Chapter 4

Autoregulation of Nodulation in Soybean Plants

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1. Introduction

Soybean (*Glycine max* [L.] Merr.) seeds contain a high concentration of protein and oil. Therefore, soybean is an important source of protein and calories for humans and livestock in the world. Although to cultivate soybean is in need of a large amount of nitrogen, soybean plants can form root nodules which are symbiotic organs with soil bacteria bradyrhizobia. The partner bradyrhizobia fix atmospheric nitrogen in nodules, and then the plants can use the fixed nitrogen. So soybean plants can grow well even in the absence of soil nitrogen. On the other hand, the plants give photosynthate to the bradyrhizobia. Substantial amounts of photosynthate are required for nitrogen fixation activity in matured nodules. The formation of excess nodules might be a disadvantage because of decreasing the carbon supply each nodule. Therefore, the number of nodule is strictly regulated by the host soybean plant. This system is referred to as the autoregulation of nodulation. The nodule growth of later infection site is suppressed by the rapid response to the earlier rhizobial infection and subsequent nodule initiation. In this chapter, we discuss the autoregulation of nodulation in soybean plants.

2. Research of autoregulation of nodulation in soybean plants

Using a split root technique, it was shown that nodule formation of the latter infected root was systemically suppressed by a prior infected root [1]. Host plants systemically suppress excess nodulation through communications between shoots and roots, by using unknown signals [2]. It is postulated that when soybean root is infected with bradyrhizobia, infection signal is synthesized in root and is transported toward shoot. And then shoot-derived autoregulation signal is synthesized in shoot and is transported toward root. Differentiation of nodule at later infected root is suppressed by the shoot-derived autoregulation signal.

Several hypernodulation mutant lines of soybean were isolated using chemical mutagens such as ethyl-methane-sulfonate (EMS) or N-nitroso-methyl-urea (NMU) since the 1980s; nts lines (from cv. Bragg) [3], NOD lines (from cv. Williams) [4] and En6500 line (from cv. Enrei) [5] were obtained. The mutant lines can form profuse nodulation in the presence or absence of nitrate compared with their parents. These mutants are thought to lack a part of the autoregulation of nodulation. Reciprocal grafting experiments between the hypernodulation mutant and the wild type soybean showed that the hypernodulation phenotype depends on the shoot [6], especially mature leaves [7, 8]. Hypernodulation mutant supplied a large amount of photosynthate to the nodule than the roots [9]. The specific nitrogen-fixation activity and the concentration of leghemoglobin in hypernoduation mutant lines are lower than in the parent line [10, 11, 12]. Therefore, it is reasonable that the number of nodules should be regulated to be optimum by the host plant.

Autoregulatory response is induced during after nodule meristem formation but before nodule emergence [13, 14], perhaps there are some suppressing points [2]. The control has been proposed to operate 2-4 days after inoculation in soybean [15, 16]. About 7-8 days after inoculation, nodules initially appear on roots, only which has not suppressed their growth by shoot-derived autoregulation signal. These time course is a point in the study of autoregulation of nodulation.

3. Photosynthate allocation and regulation of nodulation

One of the idea, plants might suppress the translocation of photosynthate to underground part to control the excessive nodulation. It is shown that availability of photosynthate might be involved in the control of nodule number, with approach grafting [17], and light enhancement and CO_2 enrichment study [18]. From the split root experiment, the inoculation treatment of bradyrhizobia appeared to stimulate the allocation of photosynthate to the inoculated root [19, 20]. However, photosynthate requirement during nodule initiation was not known. In general, growth point has a strong localized sink activity of photosynthate, which is the energy source as well as structural resources. So we investigated whether a requirement of carbon source is increased or not in early stages of nodule formation. The early stage of nodule formation (a period of 2-8 days after inoculation in this study) is important, because autoregulation of nodulation is already activated in this period.

Current photosynthate allocation of soybean cv. Williams was conducted in relation to the nodule initiation [21]. Whole shoots were exposed to $^{14}CO_2$ for 120 min, and the distribution of radioactivity in each organ was determined. During the early stage of nodule formation (4, 6, 8 days after inoculation) the ^{14}C distribution to the inoculated roots did not increase in comparison with uninoculated control roots. ^{14}C respired by underground parts was also similar between the inoculated and control roots. Visualized Images of the distribution of ^{14}C indicated that the shoot apex and root apex, which were growing point in above and underground parts, showed a high radioactivity, but the intense signals were not seen in the expected nodulating parts of roots. These results indicate that current photosynthate allocat-

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ed to the inoculated roots did not increase in comparison with uninoculated roots, in the early stage of nodule formation.

After the emergence of nodules (10 and 12 days after inoculation), the inoculated roots gradually had priority of photosynthate allocation compared with the uninoculated control roots. At 12 days after inoculation, ^{14}C distribution of inoculated root was statistically increased in comparison with the uninoculated control roots. Also, consumption of current photosynthate by the respiration of underground parts increased at day 12 after inoculation. The radioactivity per dry weight was higher in the nodules than that of the growing point of shoots (i.e. shoot apex). Thus, the underground part after emergence of the nodule is already a high consumer of energy (Figure 1).

