Plant Nitrogen Levels and Photosynthesis in the Supernodulating Soybean (*Glycine max* L. Merr.) Cultivar ‘Sakukei 4’

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Abstract: Supernodulating soybeans, having several times more nodules than normal genotypes, are expected to produce higher dry matter and grain yields through a higher nitrogen fixation potential. However, their growth and yield have been inferior to those of normal genotypes. We have recently developed the supernodulating genotype ‘Sakukei 4’ (formerly ‘En-b0-1-2’, presently ‘Kanto 100’), with improved growth and yield and a high nitrogen fixation potential. The objective of the present study was to examine the time course of changes in plant nitrogen content, leaf chlorophyll content (SPAD value), and photosynthetic rate of Sakukei 4 to reveal the source of its advantages in growth. The leaf nitrogen content after the flowering stage and the stem nitrogen content after the seed-filling stage were higher in Sakukei 4 than in the normal cultivar ‘Enrei’. The SPAD values in Enrei and another normal cultivar, ‘Tamahomare’, decreased rapidly after the seed-filling stage, whereas that in Sakukei 4 stayed high until the late seed-filling stage. Differences in photosynthetic rate and leaf SPAD value between Sakukei 4 and Enrei were negligible at the beginning of podding but became very clear at the seed-filling stage because of the drop in the values for Enrei. In Sakukei 4, a large amount of nitrogen might not be translocated from leaf to seed during the seed-filling stage because of the higher capability of the nodules to send fixed nitrogen to the growing seeds. Sakukei 4 could thus maintain a high photosynthetic rate and grain growth during the seed-filling stage.

Key words: Chlorophyll content, *Glycine max* L. Merr., Nitrogen content, Nitrogen fixation, Nodule, Photosynthesis, Soybean, Supernodulation.

Supernodulating soybeans, having several times more nodules than normal genotypes, have been generally inferior to normal genotypes in terms of growth and yield, even though their greater number and mass of nodules imply a higher potential to fix nitrogen, and thus higher dry matter and grain yields (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). We have recently developed the supernodulating genotype ‘Sakukei 4’ (formerly ‘En-b0-1-2’, presently ‘Kanto 100’), with improved growth and yield (Takahashi et al., 2003a; 2003b; Yamamoto et al., 2004). Sakukei 4 has the potential to produce higher yields, especially in fields with low nitrogen fertility (Takahashi et al., 2003a).

The capability of Sakukei 4 to fix nitrogen is significantly higher than that of the normal nodulating cultivar ‘Enrei’ and other supernodulating lines, especially during the later growing stages, or seed-filling stage (Takahashi et al., 2005). As soybeans generally need a large amount of nitrogen during the seed-filling stage, nitrogen in the leaves and stems is transferred to the growing seeds to make up for nitrogen deficits (Sinclair and de Wit, 1975). Because of its higher nitrogen-fixing capability, Sakukei 4 should maintain a higher photosynthetic rate during the seed-filling stage. The study reported here was conducted to test this hypothesis by comparing temporal changes in the nitrogen contents of different plant parts, in leaf chlorophyll content, and in photosynthesis between supernodulating genotype Sakukei 4 and normal nodulating genotypes.

Materials and Methods

1. Plant materials

We examined the supernodulating soybean cultivar Sakukei 4 and the normal cultivar Enrei in 1998 and 1999. Sakukei 4 was selected among the progenies from the Enrei/‘En6500’//‘Tamahomare’ cross (Takahashi et al., 2003a; 2003b; Yamamoto et al., 2004). En6500 is a supernodulating mutant line derived from Enrei by means of artificial mutation (Akao and Kouch, 1992). Recently, we have found that a natural crossing occurred in the early process of Sakukei 4 breeding, and that the pollen parent...
of Sakukei 4 must be the cultivar Tamahomare (Yamamoto et al., 2004). Therefore, we also used a normal cultivar Tamahomare for measuring leaf chlorophyll content and photosynthetic rate in 2004.

2. Cultivation method
Soybean seeds were inoculated with Bradyrhizobium japonicum strain A1017 and were sown in pots (16 cm diameter, 35 cm depth, 7 L volume) containing 6 kg of Low-humic Andosols (Classification Committee of Cultivated Soils, 1995) on 30 June 1998 and 2 July 1999, or in pots (25 cm diameter, 25 cm depth, 10 L volume) containing 8 kg of Low-humic Andosols on 29 March 2004. In all years, fertilizer was supplied at 0.6 g N, 5 g P2O5, and 2 g K2O per pot. Eight seeds were sown in each pot, and seedlings were thinned to one per pot when the first trifoliate leaf had fully expanded. We grew 33 plants per genotype in 1998, 8 plants in 1999, and 5 plants in 2004. In 1998 and 1999, plants were usually grown in the open air but were moved into a glasshouse when it rained. In 2004, plants were grown in a greenhouse with light supplement (0600-2000) where the air temperature was allowed to vary between 27 and 35˚C. In all years, sufficient water was applied every few days during the early growth stages, and once or twice per day after the middle growth stages.

