Allocation of Photosynthetic Products in Hypernodulation Mutant of Soybean NOD1-3 in the Early Stage of Nodule Formation

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Summary

We reported previously that photosynthate allocation for nodule formation did not increase during early stage of nodule formation in soybean (*Glycine max* [L.] Merr.) cv. Williams (Ito *et al*., 2006). In this study, current photosynthate allocation of hypernodulation mutant of soybean NOD1-3 isolated from Williams was examined in early stage of nodule formation. Whole shoots were exposed to $^{14}CO_2$ for 120 min and the distribution of radioactivity in each organ was determined. The ^{14}C distribution in the roots of 8 days after inoculation did not increase when compared with uninoculated control plants. In visualized images of radioactivity by imaging plate, nodules were observed as strong signal spots in the underground organ. These results indicate that current photosynthate allocation to the inoculated root did not increase markedly during the early stages of nodule formation, while small nodules had already strong sink activity than root in 8 days after inoculation different from Williams. To investigate the carbon status of plants at 8 days after inoculation, the starch and sugar concentration in the plants were analyzed. It was shown that the accumulation of starch and sugar was similar in both inoculated and uninoculated plants. We concluded that appreciable amount of photoassimilate is not required for nodule initiation in NOD1-3 in 8 days after inoculation.

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Key words : ^{14}C distribution, nodule formation, hypernodulation mutant, soybean

Leguminous plants can fix and assimilate atmospheric nitrogen by root nodules as symbiotic organs with soil bacterium rhizobia. In soybean (*Glycine max* [L.] Merr.), it is well known that nodulation is systemically suppressed by the rapid response to the initial rhizobial infection. Nodule number is essentially controlled by host plant because substantial amounts of photoassimilates are required for nodule growth and the maintenance of nitrogen fixation including nitrogen assimilation and transport. The negative regulating mechanism is referred to as "autoregulation of nodulation", which is controlled by shoot through the exchange of unknown signal molecule(s) between shoot and root (Olsson *et al*., 1989; Caetano-Anolles and Gressoff, 1991; Francisco and Harper, 1995; Sato *et al*., 2002). About 7-8 days after inoculation, nodules initially appear on the roots. The early stage of nodule formation is important to determine the development of infection sites, because the infection sites are destined by their fate to develop into nodules or remain in the stage just after infection followed by cell division.

Since the 1980s, hypernodulation mutants of soybean have been isolated from several soybean cultivars following chemical mutagenesis (Carroll *et al*., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992). The fact that hypernodulation phenotype is decided by shoot genotype of hypernodulation mutant, not root genotype; hypernodulation mutants lack or decline a part of the communication between shoot and root of autoregulation of nodule formation. Plant growth of hypernodulation mutant is less vigorous than wild type (Carroll *et al*., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992); however, it is unclear that inferior growth is appeared by gene mutation or secondary effect of hypernodulation trait.

It is well known that substantial amounts of photosynthate are required for nitrogen fixing activity in mature nodule (Fujikake *et al*., 2003). In the cv. Williams, we reported that photosynthate allocation for nodule formation was not markedly increased during the early stage; the inoculated roots gradually had priority of photosynthate allocation after the emergence of nodules at day 10 after inoculation (Ito *et al*., 2006). The objective of this study is to clarify how is the photosynthate allocation in hypernodulation mutant. So we investigated current photosynthate allocation for nodule formation during the early stage in hypernodulation mutant NOD1-3, which is isolated from cv. Williams (Gremaud and Harper, 1989).

