific to nodulation.

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Key words : autoregulation of nodulation, hypernodulation mutant, leaf growth, *Lotus japonicus*

Leguminous plants have the ability to form root nodules, which are symbiotic organ with rhizobia. Symbiotic rhizobia fix atmospheric nitrogen and then the fixed nitrogen can be used by host plant. But this process is energetically expensive and so legumes strictly control the number of nodules they form. This negative regulation system is referred to as the autoregulation of nodulation (Caetano-anolles and Gresshoff 1991; Oka-Kira and Kawaguchi 2006).

Following mutagenesis, several hypernodulation mutant lines were isolated in soybean (*Glycine max* [L.] Merr) (Carroll *et al.* 1985; Gremaud and Harper 1989; Akao and Kouchi 1992) and in *Lotus japonicus* (Wopereis *et al.* 2000; Kawaguchi *et al.* 2002). Reciprocal grafting experiment between hypernodulation mutant and the wild type showed that control of nodule number depends on the shoots rather than the root. Autoregulation of nodulation looks like to be controlled by the shoot through the exchange of signal molecule(s) between the shoot and roots. It is postulated that when a plant root is infected with rhizobia, an infection signal is synthesized in the root and is transported toward shoot. And then a shoot-derived autoregulation signal is synthesized in the shoot and is transported toward the roots. Differentiation of nodule at later infected roots is suppressed by the shoot-derived autoregulation signal. In determinate type of nodules (e.g. in soybean, *L. japonicus*), this suppression acts already initiated primordia from developing into a nodule, whereas in indeterminate type of nodules (e.g. in *Medicago truncatula*), it blocks the formation of new nodule primordia (Caetano-anolles and Gresshoff 1991).

HAR1 and *GmNARK*, which play an important role in the autoregulation of nodulation, were identified and shown to encode a receptor-like kinase protein that contain leucine-rich repeat in *L. japonicus* and soybean, respectively (Krusell *et al.* 2002; Nishimura *et al.* 2002; Searle *et al.* 2003). These legume genes are homologous to Arabidopsis *CLAVATA1* (*CLV1*), which is involved in control of cell proliferation in shoot apical meristem (SAM) (Clark *et al.* 1997). It is interesting that *HAR1/GmNARK* is expressed in most tissue except SAM (Nishimura *et al.* 2002; Searle *et al.* 2003), whereas *CLV1* is expressed in SAM (Clark *et al.* 1997). It seems that HAR1/GmNARK protein is involved in the perception of a root derived infection signal.

Recently, it was indicated that less total dry matter accumulation of hypernodulation mutant lines than the wild type may be the secondary effect due to the large number of nodules, while the hypernodulation trait is a primary effect of the mutated gene in soybean (Ito *et al.* 2007). It was also indicated that leaf growth of hypernodulation mutant was different from the wild type; the expanded leaf was smaller but the leaf emergence rate was faster compared with wild type. Microscopic study showed that hypernodulation mutant soybean lines produced small-size leaves due to the smaller number of leaf cells, compared to wild type (Ito *et al.* 2008). So the autoregulation of nodulation is related to the control system of leaf cell proliferation in soybean. However, the relationship in *L. japonicus* was not known. In this study, the

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relationship between autoregulation of nodulation and leaf cell proliferation in *L. japonicus* was investigated.

Materials and Methods

In this study, plants were cultured as follows. Surface-sterilized seeds of *L. japonicus* B-129 Gifu and the hypernodulation mutant *har1-4* were sown onto agar medium plate without inoculation and grown in a growth chamber under the following conditions: 16-h photoperiod at 23℃ / 8-h dark period at 23 ℃ . 6 days after sowing, test plants were transferred to hydroponic of sterilized B&D medium with 5 mM NO_3^-. The culture solution was continuously aerated by an air pump and changed every 3 days. At 14 days after sowing, test plants were sampled, and fresh weight and leaf area was analyzed.