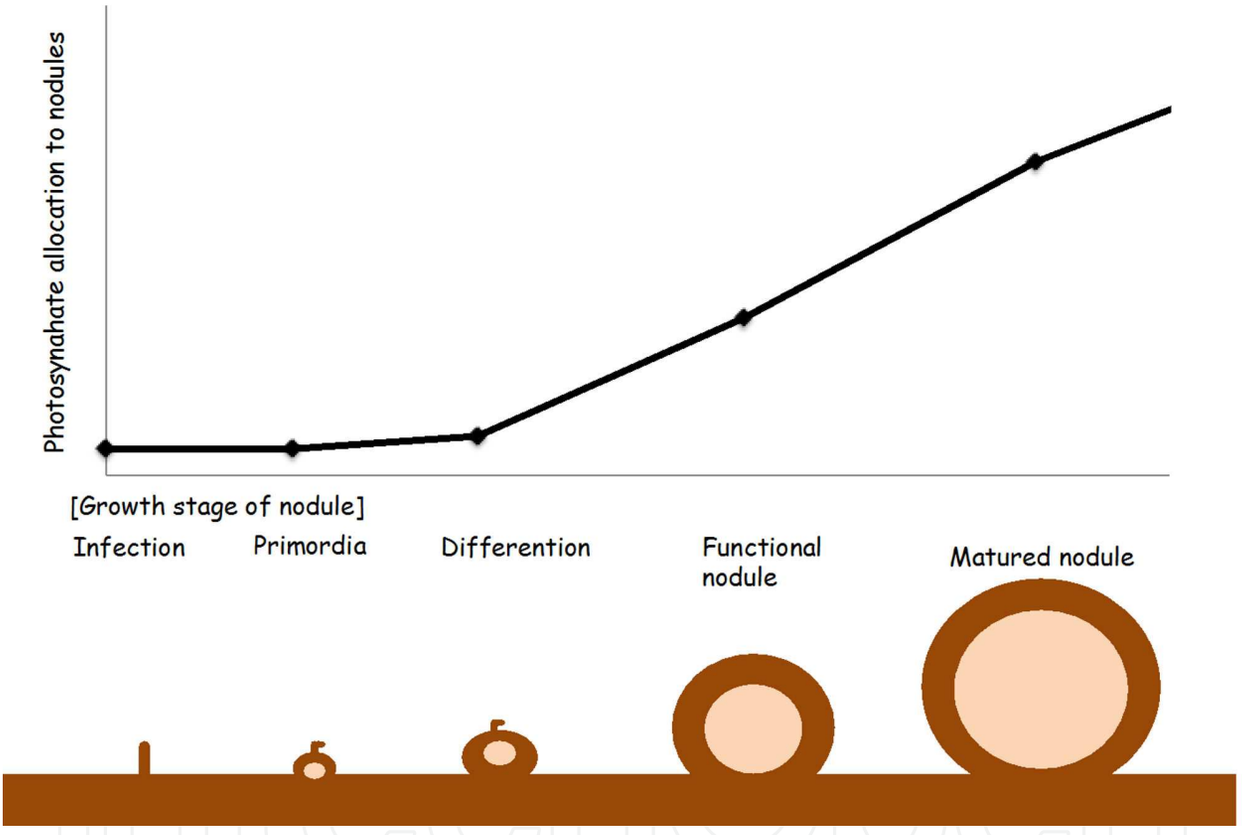


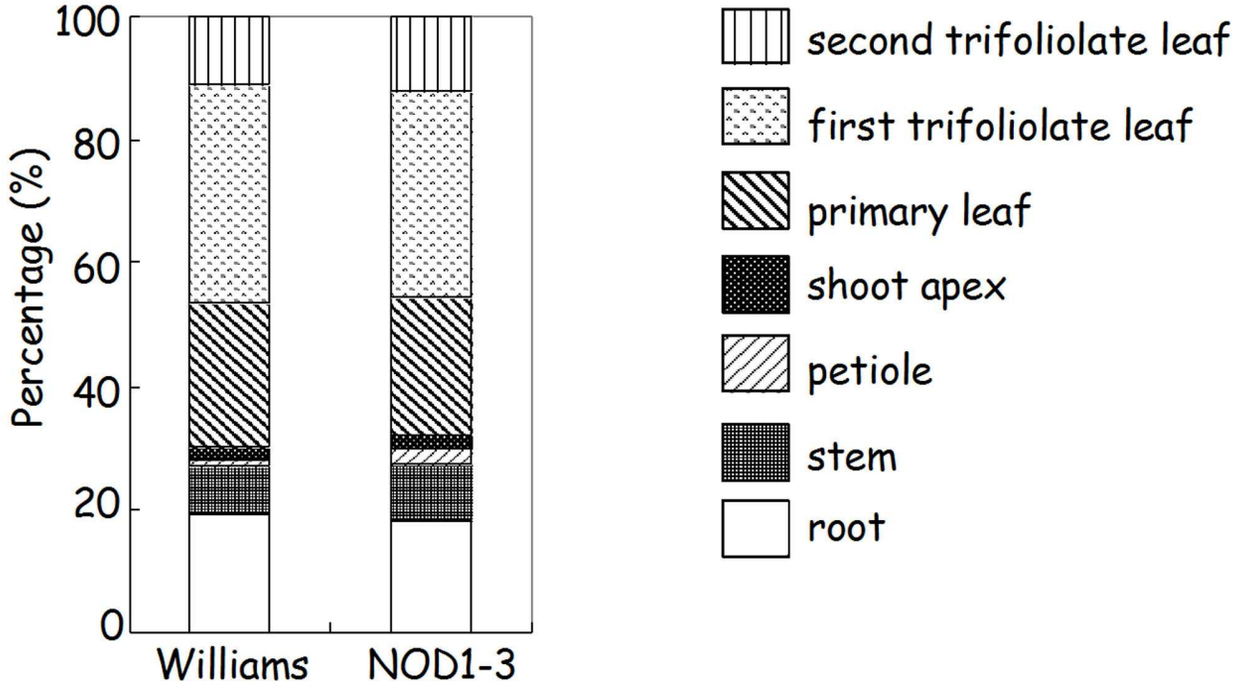
Figure 1. A model for the requirement of photosynthate of nodules during initial stages of nodule formation.