MATERIALS AND METHODS

Plant Culture

Soybean (*Glycine max* [L.] Merr.) hypernodulation mutant NOD1-3, which was mutagenized from Williams, seeds were sterilized and were thoroughly washed with water. They were sown on vermiculite bed sterilized by autoclaving (121℃,

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Table 1. Dry weight of each part (top) and starch and sugar concentration of underground, primary leaf and first trifoliolate leaf (bottom) of soybean hypernodulation mutant NOD1-3 at day 8 after inoculation.

	underground part	stem	petiole	primary leaf	first trifoliolate leaf	second trifoliolate leaf	shoot apex	total
				Dry Weight (mg/plant)				
control	194 (11)	89 (10)	17 (2)	94 (12)	117 (6)	41 (4)	9 (1)	561 (20)
inoculation	209 (18)	86 (12)	15 (1)	99 (11)	108 (7)*	33 (10)	7 (2)	557 (38)

	underground part	primary leaf	first trifoliolate leaf
	Starch (mg/gD.W.)		
control	44.2 (4.5)	297 (21)	184 (31)
inoculation	54.8 (13.0)	305 (29)	197 (17)
	Sugar (mg/gD.W.)		
control	33.1 (1.7)	26.1 (3.2)	29.7 (2.5)
inoculation	34.8 (1.8)	25.8 (2.3)	27.5 (1.5)

The number within parentheses indicates standard deviation (n=6). *Significantly different at 5% level compared with control.

15min), and grown in a growth chamber (Biophotochamber LX-3000, TAITEC, Japan) under the following conditions: 16h photoperiod at 28 ℃ and 8h dark at 18 ℃ . At day 7 after sowing, they were transferred aseptically to an glass bottle with 800mL rhizobium-free nutrient solution (K_2SO_4 109, K_2HPO_4 8.5, KCl 0.935, $CaCl_2 \cdot 2H_2O$ 183.0, $MgSO_4 \cdot 7H_2O$ 123, H_3BO_4 0.367, $CuSO_4 \cdot 5H_2O$ 0.032, $MnSO_4$ 0.189, $ZnSO_4 \cdot 7H_2O$ 0.144, $(NH_4)_6Mo_7O_{24}$ 0.004, $CoSO_4$ 0.028, $NiSO_4 \cdot 6H_2O$ 0.0035, EDTA · 2Na 18.6, $FeSO_4 \cdot 7H_2O$ 13.9 mg L^{-1}, pH 6.0). The culture solution was continuously aerated by an air pump, and changed three times a week. At day 10 after sowing, the roots were inoculated with *Bradyrhizobium japonicum* strain USDA 110 (10^9 cells per plant). The control plants were not inoculated. All the plants were harvested at 18 days after sowing.

Analysis of starch and sugar

In this study, the seeds were carefully selected by their weight for uniformity of seed reserves. The plants were grown under the conditions described above, with inoculation or uninoculation (control). The plants at 18 days after sowing were harvested. They were separated into each part and frozen in liquid N_2, and subsequently freeze-dried. After the dry weight was measured, the underground parts (roots plus nodules), primary leaves, and first trifoliolate leaves were ground into a fine powder. Sample powder was extracted with 80%(v/v) ethanol. Sugar in 80% ethanol-soluble fraction was determined by the phenol-H_2SO_4 method. The 80% ethanol-insoluble fraction was extracted with 4.6M $HClO_4$, and the starch in 4.6M $HClO_4$-soluble fraction was determined by the Anthrone method.

Analysis of ^{14}C distribution

Whole shoots of plants at 18 days after sowing were enclosed in a transparent plastic chamber, and it was connected to a closed gas circuit system with an air pump (**Fig. 1**). The $^{14}CO_2$ was generated by the injection of 2 mL of 60% $HClO_4$ into a test tube containing 740 KBq of ^{14}C-labelled sodium carbonate. The plants were exposed to $^{14}CO_2$ for 120min. The plants were wrapped in polyvinylidene chloride film immediately after loosing the enzymatic activities by heated ironing, and they were exposed to an imaging plate (IP) over night. The IP was visualized using an imaging analyzer (Typhoon9210, Amersham Bioscience, USA). Plants were separated into underground parts (roots plus nodules), stems, petioles, primary leaves, first trifoliolate leaves, second trifoliolate leaves, and shoot apexes, and they were dried in an oven at 70℃ . The 5mg dry weight of powder of each sample was mixed in 170 μ L of 30% H_2O_2 plus 330 μ L of 60% $HClO_4$ at 70℃ , and 4.5mL of a liquid scintillator (Clear-sol Ⅱ , Nacalai Tesque, Japan) was added. The radioactivity in these parts was determined using the Liquid Scintillation Counter (LSC-6000, ALOKA, Japan). The percentage distribution of ^{14}C of inoculated roots was compared with that of uninoculated control.