As a microscopic study, we used the method described by Horiguchi *et al.* (2005). The leaves were fixed with FAA (formaldehyde - acetic acid - ethanol) and were cleared with a chloral solution (chloral hydrate 500 g, glycerol 50 g, and H_2O 0.125 L). The leaf cells were observed using an optical microscope (BX-60, OLYMPUS, Tokyo Japan). The palisade cells in the subepidermal layer per unit area in the center of the central leaf blade of the first trifoliolate leaf were determined. The value of the palisade cell number per leaf and the cell size were calculated.

Results

Fig. 1 shows fresh weight of *har1-4* and wild type. The total fresh weight of *har1-4* (35.2 mg) was similar to wild type (35.2 mg). Fresh weight of stem of *har1-4* (2.2 mg) was significantly lower than that of wild type (3.0 mg). Fresh weight of root of *har1-4* (13.6 mg) tended to be high compared with the wild type (12.3 mg), while it was not significant difference. The root of *har1-4* was short, but the volume is large because of the increased number of lateral roots. Fresh weight of leaf of *har1-4* (19.3 mg) was similar compared with the wild type (19.9 mg). The growth of the underground part tended to have priority than shoot in *har1-4*.

Leaf area of primary and 1st and 2nd trifoliolate leaf on *har1-4* was similar with that of wild type (Fig. 2). Leaf area of 3rd trifoliolate leaf of *har1-4* was slightly smaller than that of wild type. A microscopic study showed that cell number per leaf of 1st trifoliolate leaf of *har1-4* tended to be lower than that of wild type, but it was not significantly difference (Fig. 3A). On the other hand, cell area of *har1-4* tended to be larger than that of wild type (Fig. 3B).

Discussion

In soybean plants, growth of hypernodulation NOD mutant lines was similar with the wild type when they were grown without inoculation of bradyrhizobia (Ito *et al.* 2007). Also in *L. japonicus*, fresh weight of a whole plant of *har1-4* was similar with the wild type (Fig. 1). This result indicated that less total dry matter accumulation of nodulated hypernodulation mutant line than the nodulated wild type may be the secondary effect due to the large number of nodules also in *L. japonicus* as well as soybean. However, fresh weight of stem of *har1-4* was smaller than the wild type. Similar results were obtained in soybean hypernodulation mutant NOD3-7 seedling (Ito *et al.* 2006). Growth of stem in *L. japonicus har1-4* and in soybean NOD3-7 might be inferior compared with their wild type. It is indicated that the shoot growth of *har1-4* might be different from the wild type.

Previous study showed that the fully expanded leaves of hypernodulation soybean mutant NOD1-3 and NOD3-7 lines

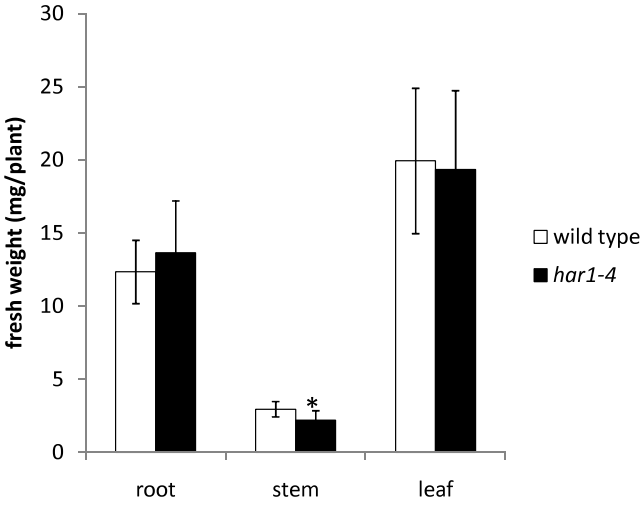

Fig. 1. Comparison of fresh weight of hypernodulation mutant *har1-4* and wild type (Gifu) in *Lotus japonicus*. Plants were grown without inoculation under 5 mM NO_3^- supply (n=7).
* represent significant differences between *har1-4* and wild type at $P<0.05$.

