Correlation of Leaf Nitrogen, Chlorophyll and Rubisco Contents with Photosynthesis in a Supernodulating Soybean Genotype Sakukei 4

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Abstract: Soybean requires more nitrogen (N) than gramineous crops because it accumulates a large amount of N in seeds, and its photosynthetic rate per leaf N is low. The supernodulating genotype Sakukei 4 has a superior symbiotic N2 fixation capability, and thereby is potentially high-yielding. In our previous study, Sakukei 4 was characterized by having a superior ability to maintain high leaf N content and high photosynthetic rate. The objectives of this study were to know photosynthetic characteristics of Sakukei 4 in detail, especially, the responses to CO2 concentration and light intensity, and to elucidate how the photosynthetic characteristics of Sakukei 4 are associated with the amounts of photosynthesis-related N compounds (chlorophyll and Rubisco). The three genotypes (Sakukei 4 - supernodulating cultivar derived from Enrei, Enrei - normally nodulating cultivar, En1282-non-nodulating line derived from Enrei) were grown at various N levels in this study. The CO2 exchange rate (CER) in Sakukei 4 was higher than, or equal to that in Enrei at wide ranges of CO2 concentrations (150-700 µmol mol⁻¹) and light intensities (200-1,500 µmol m⁻² s⁻¹ PPFD). Sakukei 4 had higher leaf N (NL), chlorophyll (ChlL) and Rubisco (RubL) contents per leaf area, but lower chlorophyll and Rubisco contents per leaf N content (ChlL/NL, RubL/NL) than Enrei. The specific leaf weight (SLW) and leaf area trended to be lower in Sakukei 4 than in Enrei. These results indicate that the superior photosynthetic rate in Sakukei 4 is attributed to higher total N, chlorophyll and Rubisco contents per leaf area, but not to high rate of allocation of total N to these N compounds.

Key words: Chlorophyll, Glycine max, Leaf nitrogen, Photosynthesis, Rubisco, Soybean, Supernodulation.

Soybean (Glycine max L.) accumulates a large amount of protein in seeds, therefore it requires a large quantity of nitrogen (N) compared with other major crops. Nitrogen accumulated in vegetative tissues during flowering and pod-expanding stages is translocated to seeds at seed-filling stage, and about 50% of the N required for seed protein synthesis derives from N mobilized in vegetative tissues (Hanway and Weber, 1971; Loberg et al., 1984; Warembourg and Fernandez, 1985; Shibies and Sundberg, 1998). At the seed-filling stage when N demand for seeds markedly increases, N in leaf is translocated to seeds, leading to a rapid decline in chlorophyll and Rubisco contents in leaf. This self-destructive N mobilization from leaf to seeds is likely to harm the capability of photosynthesis and restrict the yield of soybean (Sinclair and de Wit, 1975). Therefore, the enhancement of N-absorbing capability is essential for the improvement of photosynthetic ability of soybean.

Makino et al. (1988) found that photosynthetic rate, Rubisco contents and stomatal conductance per leaf N content in the most upper leaf and the 4th or 5th leaf from the top of soybeans were significantly lower than those in the flag leaf of wheat and rice, and that the efficiency of photosynthesis per leaf N content in soybean was lower than that in wheat and rice. Similarly, Sinclair and Horie (1989) showed that the photosynthetic rate per leaf N content in soybean is lower than that in rice and maize.

Soybean has three N sources: fertilizer N, soil N and symbiotically fixed N2. Since application of fertilizer N hinders the activity of rhizobia and reduces symbiotic N2 fixation, total N absorption is not likely to be enhanced by increasing fertilizer N (Harper, 1987). Therefore, it is difficult to promote total N absorption and improve the yield of soybean by the increased application of N. In addition, the overuse of N fertilizer causes environmental problems. The genetic improvement of N2 fixation is an option to solve these problems. The practical use of supernodulating...
genotypes is a feasible way to enhance N₂-fixing capability, since supernodulation soybean has superior symbiotic N₂ fixation capability and tolerance to nitrate (Kokubun, 2001).

