Characteristics of Nodulation and Nitrogen Fixation in the Improved Supernodulating Soybean (\textit{Glycine max} L. Merr.) Cultivar ‘Sakukei 4’

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\textbf{Abstract}: Supernodulating soybean lines have more than several times as many nodules as normal cultivars. They are expected to have high nitrogen-fixing ability and enhanced productivity, but their yields have been inferior to those of normal genotypes. We have recently developed a new supernodulating cultivar, ‘Sakukei 4’ (formerly ‘En-b0-1-2’, presently ‘Kanto 100’), with improved growth and yield. The objective of the present study was to identify the characteristics of the nodulation and nitrogen-fixing ability of Sakukei 4. In pot trials, the nodule number of Sakukei 4 was 8.3 times that of a normal cultivar, ‘Enrei’, and the nodule weight per plant was 2.3 to 2.8 times the value for Enrei. The acetylene reduction activity per plant in Sakukei 4 was higher than that in Enrei and conventional supernodulating genotypes, especially during the late growth stage. Compared with conventional supernodulating lines, the improved vegetative growth in shoots and roots of Sakukei 4, especially after flowering, probably enhanced its nitrogen-fixing ability per plant. We consider that its high nitrogen-fixing ability at the seed-filling stage, would help increase its yield in fields with low nitrogen fertility.

\textbf{Key words}: \textit{Glycine max} L. Merr., Growth, Nitrogen fixation, Root nodule, Soybean, Supernodulation.

Supernodulating soybean lines have more than several times as many nodules as normal cultivars. Therefore, they have been expected to have high nitrogen-fixing ability and enhanced productivity, but their growth and yields have been inferior to those of normal genotypes in general (Herridge et al., 1990; Wu and Harper, 1991; Hussain et al., 1992; Pracht et al., 1994; Song et al., 1995; Zhao et al., 1998; Herridge and Rose, 2000). Some super- or hyper-nodulating soybean genotypes showed high nitrogen-fixing ability only in the early growth stage (Day et al., 1986; Eskew et al., 1989; Wu and Harper, 1991).

We previously developed the supernodulating cultivar ‘Sakukei 4’ (formerly ‘En-b0-1-2’, presently ‘Kanto 100’), having improved growth and yield, from crosses a supernodulating line ‘En6500’, a mutant of cultivar ‘Enrei’, with normal nodulating cultivars (Takahashi et al., 2003a; 2003b; Yamamoto et al., 2004). The yield of Sakukei 4 in field trials was much higher than that of En6500 and similar to that of Enrei (Takahashi et al., 2003a; 2003b). When overall yields were low, Sakukei 4 tended to yield more than Enrei.

The objective of this research was to identify the characteristics of the nodulation and nitrogen-fixing ability of Sakukei 4 in comparison with the normal nodulating check genotype Enrei and conventional supernodulation lines En6500 and ‘En-b0-1’. This will help to reveal the cause of the differences in growth and yield between this new supernodulating cultivar and conventional ones.

\textbf{Materials and Methods}

1. \textbf{Plant materials}

We used the normal nodulating soybean cultivar Enrei, the supernodulating lines Sakukei 4, En-b0-1, and En6500, and the non-nodulating line ‘En1282’ for pot trials in 1997. Sakukei 4 was selected among progenies from the cross of Enrei/En6500 // Tamahomare (Takahashi et al., 2003a; 2003b; Yamamoto et al., 2004). En-b0-1 resulted from an Enrei/En6500 cross (Takahashi et al., 2003a; 2003b). En6500 and En1282 were artificial mutants of Enrei (Akao and Kouch, 1992; Francisco and Akao, 1993). In 1998, we limited the genotypes to Enrei, Sakukei 4, and En1282 to further investigate the differences between the genotypes.

2. \textbf{Cultivation method}

Plants were grown in 1/5000 a deep Wagner pots (16 cm diameter, 35 cm depth, 7 L volume) that contained 6 kg of Low-humic Andosols (Classification Committee of Cultivated Soils, 1995). Fertilizer was supplied at
0.6 g N, 5 g P₂O₅, and 2 g K₂O per pot. Seeds were inoculated with *Bradyrhizobium japonicum* strain A1017 and sown on 3 July 1997 and 30 June 1998. Eight seeds were sown per pot, and seedlings were thinned to two per pot in 1997, and one per pot in 1998. We grew 40 plants (20 pots) of each genotype in 1997 and 33 plants (33 pots) in 1998. Plants were usually grown in the open air but were moved to a glasshouse when it rained. Sufficient water was applied every few days during the early growth stages, and once or twice per day after the middle growth stages.