RESULTS

Analysis of starch and sugar

We analyzed the starch and sugar concentrations in the plants as the indicators of relatively long term C status of the plants at day 8 after inoculation compared with uninoculated control, those of Williams were also examined before (Ito *et al*., 2006). Primary leaf and first trifoliolate leaf, which completed fully expansion and became source organ, and underground part was analyzed.

Table 1 (top) shows dry weight of each part in this

Fig. 1. Photograph of the plants, which were enclosed in plastic chamber. Four plants (two of inoculated plants and two of control plants) were enclosed together.

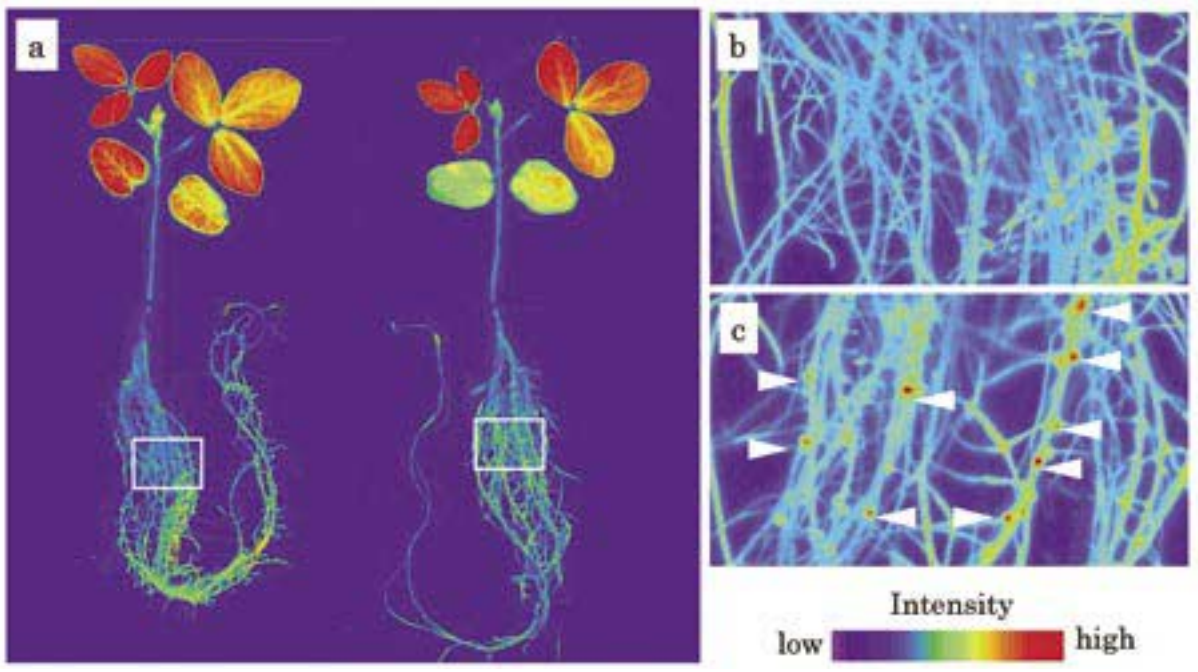

Fig. 2. Images of ^{14}C distribution of soybean hypernodulation mutant NOD1-3 using imaging plates. a; whole plant of control (left) and inoculation treatment (right), b; close-up of underground part (white squared) of control plant, c; close-up of underground part (white squared) of inoculated plant. Nodule images were indicated by white arrowhead.