It was shown that an appreciable amount of photoassimilate is not required for nodule initiation before emergence in Williams. What about in hypernodulation mutant lines? The availability of photosynthate might be involved in autoregulation of nodulation. For example, if photosynthate allocation to nodulation in the early stages of nodule formation is markedly increased in NOD1-3, autoregulation of nodulation might be involved in the availability of photosynthate.

To demonstrate this idea, a current photosynthate allocation of hypernodulation mutant of soybean NOD1-3 (isolated from Williams) was examined at 8 days after inoculation using

$^{14}CO_2$ as shown above [22]. The results showed that the ^{14}C distribution in the roots on 8 days after inoculation did not increase when compared with uninoculated control plants in NOD1-3. In visualized images of radioactivity by an imaging plate, the nodules were observed as the strong signal spots in the underground organ. It was concluded that appreciable amount of photoassimilate is not required for the nodule initiation in NOD1-3 in 8 days after inoculation. It was also indicated that the growth of nodule in NOD1-3 is slightly early, compared with Williams; because nodule at early stage had already high sink activity in NOD1-3.

These results indicate that an appreciable amount of photoassimilate is not required for nodule initiation, irrespective of wild type and hypernodulation mutant. It is considered that photosynthate allocation to the nodulated root is not related to autoregulation of nodulation. However, it is unclear whether photosynthate allocation to root in the uninoculated NOD1-3 is similar with the uninoculated Williams or not. If photosynthate allocation to root in uninoculated NOD1-3 is higher than that in uninoculated Williams, cause of hypernodulating trait in NOD1-3 might be attributed to the photosyanthate allocation. So we compared ^{14}C partitioning in the uninoculated plants between Williams and NOD1-3 (unpublished data). Results show that there was no difference between NOD1-3 and Williams in ^{14}C distribution per plant parts (Figure 2). It is strongly suggested that control of nodule formation may be independent of allocation of photosynthetic product.



They were cultured without inoculation of bradyrhizobium under –N condition. At 18 days after sowing, whole shoots were exposed to $^{14}CO_2$ for 120min, and the distribution of radioactivity in each organ was determined.

Figure 2. Percentage distribution of radioactivity of ^{14}C in whole plants in uninoculated Williams and NOD1-3.

4. Role of autoregulation system

While it is known that substantial amounts of photosynthate are required for the nitrogen-fixing activity in mature nodules, real-time analysis of photosynthate allocation in hypernodulation has not been reported. Time course study was performed with the aim to clarify the real-time allocation of photosynthetic products in relation to excess nodulation in soybean plants [23]. Allocation of photosynthates to underground part in soybean plants was analyzed using $^{11}CO_2$ and positron-emitting tracer imaging system (PETIS). PETIS can monitor 2D-distribution of positron-emitting tracer like ^{11}C, and a PETIS imaging provides an animation movies of radioactivity non-invasively. Soybean plants were inoculated with *B. japonicum* when they were sown and were grown hydroponically. Whole shoots of the plants at 35 days after sowing were exposed to $^{11}CO_2$, and the ^{11}C imaging was performed for 180 min. In this study, the distribution of ^{11}C-photosynthates in the nodule of hypernodulation NOD1-3 was characterized by comparison to wild type Williams. Results showed that both in the NOD1-3 and Williams, ^{11}C-photosynthates were transported to the root base within about 20 min after feeding of $^{11}CO_2$ and to the root tips within one hour. Most of ^{11}C-photosynthates in the underground part were localized to the root base where many nodules are formed. It was shown that a larger amount of ^{11}C-photosynthates was transported into the nodules on the root base than into those on distal root regions, both per nodule and per volume of nodule (Figure 3). This suggested that the basal nodules may have higher activity for nitrogen fixation both in the mutant and wild type, and such position of a nodule may be a dominant determining factor for the activity. Surprisingly, there was no difference between the mutant and wild type in the amount of ^{11}C-photosynthates accumulated into the nodules per volume of nodule, both in basal and distal regions (Figure 3). These results suggested that the reduced activity of nitrogen fixation in the mutant might be generally caused by the increased proportion of the distal nodules which are poorly fed with photosynthates. So regulation of nodulation on distal region of root is thought important for efficient activity of nitrogen fixation. It might be that shoot-derived autoregulation signal regulate nodulation of distal region of root (Figure 4).