3. Acetylene reduction assay

Acetylene reduction activity (ARA), which is a good index of nitrogenase activity, was measured 4 or 5 times after flowering (growth stage R2-R2.5 of Fehr et al., 1971). At each measurement, we used 3 pots per genotype in 1997 and 4 pots in 1998. The plants were pulled from the pots and the soil was immediately removed. The aboveground parts were detached in 1997 but not in 1998. The root system was inserted into an open-flow gas exchange chamber (Minchin et al., 1983). To avoid the decline of ARA with time, every measurement was started within 5 min of exposure of the roots. The measurement was conducted at 25˚C.

The capacity of the chamber was 0.9 L, and the flow rates of air and acetylene gas into the chamber were regulated by a computer-controlled mass flow meter. After carbon dioxide was removed by passage through soda lime, air containing acetylene gas at 0.1 L L⁻¹ was introduced at a flux of 1 L min⁻¹. The air leaving the chamber was sampled with syringes. The amount of ethylene generated from the acetylene by the nodules was measured by means of gas chromatography (GC7A; Shimadzu Corp., Kyoto, Japan, with a Porapak N column), and the acetylene reduction rate was calculated. Since the ethylene generation rate reached a maximum within 4-6 min after the acetylene introduction in a preliminary experiment, measurements were recorded every minute for 6 min, and ARA was calculated from the maximum value.

4. Measurement of the respiration rate of roots and nodules

The respiration rate of the underground parts (roots + nodules) of the plants at 25˚C was measured at the same time as the ARA measurement. The carbon dioxide concentration in the gas flowing out from the root chamber was measured with an infrared gas analyzer (SPB-H2; Shimadzu Corp., Kyoto, Japan) and recorded in a personal computer connected to the analyzer. The respiration rate of the underground parts was calculated from the value 5 min after acetylene introduction, when the value became stable. We found that the respiration rate of the roots was in direct proportion to the root mass in the non-nodulating genotype En1282 at each growth stage in 1998. Therefore, we estimated the respiration rate of the roots in each genotype by assuming that the same relationship is applied to the other genotypes. The respiration rate of the nodules was calculated by subtracting the value of the roots from that of the underground parts.

5. Measurement of dry matter of each part of plants

After ARA was measured, plant samples were divided into leaf, stem + petiole, pod + seed, root, and nodule.
dried for 48 h at 75°C, then weighed.

**Results**

1. **Progression of growth stages**

   Though Sakukei 4 flowered only one day later than Enrei, it matured 2 weeks later than Enrei (Table 1). In Sakukei 4, the period from flowering (growth stage R2) to the start of podding (R3) and from seed-filling (R6) to maturity (R8) tended to be longer than those in other genotypes.

2. **Nodulation**

   Nodule number per plant reached a maximum 58 days after sowing (DAS) (R4.5) in Enrei and 68 DAS (R5) in Sakukei 4 (Fig. 1). Sakukei 4 had 8.3 times as many nodules as Enrei at 65 DAS in 1997 (Table 2) and 68 DAS in 1998 (Fig. 1). En6500 had 2.9 times and En-b0-1 had 4.7 times as many nodules as Enrei at 65 DAS (R5.5) in 1997 (Table 2).

   The nodule weight of Enrei reached a maximum at 58 DAS (R4.5) (Fig. 2). The nodule weight of Sakukei 4 reached 3.4 g at 68 DAS (R5), and continued increasing slowly. Among the supernodulating lines, the ratio of nodule weight to that of Enrei was 2.3 in Sakukei 4, 1.4 in En-b0-1, and 0.84 in En6500 (Table 2). However, the individual nodule weights of all supernodulating lines were about 30% of that of Enrei (Table 2).

3. **Temporal changes in acetylene reduction activity per plant**

   The ARA per plant of Sakukei 4 was higher than that of Enrei throughout the growing season (Figs. 3A, B).

### Table 2. Nodule number, nodule weight, and acetylene reduction activity per unit of nodule respiration rate of the soybean genotypes 65 days after sowing (1997).

| Genotype | Nodule number (plant⁻¹) | Nodule weight (g plant⁻¹) | Single nodule weight (mg) | Nodule weight per unit of shoot dry weight (mg g⁻¹) | ARA per unit of nodule respiration rate (µmol C₂H₄/µmol CO₂) |
|----------|-------------------------|---------------------------|---------------------------|--------------------------------------------------|--------------------------------------------------|
| Sakukei 4| 3242 a                  | 2.363 a                   | 0.739 b                   | 98 a                                             | 0.237 a                                           |
| Enrei    | 389 d                   | 1.011 b                   | 2.607 a                   | 27 c                                             | 0.338 a                                           |
| En6500   | 1118 c                  | 0.845 b                   | 0.757 b                   | 70 b                                             | 0.268 a                                           |
| En-b0-1  | 1834 b                  | 1.426 b                   | 0.784 b                   | 61 b                                             | 0.266 a                                           |

1) Within the same column, means not followed by the same letter are significantly different at P≤0.05 based on Tukey’s HSD test.