study. Total dry weight of the inoculated plant with 557 mg was similar to the control plant with 561 mg. Data of this experiment was carefully carried out to minimize environmental fluctuations, so we concluded that total dry weight of plants at day 8 after inoculation was not decreased compared with uninoculated plants. The dry weight of underground part was not significantly different between control and inoculated plants. Dry weight of first trifoliolate of inoculation treatment was slightly smaller than that of control. Leaf growth of inoculated plant may be a little bit slower than control.

Table 1 (bottom) shows starch and sugar concentration in underground part, primary leaf and first trifoliolate leaf. This indicates that starch and sugar concentrations of inoculated plant were not decreased compared with control plant due to high consumption of carbohydrate for nodule initiation. The starch concentration in underground part of inoculated plant was slightly higher than control plant (not statistically significant); this trend was the same as in the case of Williams (Ito *et al*., 2006), so it was not because of the greater number of nodule formations.

Analysis of ^{14}C distribution

The $^{14}CO_2$ experiments was done to investigate the short term C flux for 120 min $^{14}CO_2$ exposure to whole shoot.

Fig. 2 shows that the distribution of radioactivity analyzed using the IP. Shoot was observed as strong signal than underground part in both inoculation and control plants, because whole shoot was exposed to $^{14}CO_2$. Young leaves, which is source organ as well as sink organ, were indicated as very strong signal. Underground part shows relatively weak signal. Some root apexes were indicated as slightly higher signal as well as shoot apex; in general, the growing point has strong signal activity for energy source as well as the structural materials. Partially strong signals were, however, observed in intermediate area of inoculated underground and they were identified signal of nodules (**Fig. 2c** arrowhead). Images of IP indicate that nodules of NOD1-3 have already high sink activity than root at day 8 after inoculation. In the case of cv. Williams, the parent of NOD1-3, the nodules did not have high sink activity at day 8 after inoculation (Ito *et al*., 2006). These results indicate that nodules of NOD1-3 might have faster growth than that of Williams.

Table 2 shows that the dry weight and ^{14}C radioactivity of each part of inoculation treatment and uninoculation treatment (control) of NOD1-3. The inoculated plants at 8 days after inoculation were in the stage when the nodules were initially recognized by eye. In this stage, we could not separate nodules from roots so we analyzed both parts as "underground part", which include roots and small nodules. Total radioactivity (KBq/plant) and specific radioactivity (KBq/gD.W.) of inoculated underground part was not increased but rather decreased in comparison with uninoculated one. Distribution of ^{14}C activity also indicates that inoculated underground part with 11.2% was similar to control with 13.8% (**Fig. 3**). These results indicate that current photosynthate allocation to the inoculated roots in the early stages of nodule formation did not increase in comparison to uninoculated plants, even in hypernodulation mutant NOD1-3. This was the same as in Williams (Ito *et al*., 2006). In the shoot, specific radioactivity (KBq/gD.W.) of first trifoliolate leaf and second trifoliolate leaf of inoculated plant was higher than that of control plant, while total radioactivity (KBq/plant) of these leaves were similar between both treatments (**Table 2**). In this study, dry weight of inoculated

Table 2. Dry weight and ^{14}C activity of each part of soybean hypernodulation mutant NOD1-3 at day 8 after inoculation.