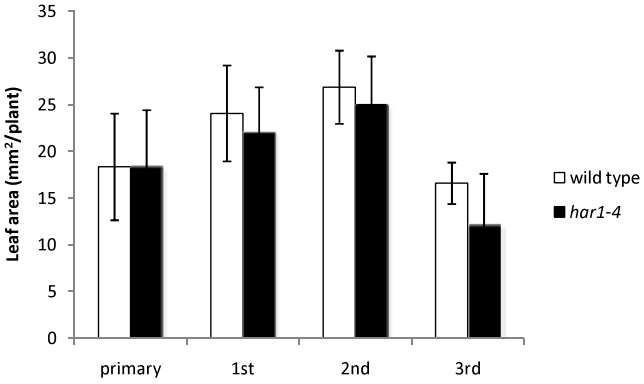

Fig. 2. Leaf area of hypernodulation mutant *har1-4* compared with wild type (Gifu) in *Lotus japonicus*. Plants were grown without inoculation under 5 mM NO_3^- supply (n=7).

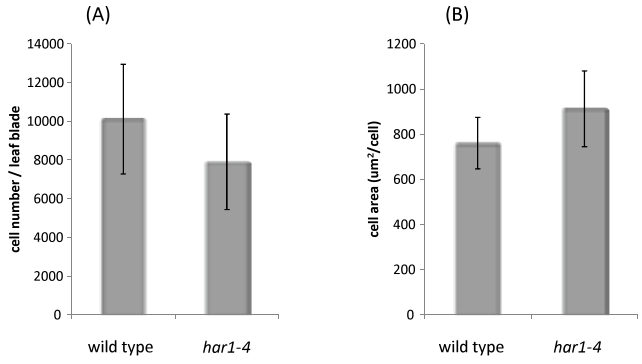

Fig. 3. Leaf phenotypes of hypernodulation mutant *har1-4* compared with wild type (Gifu) in *Lotus japonicus*. Plants were grown without inoculation under 5 mM NO_3^- supply (n=7). Comparison of palisade cell number per leaf blade (A), and calculated cell area (B) of the first trifoliolate leaves.

tended to be small, but the expanding new leaves were larger than those of the wild type (Ito *et al.* 2007). Microscopic study showed the cell number per leaf of NOD1-3 and NOD3-7 was lower than the wild type (Ito *et al.* 2008). It was thought that small size of leaves due to small number of leaf cells is the non-symbiotic phenotype of NOD1-3 and NOD3-7. In *L. japonicus*, it was indicated that leaf growth of hypernodulation mutant *har1-4* was similar with the wild type (Fig. 2). Cell number of leaf of *har1-4* tended to be lower than that of wild type, while cell area of *har1-4* tended to be larger than that of wild type (Fig. 3). Increased cell area of *har1-4* might be caused by "compensation effect" of decreased cell number (Tsukaya 2008), so leaf area of *har1-4* was similar to that of wild type. These results indicate that a part of autoregulation system might be related to the control system of leaf-cell proliferation also in *L. japonicus*, although the mechanism could not be clarified in this study. In soybean, it was shown that a part of the autoregulation of nodulation might have the common pathway with the control system of cell proliferation in leaves (Ito *et al.* 2008). Also in *L. japonicus*, a part of the autoregulation of nodulation might be related to cell proliferation in leaves. The system of autoregulation of nodulation in legume should be a part of general plant mechanisms rather than specific to nodulation.

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ミヤコグサ根粒超着生変異株 *har1-4* の葉の生育

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要　約

ダイズの根粒超着生変異株は親株と比較し、葉面積が小さく、葉の細胞数が少ない。そのため、ダイズの根粒形成自己制御機構は葉の細胞増殖と関連することが示唆されている。本報告では、マメ科モデル植物であるミヤコグサの根粒超着生変異株 *har1-4* の葉の生育を親株と比較した。*har1-4* の葉面積は、親株と同程度であった。*har1-4* の葉の細胞数は親株より少ない傾向にあり、*har1-4* の細胞面積は、親株よりも大きい傾向にあった。以上の結果から、ミヤコグサにおいても根粒形成自己制御機構は葉の細胞増殖と関連する可能性が示唆された。マメ科植物の根粒形成自己制御機構は、根粒形成に特異的な機構というよりは、植物の一般的なメカニズムの一部を利用した機構であるのかもしれない。

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