Generally, hypernodulation mutant lines tend to show an inferior growth and seed yield compared with the parent. The peculiar supernodulating variety of soybean "Sakukei 4" showed the improved growth [24], so it was expected high yield. However, Sakukei 4 could not produce higher seed yield compared with the parental varieties Enrei or Tamahomare, because N use efficiency of Sakukei4 was low [25]. One of the reason of low N efficiency might be nodule formation on distal region of root in Sakukei 4.

5. The growth of hypernodulation mutant

Hypernodulation mutants form profuse nodules compared with their wild type parents. Physiological characterization of hypernodulation mutants showed some features of plant growth in addition to the hypernodulation trait. Especially, less vigorous plant growth had

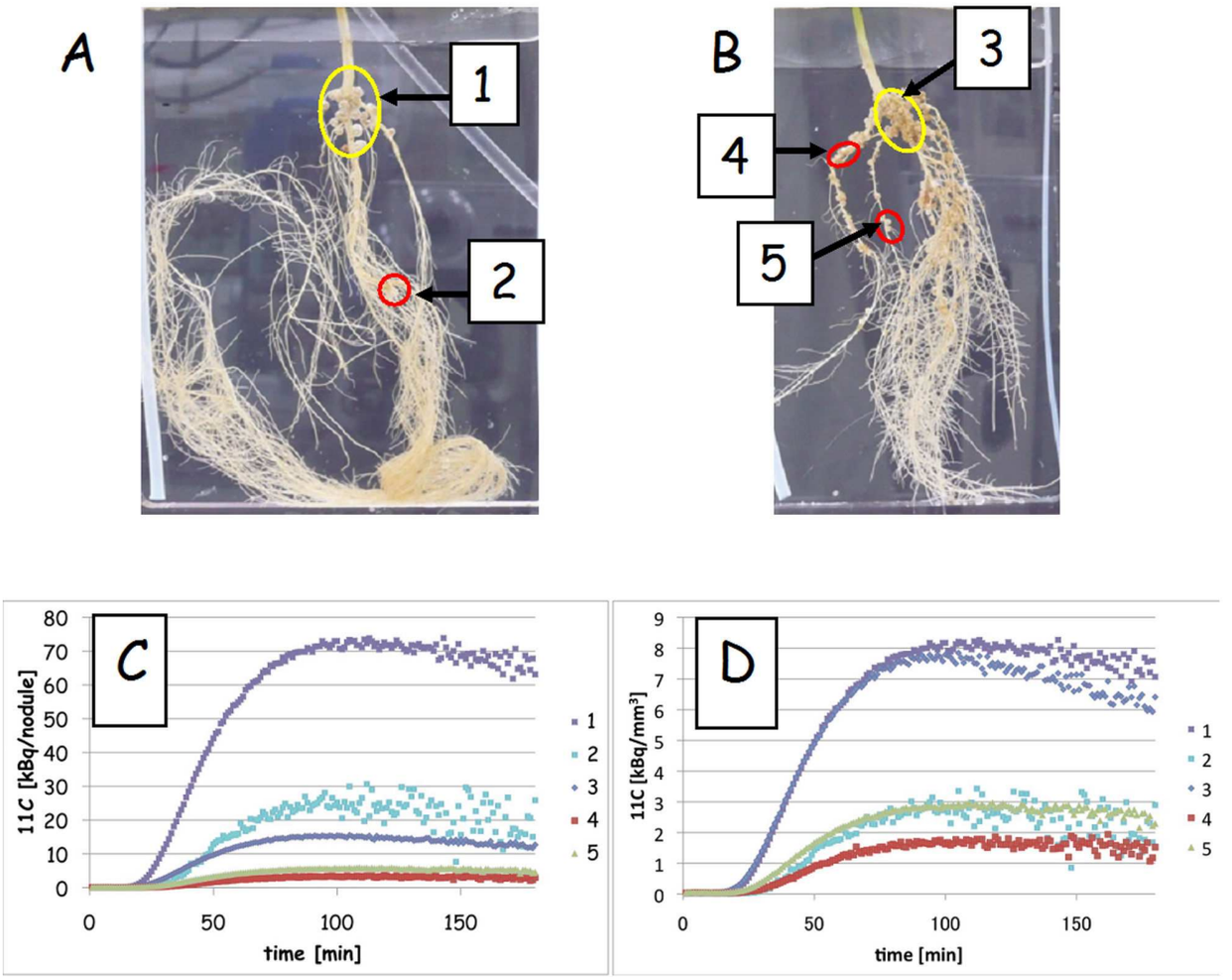


Figure 3. Accumulation of ^{11}C-photosynthates into nodules. (from reference [23]) (A, B) Photographs of underground part in wild-type (A) and NOD1-3 (B). Yellow ellipses indicate regions of interest (ROIs) for the basal nodules and red ellipses indicate ROIs for distal nodules. (C) Time activity curves (TACs) per nodule. (D) TACs per volume of nodules.

been reported [3, 4, 5]. In general, it is because of strong requirement for carbon source of nodules. But it was unclear whether inferiority of growth occurs as a secondary effect of hypernodulation trait or directly due to gene defects by mutation. The details of the growth characteristics of hypernodulation mutant lines may be important to understand the systemic features of the autoregulation mechanism.