3. Dry matter measurement of plant parts
Plants were sampled six times during the period from flowering (growth stage R2-R2.5 of Fehr et al., 1971) to maturity (R8) in 1998. Four plants were sampled per treatment, and were divided into leaf, stem+petiole, pod+seed, root, and nodule. The parts were dried for 48 h at 75˚C and weighed.

4. Nitrogen content in plants
After grinding, the nitrogen contents of the dried plant organs were determined with an N-C analyzer (Sumigraph NC-800; Sumika Chemical Analysis Service Ltd., Osaka, Japan).

5. Photosynthetic rate and chlorophyll content of leaves
The photosynthetic rate of the second uppermost fully expanded leaf of the main stem was measured with a portable photosynthesis and transpiration analyzer (SPB-H2; Shimadzu Corp., Kyoto, Japan) at the seed-filling stage (75 days after sowing [DAS]; R5.5-R6) in 1998; at the podding (61 DAS; R4-R5) and at the seed-filling stage (83 DAS; R6) in 1999; and several times after podding in 2004. All measurements were taken from 0900 to 1200 on cloud-free days when the photosynthetic photon flux density was higher than 900 $\mu$mol m$^{-2}$ s$^{-1}$.

When the leaf photosynthetic rate was measured, the chlorophyll content index (the SPAD value) of the same leaf was measured with a chlorophyll meter (SPAD-502; Minolta Co. Ltd., Osaka, Japan). The SPAD value was measured several times more, independent of the measurements of photosynthetic rate.

6. Yield and yield components
Six plants (in 1998 and 1999) and five plants (in 2004) were harvested at maturity. Pod number per plant, seed number per pod, 100 seed weight, and seed yield per plant were examined. The seed yield and the 100 seed weight were expressed on the basis of 150 g moisture kg$^{-1}$.

**Results**

1. Progression of growth stages
Sakukei 4 flowered 1-3 days later and matured 12-17 days later than Enrei (Tables 1, 2). On the other hand,
the growth stage of Sakukei 4 progressed like that of Tamahomare until R4, while it was earlier than that of Tamahomare after R5 (Table 2). Therefore, the maturity of Tamahomare was 8 days later than that of Sakukei 4. In 2004, the period from R7 to R8 was exceptionally long in all three genotypes, because those showed delayed stem maturation.

2. Temporal changes in nitrogen content

Although leaf nitrogen content decreased with time in both genotypes after 41 DAS (R2-R2.5), it was always higher in Sakukei 4 than in Enrei (Fig. 1A). The leaves of Sakukei 4 had 40 mg g\(^{-1}\) nitrogen at 88 DAS (R6.5), whereas those of Enrei rapidly lost nitrogen after 68 DAS (R5.5).

Genotypic differences in stem nitrogen content became clear after 58 DAS (Fig. 1B). The stem nitrogen content of Enrei started to decline after 58 DAS, whereas that of Sakukei 4 increased up to 79 DAS and decreased thereafter. In Enrei, the nitrogen content of pod+seed increased gradually to 56 mg g\(^{-1}\) at maturity (Fig. 1C). In Sakukei 4, it increased 8 days later than in Enrei and reached 60 mg g\(^{-1}\) at maturity. The seed nitrogen content of Sakukei 4 at maturity (79 mg g\(^{-1}\)) was higher than that of Enrei (76 mg g\(^{-1}\)) (data not shown).

3. Temporal changes in dry weight

The aboveground dry weight of Sakukei 4 increased more slowly than that of Enrei, especially during the early to middle growth stages, but Sakukei 4 had a dry weight at maturity similar to that of Enrei (Fig. 2). The leaf dry weight of Enrei increased up to 68 DAS,
attaining a maximum value of 15.1 g, and decreased after then. On the other hand, Sakukei 4 maintained a heavy leaf dry weight until 88 DAS, after reaching a maximum value of 11.6 g at 58 DAS. Although the pod + seed dry weight of Sakukei 4 was obviously lighter than that of Enrei before 79 DAS, the final pod + seed dry weight of Sakukei 4 was 40.0 g, slightly heavier than that of Enrei, 38.5 g.

Sakukei 4 had a lighter root dry weight, but a markedly heavier nodule dry weight than Enrei (Fig. 3). Overall, the weight of total root system in Sakukei 4 was similar to that in Enrei.