	underground part	stem	petiole	primary leaf	first trifoliolate leaf	second trifoliolate leaf	shoot apex	total
Dry Weight (mg/plant)								
control	228 (17)	152 (45)	20 (1)	116 (7)	160 (12)	57 (2)	14 (1)	747 (26)
inoculation	232 (14)	110 (12)	16 (2)*	108 (10)	134 (12)*	44 (7)*	9 (1)*	656 (49)*
Total Radioactivity (KBq/plant)								
control	18.5 (8.3)	10.4 (3.3)	2.5 (0.3)	31.1 (7.8)	48.1 (5.2)	17.7 (2.6)	3.9 (0.7)	132 (16)
inoculation	12.4 (3.5)	8.7 (1.8)	1.8 (0.3)*	20.1 (3.2)	48.9 (5.0)	17.0 (2.2)	2.5 (0.8)*	112 (6)
Specific Radioactivity (KBq/gD.W.)								
control	80 (34)	73 (32)	124 (17)	268 (57)	302 (37)	312 (52)	273 (42)	178 (25)
inoculation	54 (16)	78 (9)	109 (9)	195 (27)	358 (22)*	390 (49)	290 (89)	171 (7)

The number in the parentheses indicate standard deviation (n=4). *Significantly different at 5% level compared with control.

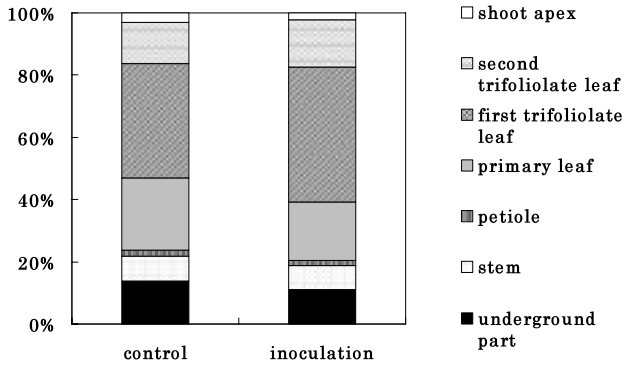

Fig. 3. Percentage distribution of radioactivity of ^{14}C in soybean hypernodulation mutant NOD1-3 at day 8 after inoculation. Radioactivity in each part was determined using the Liquid Scintillation Counter.

plant was slightly lower than that of control, which was different from "starch and sugar experiment" (**Table 1**). High specific radioactivity (KBq/gD.W.) of first trifoliolate leaf and second trifoliolate leaf might be cover decreased shoot growth of inoculated plant in this experiment. So we concluded that these differences of photosynthate allocation of shoot were not caused by the effect of inoculation treatment.

DISCUSSION

Early stage of nodule formation is very important period to control nodule formation in soybean. In this study, it was shown that current photosynthate allocated to the inoculated roots at the early stage of nodule formation did not increase in comparison with uninoculated control in hypernodulation soybean mutant NOD1-3. The results were essentially the same as Williams parent, so we concluded that appreciable amount of photosynthate is not required for initial stage of nodulation irrespective of hypernodulation or the parent. However, the IP images of the distribution of ^{14}C show that nodules of NOD1-3 at day 8 after inoculation has already high sink activity than root; it was observed in the nodules of Williams at day 10 after inoculation. Sato et al. analyzed four major leghemoglobin components of nodules, and it was indicated that leghemoglobins of NOD1-3 were initially appeared a few days earlier than those of Williams (Sato *et al*., 2001). These results suggest that initial growth of nodule of NOD1-3 might be earlier than that of Williams.

In general, plant growth of hypernodulation mutant is less vigorous than wild type (Carroll *et al*., 1985; Gremaud and Harper, 1989; Akao and Kouchi, 1992); but it is not clear that is secondary effect of hypernodulation trait or not. In Williams, after the emergence of the nodules, photosynthate allocation to the inoculated roots gradually increased after day 12 (Ito *et al*., 2006). It was shown that photosynthate allocation to the underground part of hypernodulation mutant is not increased at early stage of nodule formation at day 8 after inoculation in this study. It's just conceivable that photosynthate allocation to underground part after the emergence of nodules in the mutant may be higher than these in wild type. From the results obtained, the higher allocation of photosynthate to underground part is not the main reason to control nodule number in soybean during nodule initiation.