Several supernodulating soybean mutants have been isolated in the 1980s (Carroll et al., 1985a, 1985b; Gremaud and Harper, 1989; Akao and Kouchi, 1992). Although these mutants form a large number of nodules, they are agronomically inferior to conventional cultivars (Herridge and Rose, 2000; Kokubun, 2001). Recently, a supernodulating cultivar, “Sakukei 4” was bred from its parental cultivar “Enrei” by mutation breeding and backcross method (Takahashi et al., 2003a). This genotype, the yield of which can be comparable to Enrei under certain environments, is the only supernodulating soybean cultivar which can be grown practically (Takahashi et al., 2003a, 2003b). Previously, we found that Sakukei 4 has a superior ability to maintain high N content in the leaf and high photosynthetic rate until the later growth stage compared to its parental cultivar Enrei (Maekawa et al., 2003). These observations raise the following question. How is the photosynthetic rate in supernodulating soybean Sakukei 4 associated with the amount of photosynthesis-related N compounds (chlorophyll, Rubisco) in leaf? Another interest is how the photosynthetic rate in Sakukei 4 responds to the change of CO₂ concentration or light intensity, because the photosynthetic rate is decisively dependent on these environmental variables in the field.

The objectives of this study were to know photosynthetic characteristics of Sakukei 4 in detail, especially, the responses to CO₂ concentration and light intensity, and to elucidate how the photosynthetic characteristics of Sakukei 4 are associated with the amounts of photosynthesis-related N compounds (chlorophyll and Rubisco).

Materials and Methods

Three experiments (Exp. 1-3) were conducted in 2001, 2002 and 2003. The genotypes used were the same in the three experiments, but cultural practices were different among the experiments (Table 1).

1. Plant materials

Three contrasting genotypes: the supernodulating cultivar Sakukei 4, normally nodulating cultivar Enrei and non-nodulating line En1282 were used in this study. Sakukei 4 is an improved genotype which was selected from the progeny of Enrei × En6500 (Takahashi et al., 2003a). En6500 is a mutant of Enrei (Akao and Kouchi, 1992). A recent genetical analysis indicated that this progeny was likely to be naturally crossed, during a period of selection, with

| Table 1. Summarized information on the three experiments (Exp. 1, 2 and 3). |
|-----------------------------|-----------------------------|-----------------------------|
| Exp. 1 | Exp. 2 | Exp. 3 |
| Year | 2001 | 2002 | 2003 |
| Date of sowing | May 29 | May 28 | July 20 |
| N (g pot⁻¹) | 0, 0.2, 0.6, 1.5 | 0, 0.6, 1.5 | 0, 0.6, 1.5, 2.5 |
| P₂O₅ (g pot⁻¹) | 0.6 | 0.6 | 2 |
| K₂O (g pot⁻¹) | 0.6 | 0.6 | 2 |
| Slaked lime (g pot⁻¹) | 5 | 5 | 5 |
| Flowering stage | July 20-24 (52-56)* | July 28 (61) | Aug. 30-Sept. 2 (41-44) |
| Measurement date | | | |
| CER | July 14 (46) | July 14 (47) | Sept. 4 (46) |
| | Aug. 10 (73) | July 18 (51) | Sept. 6 (48) |
| | Sept. 12 (106) | July 31 (64) | Sept. 17 (59) |
| | Sept. 19 (113) | Sept. 1 (96) | Sept. 18 (60) |
| | | July 26 (58) | Sept. 4 (99) | Sept. 15 (57) |
| | | Sept. 15 (109) | Sept. 8 (103) | Sept. 27 (69) |
| | | July 17 (49) | July 25 (58) | Sept. 5 (47) |
| | | Aug. 14 (77) | Aug. 13 (77) | Sept. 9 (51) |
| | | Sept. 1 (95) | ---- | Sept. 16 (58) |
| | Rubisco, Chlorophyll | ---- | July 31 (64) | Sept. 6 (48) |
| | Total N | Aug. 10 (73) | July 31 (64) | Sept. 6 (48) |
| | | | | |

* Figures in parentheses indicate DAS (days after sowing).
Tamahomare, a high-yielding cultivar (Yamamoto et al., 2004). En1282 is a non-nodulating mutant derived from Enrei and the seeds were provided from Dr. AkaO, National Institute of Agrobiological Sciences, Japan.