GmNARK, which plays an important role in autoregulation of nodulation, was identified in soybean [26, 27]. The soybean hypernodulation NOD mutant lines were isolated from the Williams parent, NOD1-3 and NOD3-7 by N-nitroso-N-methylurea and NOD2-4 by ethyl methanesulfonate [4]. Allelism analysis showed that hypernodulation of all NOD mutant lines from Williams and the En6500 mutant from Enrei is controlled by a single recessive allele [28, 29]. The mutation site of En6500 and NOD3-7 has been identified in *GmNARK* [26, 30]. Therefore, the mutation site of all NOD mutant lines is thought to be in *GmNARK*. There are some different phenotypes between these NOD mutant lines [4, 31], although the reason is unclear so far. Our previous study showed that NOD1-3 had larger nodule compared

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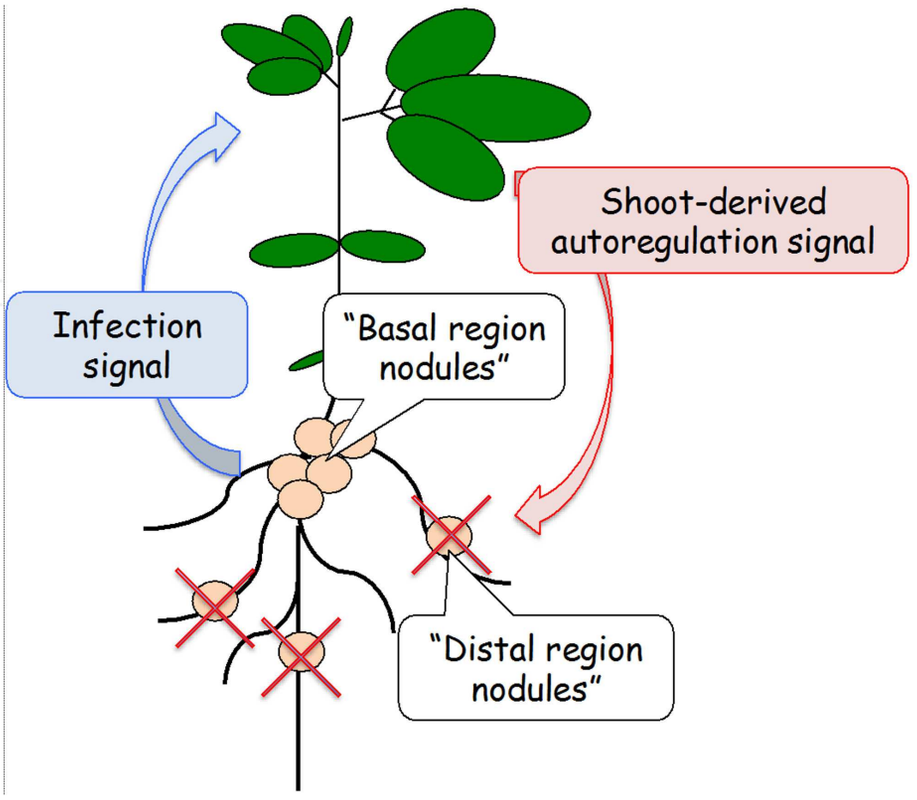


Figure 4. A model for autoregulation of nodulation in soybean plants. Nodule formation of distal region might be regulated by shoot-derived autoregulation signal.

with Williams in 8 days after inoculation [21, 22]. And images analyzed using imaging plate shows that nodules can be observed as partially strong signal in the underground organ at 8 days after inoculation, whereas strong signal of nodule in Williams was narrowly observed at 10 days after inoculation. This observation was consistent with in the literature [32], they reported that initial nodule growth of NOD1-3 was earlier than that of Williams. Also another mutant line, reference [33] suggested that supernodulating mutant nts382 growth in early stages was faster than its wild-type cv.Bragg, especially with early lateral root formation. These observations may indicate that the start of new underground organogenesis of mutant is partially faster than its parents. However, the shoot growth of the hypernodulation mutant had not been well investigated.

The objective of our study was to investigate the characteristics of the initial growth of the NOD mutant lines with the dry matter weight of each part and a whole plant. The experiment was designed within the period when the plants could grow without nitrogen supply. We investigated the phenotypes of not only inoculated plants but also uninoculated plants, and clarified whether phenotypes appeared by a secondary effect of a hypernodulation trait or not; secondary effect means the phenomenon which is caused by excess number of nodules, while a hypernodulation trait is primary effect of mutation gene. The point of this study is that soybean seeds of hypernodulation mutant lines, NOD1-3, NOD2-4, and NOD3-7, and of the Williams parent were carefully selected by uniform seed weight. If

seeds were selected randomly, seeds of NOD1-3 and NOD3-7 tend to be smaller than that of Williams and NOD2-4. Difference of seed weight affects initial growth of plants.