REFERENCES

Akao, S. and Kouchi, H. 1992. A supernodulating mutant isolation soybean cultivar Enrei. *Soil Sci. Plant Nutr*., **38**: 183-187.

Caetano-anolles, G. and Gresshoff, P.M. 1991. Plant genetic control of nodulation. *Annu Rev. Microbial*., **45**: 345-382.

Carroll, B.J., McNeil, D.L. and Gresshoff, P.M. 1985. A supernodulation and nitrate-tolerant symbiotic (*nts*) soybean mutant. *Plant Physiol*., **78**: 34-40.

Francisco, P.B. Jr. and Harper, J.E. 1995. Translocatable leaf signal autoregulates soybean nodulation. *Plant Sci*., **107**: 167-176.

Fujikake, H., Tamura, Y., Ohtake, N., Sueyoshi, K. and Ohyama, T. 2003. Photoassimilate partitioning in hypernodulation mutant of soybean (*Glycine max* (L.) Merr) NOD1-3 and its parent Williams in relation to nitrate inhibition of nodule growth. *Soil Sci. Plant Nutr*., **49**: 583-590.

Gremaud, M.F. and Harper, J.E. 1989. Selection and initial characterization of partially nitrate tolerant nodulation mutants of soybean. *Plant Physiol*., **89**: 169-173.

Ito, S., Ohtake, N., Sueyoshi, K. and Ohyama, T. 2006. Allocation of photosynthetic products in soybean during the early stages of nodule formation. *Soil Sci. Plant Nutr*., **52** (in press).

Olsson, J.E., Nakao, P., Bohlool, B.B. and Gresshoff, P.M. 1989. Lack of systemic suppression of nodulation in split root system of supernodulating soybean (*Glycine max* [L.] Merr.) mutants. *Plant Physiol*., **90**: 1347-1352.

Sato, T., Onoma, N., Fujikake, H., Ohtake, N., Sueyoshi, K. and Ohyama, T. 2001. Changes in four leghemoglobin components in nodule of hypernodulating soybean (Glycine max [L] Merr.) mutant and its parent in the early nodule development stages. *Plant and Soil*, **237**: 129-135.

Sato, T., Fujikake, H., Ohtake, N., Sueyoshi, K. and Ohyama, T. 2002. Effect of exogenous salicylic acid supply on nodule formation of hypernodulating mutant and wild type of soybean. *Soil Sci. Plant Nutr*., **48**: 413-420.

ダイズ根粒超着生変異株NOD1-3の根粒形成初期過程における光合成産物の分配

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要　約

これまでに、ダイズ（*Glycine max* [L] Merr.）栽培品種Williamsを用い、根粒菌接種8日後までの根粒形成初期過程では、根粒着生による光合成産物の分配増加が認められないことを示した（Ito *et al.* 2006）。今回は、Williamsより得られた根粒超着生変異株NOD1-3を用い、根粒形成初期過程における光合成産物の要求性に違いがあるか否かを調査した。播種18日後のダイズ植物の地上部に$^{14}CO_2$を120分間供給する実験を行った。根粒菌接種8日後の地下部への^{14}Cの分配を、根粒菌非接種の地下部への分配と比較した。接種8日後では根粒超着生変異株においても、接種した根への光合成産物の分配の増加は認められなかった。しかしイメージングプレートを用いた^{14}C分配のイメージング画像では、Williamsとは異なり接種した地下部において根粒の部分が強いシグナルとして検出された。接種8日後では地下部に送られる光合成産物量全体は非接種と変わらないが、局所的には根粒が根よりもすでに強いシンク活性を持つことを示した。また、より長期的な光合成産物の動態を調査するために、植物体内のデンプンおよび糖含量を分析した。しかし、デンプン・糖含量ともに根粒菌接種・非接種処理間に有意な差は無かった。これらの結果から、NOD1-3においても根粒形成初期過程における光合成産物の地下部への分配は顕著に増加することはないと結論づけた。

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