2. Plant culture

Four seeds per pot (16 cm in diameter, 19 cm tall) were sown and thinned to two plants per pot after emergence. Plants were grown in greenhouse until stage V1 according to Fehr et al. (1971), then they were transferred to open air with adequate irrigation. Pesticides were applied to prevent insect infestation. The amount of fertilizers differed among the three experiments. In Exp. 1 and 2, fertilizer N was applied prior to sowing at four rates (0, 0.2, 0.6, 1.5 g pot\(^{-1}\)) and at three rates (0, 0.6, 1.5 g pot\(^{-1}\)), respectively, as ammonium sulfate. Other fertilizers were applied at fixed rates: 0.6 g P\(_2\)O\(_5\) as superphosphate, 0.6 g K\(_2\)O as potassium chloride and 5 g of slaked lime per pot. In Exp. 3, fertilizer N was applied at four rates (0, 0.6, 1.5, 2.5 g pot\(^{-1}\)) as ammonium sulfate and other fertilizers were applied at fixed rates: 2 g P\(_2\)O\(_5\) as superphosphate, 2 g K\(_2\)O as potassium chloride and 5 g slaked lime per pot. Our previous studies showed that about 0.6 g N pot\(^{-1}\) was appropriate for maximal seed yield. The soil type was a fine-textured Yellow soil, clayey (Classification Committee of Cultivated Soils, 1996), containing small amounts of N and humus. It did not seem necessary to inoculate the soil with *Bradyrhizobium japonicum* in Exp. 1 and 2, because it had been used for growing soybean previously. In Exp. 3, the soil was inoculated with a strain of *Bradyrhizobium japonicum* (Tokachi-noukyouren, Obihiro, Japan).

3. Measurement of photosynthesis

The CO\(_2\) exchange rate (CER) in the most recently expanded terminal leaflets from five plants in each treatment was measured with LI-6400 Portable Photosynthesis System (LI-COR, NE, USA). The measurement was carried out during 1000 and 1300 hr at several growth stages (Table 1). The flow rate of air in the leaf chamber was adjusted to 500 \(\mu\)mol s\(^{-1}\), and the CO\(_2\) concentration to 350 \(\mu\)mol mol\(^{-1}\). The irradiance on the measured leaves (6 cm\(^2\)) was kept at 1,500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PPFD, and the temperature in the chamber at 25°C.

In addition to CER under the fixed condition, the CER under various CO\(_2\) concentrations and light intensities was also measured. The CO\(_2\) concentration and light intensity were phased down using a program of LI-6400. The concentration of CO\(_2\) was adjusted to 4-7 levels (0-1,300 \(\mu\)mol mol\(^{-1}\)), and light intensity to 4-7 levels (0-1,800 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) PPFD), and CER in the most recently expanded terminal leaflets was measured.

Except for the CO\(_2\) concentration and light intensity, other conditions for the CER measurement were the same as those under the fixed condition. Additional information on the measurement is shown in Table 1.

4. Measurement of photosynthesis-related N compounds

Immediately after the measurement of CER, the fresh weight and leaf area of the same leaflet were measured. Then, the leaflets were immediately frozen in liquid nitrogen, and stored at \(-80^\circ\)C until analysis. Rubisco (Rub\(_{L}\)), chlorophyll (Chl\(_{L}\)) and total N (N\(_{L}\)) contents per leaf area were analyzed as described by Makino et al. (1991; 1994), with modification of the homogenization buffer and preparation time. One g of the leaflets was homogenized in 9 mL of 50 mM sodium-phosphate buffer (pH 7.0) containing 0.8% (v/v) 2-mercaptoethanol, 2 mM iodoacetic acid and 5% (v/v) glycerol in a chilled mortar with a chilled pestle. Two 100 \(\mu\)L portions of this homogenate were used for the Chl determination and the Kjeldahl digestion, respectively. The Chl content was measured by the method of Arnon (Arnon, 1949). Total leaf nitrogen content was determined using Nessler’s reagent and the digested solution with sodium-potassium tartrate added (Makino, 1991; 1994).

For determination of Rubisco, Triton X-100 solution at a final concentration of 10% (v/v) was added to a 500 \(\mu\)L portion of the remaining homogenate. After centrifugation at 15,000 g for 5 min, a 200 \(\mu\)L portion of the supernatant was mixed with a lithium dodecylsulfate solution (1.0% (w/v), final concentration) and incubated at 100°C for 90 s. This mixture was stored at \(-20^\circ\)C until analysis by SDS-PAGE. The amount of Rubisco was determined spectrophotometrically after formamide extraction of Coomassie brilliant blue R-250-stained subunit bands separated by SDS-PAGE. A calibration curve was obtained using Rubisco purified from rice leaves (Makino et al., 1983). Dates of sampling are shown in Table 1.