4. Nitrogen accumulation
The amount of nitrogen accumulation was largely affected by the dry matter weight. Therefore, the amount of accumulated nitrogen in Sakukei 4 was greater than that in Enrei at maturity (Fig. 4), although it was less than that of Enrei in most of the growth stages before maturity (data not shown).

5. Temporal changes in leaf chlorophyll content
The leaf chlorophyll content (SPAD value) of Enrei decreased sharply after 68 DAS, as the single-
seed weight increased rapidly (Fig. 5). In Sakukei 4, however, SPAD value was kept high even during the period of rapid single-seed weight increase, from 68 to 88 DAS. In 2004, the SPAD value of Sakukei 4 was the highest among all three genotypes throughout the growing season (Fig. 6).

6. Photosynthetic rate
The photosynthetic rate and the SPAD values of Sakukei 4 were clearly higher than those of Enrei at the seed-filling stage in 1998 (Fig. 7A) and 1999 (Fig. 7C), although no differences between Sakukei 4 and Enrei were observed at the podding stage in 1999 (Fig. 7B). In 2004, the photosynthetic rate of Sakukei 4 tended to be higher than those of Enrei and Tamahomare after the beginning of the seed-filling stage (44 DAS) (Fig. 8).

7. Yield and yield components
In 1998, the seed yield of Sakukei 4 was slightly higher than that of Enrei, though difference was not significant (Table 3). In 1999 and 2004, Sakukei 4 yielded significantly more than Enrei. The pod number of Sakukei 4 was similar to that of Enrei in 1998 and 1999, while that of Sakukei 4 was markedly superior to that of Enrei in 2004. On the other hand, 100 seed weight of Sakukei 4 was significantly heavier than that of Enrei in 1998 and 1999, though it was lighter than that of Enrei in 2004. The difference of seed number per pod between Sakukei 4 and Enrei was small, while there was significant difference in 1999.

Compared with Tamahomare, Sakukei 4 tended to yield more, though the difference was not significant (Table 3). One hundred seed weight of Sakukei 4 was larger than that of Tamahomare, while no significant differences were observed in pod number and seed number per pod between the genotypes.

Discussion
We have previously reported that the nitrogen fixation activity per plant was higher in the supernodulating genotype Sakukei 4 than in the normal genotype Enrei throughout growing season, and that the difference in nitrogen fixation activity was especially clear during seed-filling stage (Takahashi et al., 2005). The nitrogen demand of soybeans during the seed-filling stage is the highest among all crops, and any nitrogen shortage in growth of soybean seeds must be compensated by the translocation of nitrogen from leaf and stem to the seed in a process of self-destruction (Sinclair and de Wit, 1975). Self-destruction may bring about decline of photosynthetic rate and nitrate reductase activity in leaves, leading in turn to decreases in other physiological activities such as nitrogen fixation. The high nitrogen-fixing ability of Sakukei 4, especially during the later growth stages, should lessen the magnitude of this self-destruction. Several results of the present study seem to support
this hypothesis.

First, the leaf nitrogen content of Sakukei 4 was higher than that of Enrei after the flowering time (41 DAS, R2-R2.5), especially after the onset of seed filling (68 DAS, R5-R5.5), when leaf nitrogen content rapidly decreased in Enrei. This suggests that self-destruction was much weaker in Sakukei 4 than in Enrei (Fig. 1A). In fact, the leaf nitrogen content of Sakukei 4 stayed high until 88 DAS, 20 days longer than that of Enrei. The single-seed weight of Sakukei 4 increased about 8 days later than that of Enrei (Fig. 5). This 8-day delay in Sakukei 4 is probably attributed to the character of the pollen parent, Tamahomare (Table 2). If this 8-day delay in the growth stage is subtracted from the 20 days to adjust the growth stage, Sakukei 4 retained a high leaf nitrogen level for about 12 days longer than Enrei did.

Second, the leaf dry weight of Enrei began to decrease after 68 DAS, whereas that of Sakukei 4 stayed near its maximum until 88 DAS (Fig. 2).

Third, the stem nitrogen content of Sakukei 4 increased during the podding stage (Fig. 1B). Stem nitrogen content is also known to increase when the nitrogen demand was decreased by poor pod setting and seed growth. However, the pod number of Sakukei 4 was close to that of Enrei in 1998 (Table 3), indicating that normal pod setting occurred in Sakukei 4. Thus, the high stem nitrogen content of Sakukei 4 in this experiment suggests a higher nitrogen-fixing ability of Sakukei 4 during that stage.