First, characteristics of the initial growth of hypernodulation NOD mutant lines were compared with that of Williams with or without inoculation of *B. japonicum* at 7 or 8 days after sowing [34]. When plants were grown without inoculation of bradyrhizobia, the total dry weight of each hypernodulation mutant seedling was not significantly different from that of Williams. Also in inoculated condition, the plant dry weight was not different between hypernodulation mutants and Williams at this stage. The decrease in the growth of hypernodulation mutant is thought to become evident after this growth stage. Some characteristics of each mutant line were observed in this study. Nodule number of NOD1-3 was the largest in all lines in inoculated condition. On the other hand, nodule number of NOD3-7 was similar with that of Williams in this growth stage. It might be that hypernodulation trait of NOD3-7 is not appeared when they are seedling at 8 days after sowing. It is also characterized that stem length of NOD3-7 seedling was shorter than other lines, so the shoot growth of NOD3-7 might be different from other lines. Seedling growth of NOD2-4 was very similar with that of Williams, except for nodule number.

Next, characteristics of the initial growth of NOD mutant lines were compared with Williams at 17 or 18 days after sowing [35]. The plants were grown with or without seed-inoculation of bradyrhizobia, and in the absence or presence of nitrate in the culture solution. When the plants were grown without inoculation, the total dry weight of all mutant lines was not different statistically from Williams, both in the absence and presence of nitrate. When they were grown with inoculation of bradyrhizobia, however, the total dry weight of each mutant line was significantly lower than that of Williams, both in the absence and presence of nitrate. These results 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. When the plants were grown with inoculation, the nodule number was decreased by the presence of nitrate in Williams, NOD1-3 and NOD2-4, but not in NOD3-7. NOD3-7 may be the most tolerant to nitrate inhibition of nodulation among NOD mutant lines. Growth of each leaf of NOD3-7 and NOD1-3 was different from the wild type and NOD2-4; the expanded leaf was smaller but the new leaf was larger compared with Williams under all conditions (Figure 5). This indicates that NOD3-7 and NOD1-3 might decrease the ability for leaf expansion and have a faster leaf emergence rate. In order to compare the growth rate of the leaves, study was conducted to measure leaf length of the plants daily between 6 and 18 days after sowing (unpublished data). Plants were grown in bradyrhizobium-free nutrient solution without N supply. Figure 6 shows the increase in the length of each leaf blades with time, of Williams and NOD mutant lines. In Williams, an expansion of new leaf started after growth of the previous leaf had been completed. The leaf growth pattern of NOD2-4 was similar with the Williams. In NOD3-7, expansion of new leaf was started earlier before the previous leaf finished expansion. The graph of NOD1-3 shows intermediate pattern between Williams and NOD3-7. The emergence rate of new leaf of NOD3-7 was fastest in all lines. In addition, leaf shape of NOD1-3 and of NOD3-7 was narrower compared with wild type; the

leaf index (the ratio of the leaf length to the leaf width) of NOD1-3 and of NOD3-7 was increased than the wild type. It was indicated that NOD3-7 and NOD1-3 had a rapid emergence rate of leaves, while the final size of their expanded leaves was smaller than that of Williams and NOD2-4.

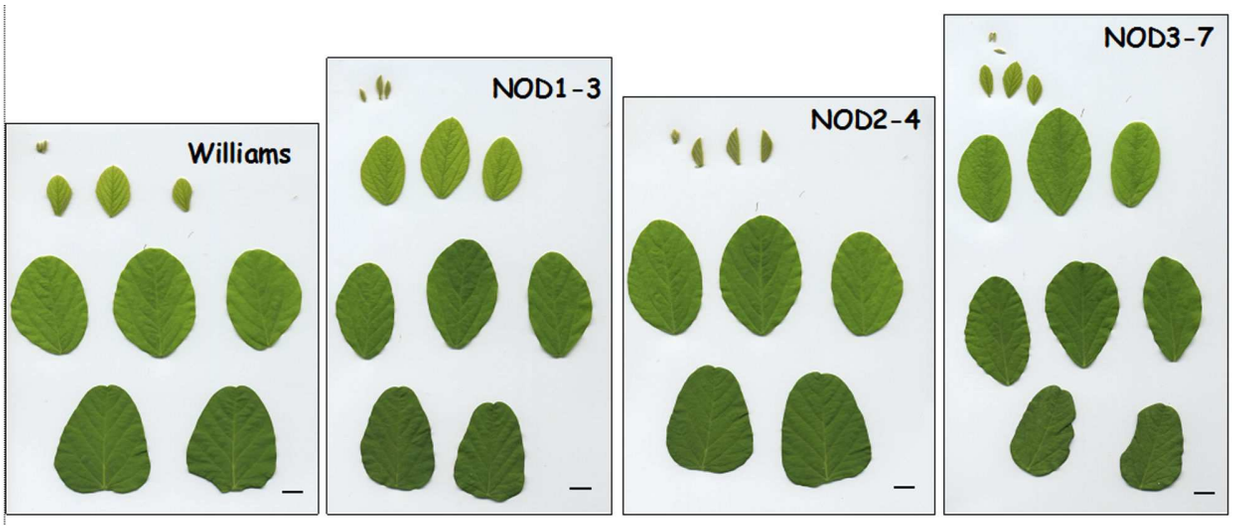


Figure 5. Photos of leaves of hypernodulation mutant lines, NOD1-3, NOD2-4 and NOD3-7, and their parent Williams. Plants were grown without inoculation of bradyrhizobium and under -N condition. In order of primary leaf, 1st trifoliolate leaf, 2nd trifoliolate leaf, and 3rd trifoliolate leaf are indicated from the bottom in each line. The bar indicates 1 cm.