**Fig. 2.** Temporal changes in nodule dry weight of the soybean genotypes (1998). Vertical bars represent standard errors of the means based on four replications.

**Fig. 3.** Temporal changes in acetylene reduction activity per plant in the soybean genotypes (A, 1997; B, 1998). Vertical bars represent standard errors of the means based on three (in 1997) or four (in 1998) replications.
Especially after 79 DAS (R6), the ARA of Sakukei 4 was remarkably superior to that of Enrei (Fig. 3B). By contrast, En6500 had a lower ARA than Enrei in all but the early stage of growth (39 DAS, R2.5), and En-b0-1 also had an ARA similar or inferior to that of Enrei in all but the early stages (Fig. 3A).

4. Specific nodule activity
Specific nodule activity (ARA per unit dry weight of nodules) decreased after 39 DAS (R2-R2.5) in all genotypes in 1997 (Fig. 4A). Enrei showed the highest specific nodule activity among the genotypes. Although the differences among the supernodulating genotypes were small, Sakukei 4 tended to have a little higher specific nodule activity than the other supernodulating lines. The result in 1998 was similar to that in 1997, except that after 79 DAS (R6), the activity of Enrei decreased further than that of Sakukei 4 did (Fig. 4B).

5. Respiration rate of underground parts
The respiration rate of the underground parts (roots and nodules) was highest in Sakukei 4, intermediate in Enrei, and lowest in En1282 throughout the growing season (Fig. 5). Temporal changes in the estimated value of the nodule respiration rate (Fig. 6) were very similar to those of the ARA per plant (Fig. 3B). The ARA per unit of respiration rate (i.e., the energy efficiency of nitrogen fixation) tended to be higher in Enrei than in Sakukei 4, but there was no significant difference between the genotypes (Fig. 7). Differences in ARA per respiration rate among the genotypes were also not clear at the 65 DAS (R5-R5.5) in 1997 (Table 2).
6. Dry matter production

The aboveground dry weight of Sakukei 4 in 1997 was about 40% of that of Enrei at 39 DAS (R2-R2.5), but it increased to 70% at 79 DAS (R6) and 97% at maturity (R8, Fig. 8). In contrast, En6500 and En-b0-1 weighed only 29% and 66% of Enrei, respectively, even at maturity (R8). Although the dry weight of pod + seed in Sakukei 4 was the lowest among the genotypes at 65 DAS, it was similar to that of Enrei at maturity (Fig. 8). Thus, the rate of increase of pod + seed weight in Sakukei 4 was low during the early reproductive stage, but high during the later stages.

The root dry weight was also highest in Enrei throughout the growing season (Fig. 9). Among the supernodulating genotypes, the root dry weight after 52 DAS (R3.5-R4.5) was highest in Sakukei 4, medium in En-b0-1, and lowest in En6500.

Discussion

The maximum nodule number of Sakukei 4 was 8.3 times that of Enrei, and the maximum nodule weight per plant was 2.3 to 2.8 times the value for Enrei (Figs. 1, 2, Table 2). The number and weight of Sakukei 4 were also larger than those of the other supernodulating genotypes, En6500 and En-b0-1. These results show the difference in nodulation among the genotypes more clearly than the results in our previous field experiment (Takahashi et al., 2003a), probably because we could collect all nodules in the present pot trials but not in the field trial. The superiority in total shoot weight of Sakukei 4 to that of En6500 and En-b0-1 could explain the abundant nodulation of Sakukei 4, but it might not be the only reason, because the nodule dry weight per unit of shoot weight was also heaviest in Sakukei 4 (Table 2).

The ARA per plant of Sakukei 4 was obviously higher than that of Enrei throughout the growing season, indicating high nitrogen-fixing ability (Fig. 3). Superior hyper-nodulating lines would be expected to have high nitrogen-fixing ability because of their abundant mass of nodules, but in some reports they did not clearly show a high ability in the late stages of growth (Day et al., 1986; Eskew et al., 1989; Wu and Harper, 1991). In this study, En6500 and En-b0-1 also had ARA similar or inferior to Enrei in all but the early growth stages. In contrast with these lines, Sakukei 4 displayed high ARA per plant throughout the growing season, especially in the late growth stage. Since soybeans demand much nitrogen during the seed-filling period, a plant’s capacity to supply nitrogen in this period is thought to be one of the major factors that restrict the seed yield of soybeans (Sinclair and de Wit, 1975). It is noteworthy that Sakukei 4 has a high capacity to fix nitrogen in this important period. Therefore Sakukei 4 should have a high yield potential.