Fourth, the chlorophyll content (SPAD value) of the upper leaf on the main stem was constantly higher in Sakukei 4 than in Enrei after the early seed-filling stage (Fig. 5). In Enrei, the SPAD value decreased rapidly from 46.2 at 68 DAS, when the mean single-seed weight reached 0.10 g, to a value of only 14.1 at 79 DAS, when the single-seed weight was 0.22 g. In contrast, the decrease of the SPAD value in Sakukei 4 was slight at 79 DAS, when the single-seed weight reached 0.14 g, and the SPAD value was still 41.7 even at 88 DAS, when the single-seed weight was 0.23 g. Sakukei 4 also showed a higher SPAD value than that of Tamahomare, its pollen parent, on the same day and at the same growth stage (Fig. 6), even though the maturing time, or the progression of growth stages of Tamahomare, was later than that of Sakukei 4 (Table 2). These results suggest a delayed or much slower self-destruction of Sakukei 4 than in the normal nodulating genotypes Enrei and Tamahomare.

Fifth, the photosynthetic rate of Sakukei 4 decreased more slowly than that of Enrei and Tamahomare (Figs. 7, 8). Although the photosynthetic rate did not differ between Sakukei 4 and Enrei at 61 DAS in 1999 (Fig. 7B), the value was much higher in Sakukei 4 than in Enrei at 83 DAS (Fig. 7C). This result suggests that the decrease in physiological activities caused by self-destruction was much more severe in the normal nodulating genotypes Enrei and Tamahomare.

Figs. 7A, 7C show that the photosynthetic rate in Sakukei 4 was high, probably because of the high chlorophyll content in leaves. Maekawa et al. (2003) and Matsunami et al. (2004) also reported the high photosynthetic rate and high nitrogen or chlorophyll content of leaves in Sakukei 4. On the other hand, in 2004 the photosynthetic rate in all three genotypes began to decrease when the SPAD value was still high (Figs. 6, 8). This discrepancy may be due to differences among the growth conditions, because we grew the plants in the greenhouse with relatively high temperature (27-35˚C) in 2004. Some factor

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Table 3. Seed yield per plant and yield components of various soybean genotypes.

| Year | Genotype   | Seed yield (g) | Pod no. | Seed no. per pod | 100 seed weight (g) |
|------|------------|----------------|---------|------------------|---------------------|
| 1998 | Sakukei 4  | 34.8 a         | 52.0 a  | 1.90 a           | 35.3 a              |
|      | Enrei      | 32.0 a         | 56.5 a  | 1.88 a           | 30.3 b              |
| 1999 | Sakukei 4  | 65.8 a         | 105.7 a | 1.84 b           | 33.8 a              |
|      | Enrei      | 54.6 b         | 104.7 a | 1.92 a           | 27.4 b              |
| 2004 | Sakukei 4  | 50.3 a         | 90.2 a  | 1.82 a           | 30.6 b              |
|      | Enrei      | 37.5 b         | 53.3 b  | 1.82 a           | 38.7 a              |
|      | Tamahomare | 42.7 ab        | 98.6 a  | 1.74 a           | 25.1 c              |

Seed yield and 100 seed weight are expressed on the basis of 150 g moisture kg⁻¹. Within the same year or column, means not followed by the same letter are significantly different at P<0.05 based on Tukey’s HSD test.
other than chlorophyll content, e.g., the content of enzymes involved in photosynthesis, might affect the decline of photosynthetic rate in 2004. Even so, the photosynthetic rate of Sakukei 4 was the highest of all the genotypes in 2004 (Fig. 8).

When we discuss the superiority in nitrogen content and chlorophyll content, we must consider the size of sink. If the sink size, e.g., the pod number per plant, is significantly small, the nitrogen may remain in the leaf and stem. In such a case, even if the nitrogen content keeps high level, we cannot expect high productivity per plant. However, in the present experiments, the pod number of Sakukei 4 was always similar to or superior to that of Enrei and Tamahomare (Table 3). In other words, the sink size of Sakukei 4 was not small. Indeed, Sakukei 4 always yielded more than other genotypes in the present experiments (Table 3).

In another study (Takahashi et al., 2005), the nitrogenase activity per plant in Enrei decreased significantly during seed filling (R6), whereas that of Sakukei 4 remained higher than the maximum level of Enrei. This high nitrogen-fixing ability in Sakukei 4 seems to explain the higher leaf nitrogen content during seed filling.

The higher nitrogen level in Sakukei 4 that was supported by the higher nitrogen fixation capability is the reason for the higher leaf chlorophyll content and photosynthetic rate, especially during the reproductive stages. In other words, the near absence of ‘self-destruction’ (Sinclair and de Wit, 1975) in Sakukei 4 is the reason why its yield potential is higher than that of the normal nodulating soybean genotypes.

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