Figure 7 shows summary of initial growth of NOD mutant lines, compared with Williams. When they were inoculated, all NOD mutant lines form great number of nodules than does Williams. But the individual nodule size of NOD mutant line was smaller than that of Williams. In the leaf phenotype, individual leaf size of NOD1-3 and NOD3-7 was smaller than that of Williams. But they had faster rate of new-leaf emergence and their leaf number per plant had tendency to increase. In NOD1-3 and NOD3-7, leaf phenotype was similar with nodule phenotype. On the other hand, leaf phenotype of NOD2-4 was similar with Williams.

6. Leaf phenotype of hypernodulation mutant

We investigated the difference of fully expanded leaf size in cell level using light microscopy when the plants were not inoculated without N supply [36]. Results showed that the cell number of NOD1-3 and NOD3-7 was significantly lower than that of Williams. Cell area of all lines was similar and there were not significant difference between each line. The cell area of NOD3-7 was a little higher than that of other lines, it maybe because of compensation effect of decreased cell number. Compensation effect is characterized by cell enragement triggered by the decrease in cellular proliferation of leaf [37]. It was indicated that NOD1-3 and NOD3-7 produced small-size leaves due to the smaller number of leaf cells,

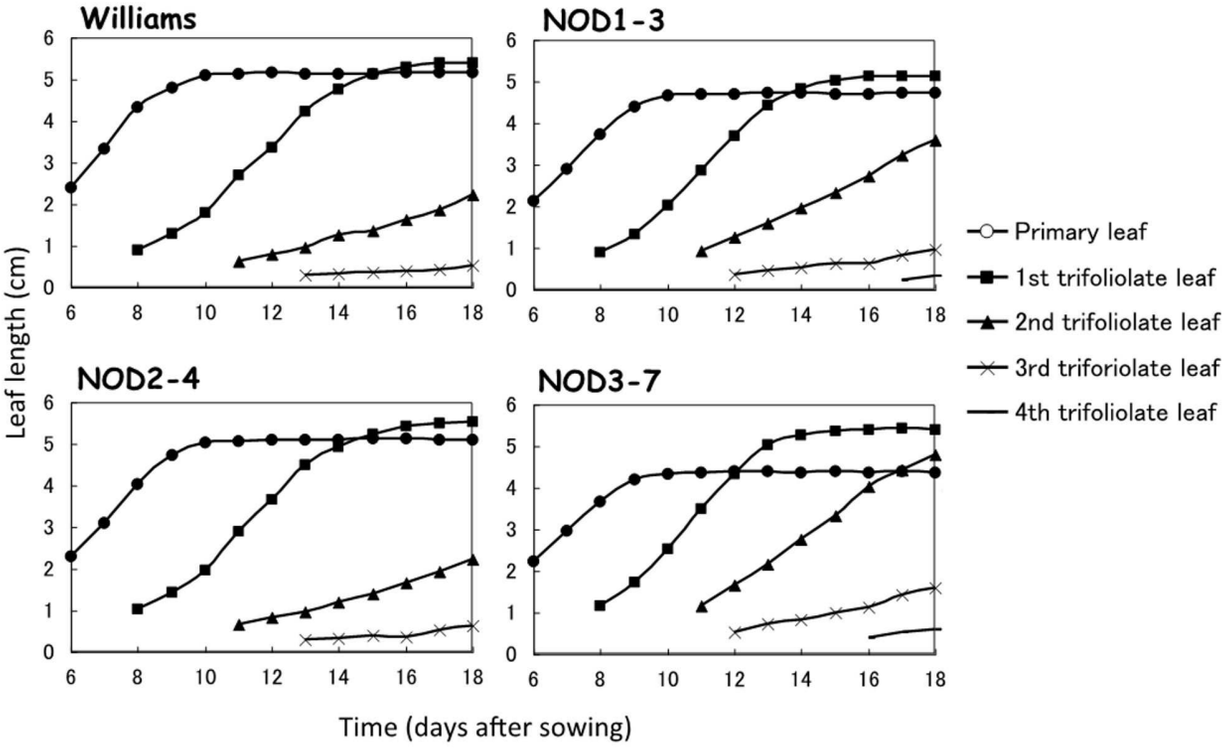


Figure 6. The increase in the length of each leaf blades with time of Williams, NOD1-3, NOD2-4 and NOD3-7. Plants were grown without inoculation of bradyrhizobium and under -N condition. Length of each leaf blade was measured daily between 6 and 18 days after sowing.