One of the reasons for high ARA per plant of...
Sakukei 4 must be its greater vegetative growth than that of En6500 and En-b0-1 (Fig. 8). The greater mass of the leaves, which are the source of photosynthates, must enable Sakukei 4 to have more nodules and higher ability to fix nitrogen. The leaf weight of Sakukei 4 at 65 DAS was 81% of that of Enrei, but those of En6500 and En-b0-1 were only 31% and 62%, respectively. In our previous field experiments, Sakukei 4 seemed to allocate more carbon assimilates to vegetative growth than to reproductive growth during the period when both vegetative and reproductive organs grew simultaneously, and such vigorous vegetative growth compensated for small vegetative growth in the earlier stages (Takahashi et al., 2003b). We confirmed this characteristic of Sakukei 4 in the present experiment, because the increase of leaf weight from 39 DAS (R2-R2.5) to 65 DAS (R5-R5.5) was greater in Sakukei 4 than those in Enrei, En6500, and En-b0-1 (Fig. 8). Recently, we demonstrated that a natural crossing occurred in the early process of Sakukei 4 breeding, and clarified that the pollen parent of Sakukei 4 must have been the cultivar Tamahomare (Yamamoto et al., 2004). Enrei and Tamahomare show many similar traits, because their paternity progenitors are identical, but they also display different traits. The active vegetative growth during the flowering period in Sakukei 4 must be inherited from Tamahomare, since we have observed in a pot trial that the increase in the number of branch nodes after the flowering stage was larger in Tamahomare and Sakukei 4 than in Enrei (unpublished data). This trait of Sakukei 4 could explain the high nitrogen fixation potential of this cultivar. The root mass of Sakukei 4 was also the largest among those of the supernodulating genotypes after 52 DAS (Fig. 9). This trait also must help the absorption of nutrients and water to support vegetative growth, photosynthesis, and nitrogen fixation.

While the specific nodule activity of Sakukei 4 was 25% lower than that of Enrei, it was slightly but consistently higher than that of other supernodulating lines (Fig. 4). The inferior mass of individual nodules and the inferior specific nodule activity of the supernodulating genotypes En6500 and nts382 were probably caused by excess numbers of nodules on plants with a relatively small shoot mass, and thus a small supply of photosynthates (Day et al., 1987; Sato et al., 1999). However, the competition among nodules for photosynthates was probably stronger in Sakukei 4 than in En6500 and En-b0-1, since the nodule weight per unit of shoot weight was larger in Sakukei 4 than in the other supernodulating lines (Table 2). Therefore, another reason may exist for the relatively high specific nodule activity of Sakukei 4 among the supernodulating lines. A prospective reason is the high nitrogen fixation activity per unit of nodule respiration (i.e., the energy efficiency of nitrogen fixation). However, no significant difference in the energy efficiency of nitrogen fixation was found among the supernodulating genotypes (Table 2). Therefore, various possibilities should be examined to clarify the reason for the relatively high specific nodule activity of Sakukei 4. For example, the cadmium concentration in the seeds of Sakukei 4 was lower than in other genotypes, including Enrei, En-b0-1, and Tamahomare, because Sakukei 4 accumulated cadmium in the roots (Arao et al., 2003). The root system of Sakukei 4 may have a high ability to accumulate elements that are needed for nitrogen fixation.

Sakukei 4 displayed high ability to fix nitrogen throughout the growing season. Compared with conventional supernodulating genotypes, the improvement of vegetative growth of shoots and roots of Sakukei 4, especially after flowering, probably enabled it to increase its nitrogen-fixing ability per plant. In our previous field experiment, the yield of Sakukei 4 tended to be greater than that of Enrei in fields with low available nitrogen (Takahashi et al., 2003a). We consider that the high ability of Sakukei 4 to fix nitrogen, especially in the seed-filling stage that we demonstrated in the present experiment explains its yield advantage in fields with low nitrogen fertility.

Acknowledgements

We gratefully thank Dr. S. Akao for providing the seeds of En6500 and En1282, and the technical staff of the National Institute of Crop Science and the National Agricultural Research Center.

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* In Japanese with English abstract or summary.
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