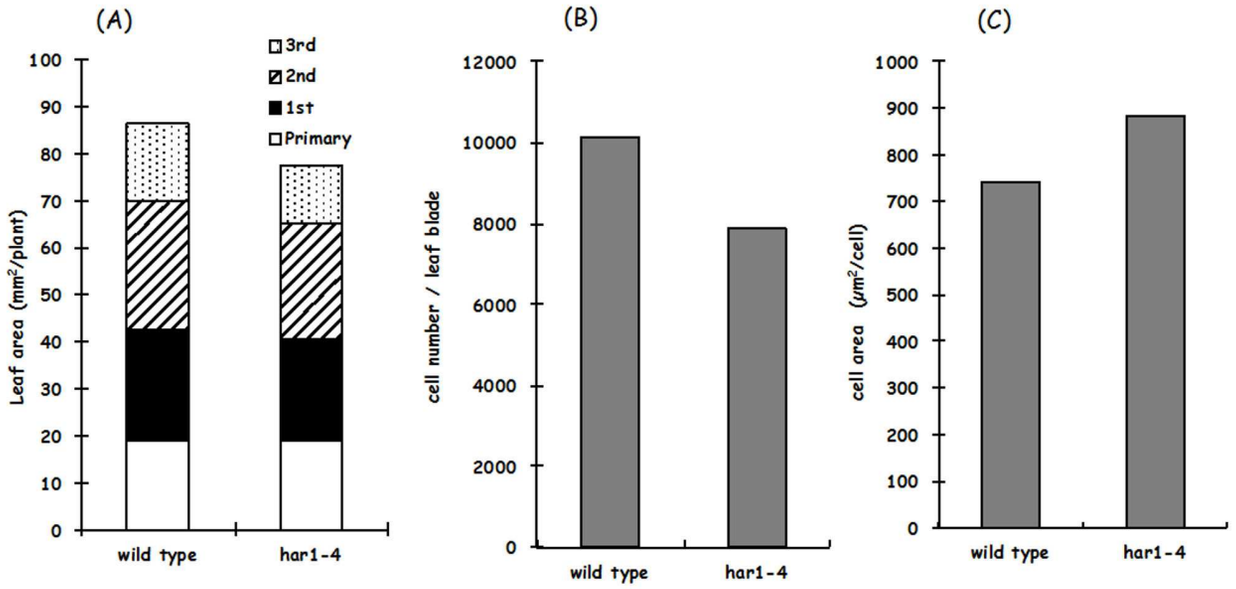
	Nodule		Leaf	
	Size	No. / plant	Size	No. / plant
NOD1-3	↘	↗	↘	↗
NOD2-4	↘	↗	similar	similar
NOD3-7	↘	↗	↘	↗

Figure 7. Summary of initial growth of NOD mutant lines, compared with Williams.

compared to Williams parent. These phenotypes were not affected by the inoculation with bradyrhizobia or nitrate supply.

Also in *Lotus japonicus,* leaf growth of hypernodulation mutant har1-4 was compared with their wild type Gifu [38]. Plants were grown in rhizobium-free nutrient solution with 5 mM nitrate supply. Sampling was done at day 14 after sowing. The total dry weight of har1-4 was similar to wild type. Leaf area of primary and 1st and 2nd trifoliolate leaf on har1-4 was

similar with that of wild type (Figure 8A). 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 was tended to be lower than that of wild type, but it was not significantly difference (Figure 8B). On the other hand, cell area of har1-4 tended to be larger than that of wild type (Figure 8C). Increased cell area of har1-4 might be caused by "compensation effect" of decreased cell number, so leaf area of har1-4 was similar that of wild type. A part of autoregulation system might be related to the control system of leaf-cell proliferation also in *Lotus japonicus*, although could not be clarified in this study.



Plants were grown without inoculation under 5 mM NO_3^- supply. Comparison of leaf area (A), palisade cell number per leaf (B), and calculated cell area (C) of the first trifoliolate leaves.

Figure 8. Leaf phenotypes of hypernodulation mutant har1-4 compared with wild type (Gifu) in *Lotus japonicus*. (from reference [38])

To investigate the relationship between nodule and leaf phenotypes, the leaf growth of seed-inoculation plants (active-autoregulation) was compared with the uninoculated plants (basal-autoregulation) in Williams [36]. Results showed that the leaf area of inoculated plants was significantly larger than that of uninoculated plants. The cell number of primary leaves of active-autoregulation plants was significantly higher than that of basal-autoregulation plants. So we concluded that the recognition of infection signal in the shoot might stimulate the cell proliferation of leaf blade. The autoregulation of nodulation system might be active at the basal level when plants are grown without inoculation. Symbiotic nitrogen fixation in root nodules requires a large amount of photosynthates. To activate cell proliferation of leaves along with the autoregulation mechanism by infection of bradyrhizobia would be reasonable.

7. Conclusion

In order to prevent a decrease in growth due to excess nodulation, especially of distal region of root, the control mechanism plays an important role. In the autoregulation of nodulation, yet-unknown signal molecules are used in the communication between shoot and root. Our study showed that control of nodule formation may be independent of allocation of photosynthetic product [21, 22]. Recently, it was indicated that the shoot-derived autoregulation signal is a downregulator of nodulation and is produced by the wild type, rather than activator produced by the mutants [39]. They also indicated that both bradyrhizobia inoculation and visible nodule formation are not essential for shoot-derived autoregulation signal synthesis [39]. Our study showed that the autoregulation of nodulation might be related to the control system of leaf-cell proliferation. Universal regulation system of plant growth might closely link to the autoregulation of nodulation. So it might be reasonable that plant hormones regulate nodule development [40]. For example, salicylic acid [41], abscisic acid [42], methyl jasmonic acid [43] and polyamine [44] are proposed to be involved in autoregulation of nodulation in leguminous plants. The autoregulation of nodulation might be regulated through interplay of several signaling compounds. The non-symbiotic phenotype of the mutants might be helpful to isolate signal molecules of autoregulation of nodulation.

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