Results

1. Effect of CO\(_2\) concentration and light intensity on CER

Fig. 1 shows the effect of CO\(_2\) concentration on CER in a leaf of three genotypes in Exp. 1 and 3. CER increased with increasing the CO\(_2\) concentration until saturation. It was the highest in Sakukei 4 followed by Enrei and En1282 in this order irrespective of CO\(_2\) concentration. The difference between Sakukei 4 and Enrei was more marked at the vegetative stage than at the reproductive stage. The saturated concentration of CO\(_2\) for CER was about 750-800 \(\mu\)mol mol\(^{-1}\) and 500-700 \(\mu\)mol mol\(^{-1}\) in Exp. 1 and Exp. 3, respectively.

As a whole, the effect of light intensity on CER was similar to that of CO\(_2\) concentration (Fig. 2). The CER was the highest in Sakukei 4 followed by Enrei and
En1282 in this order irrespective of light intensity. The difference in CER between Sakukei 4 and Enrei was slight, but CER in En1282 was much lower than that in the other genotypes. The difference in CER between Sakukei 4 and Enrei was more obvious in Exp. 1 (0.2 g N pot$^{-1}$) than in Exp. 3 (0.6 g N pot$^{-1}$). The saturated light intensity for CER was about 1,500 µmol m$^{-2}$ s$^{-1}$ PPFD in Sakukei 4 and Enrei, while it was nearly 500 µmol m$^{-2}$ s$^{-1}$ PPFD in En1282.

2. Effect of the amount of N fertilizer on CER

Fig. 3 shows the effect of N dosage on CER at flowering stage in the three genotypes. The difference between Sakukei 4 and Enrei was not obvious in Exp. 2, but the CER in En1282 was clearly lower than that in the other two genotypes. Since the CER is affected by environmental conditions during or prior to the measurement, the measurement was repeated in two or three consecutive days in Exp. 3. Fig. 3 shows the data for each day. The CER in Sakukei 4 was affected by the amount of applied N only slightly, and daily fluctuation of CER values was small. In contrast, the CER of Enrei tended to decrease with increasing amount of N, and the CER value tended to vary with the day of measurement. As a whole, CER in Sakukei 4 was higher than or similar to that in Enrei, and that in En1282 was always lower than in the other two genotypes.

3. Effect of N dosage on chlorophyll and Rubisco contents

Fig. 4 shows the effect of the N dosage on N content per leaf area ($N_L$), chlorophyll content per leaf area ($Chl_L$) and chlorophyll content per leaf N content ($Chl_L/N_L$) at the flowering stage. The $N_L$ and $Chl_L$ of Sakukei 4 tended to be lower than that of Enrei in both experiments (Exp. 2 and 3), and were affected by N dosage only slightly in all genotypes. In contrast, $Chl_L/N_L$ of Sakukei 4 tended to be lower than that of Enrei. En1282 had the lowest $N_L$ and $Chl_L$ among the three genotypes. $Chl_L/N_L$ of En1282 was lower than that of Enrei, and was similar to that of Sakukei 4.
In Sakukei 4, the Rubisco content per leaf area (RubL) tended to be higher than that in Enrei, whereas RubL/NL was lower than that of Enrei irrespective of the amount of N applied (Fig. 5). En1282 showed the lowest RubL and RubL/NL in all the measurements in Exp. 2 and in most measurements of Exp. 3. In summary, Sakukei 4 had higher N, chlorophyll and Rubisco contents per leaf area than Enrei, but N distribution to chlorophyll and Rubisco of Sakukei 4 was lower than that of Enrei.

4. Effect of the amount of N applied on SLW and leaf area

Fig. 6 shows the effect of the amount of N applied on specific leaf weight (SLW) and leaf area. In Exp. 2, SLW of Sakukei 4 was heavier than that of Enrei in the plants supplied with N (0.6 g and 1.5 g pot⁻¹), although the leaf area of Sakukei 4 was smaller than that of Enrei in the plants supplied with 0 and 0.6 g N per pot. SLW and leaf area in En1282 were generally lower than in the other two genotypes. In Exp. 3, SLW was the lowest in Sakukei 4 among the three genotypes, which was different from the result in Exp. 2. The difference in leaf area among the three genotypes was smaller in Exp. 3 than in Exp. 2.

Discussion

In this study, we examined the photosynthetic characteristics of supernodulating genotype Sakukei 4, in comparison with normally- and non-nodulating genotypes, and their association with photosynthesis-related N compounds. Sakukei 4 had higher CER than the related normally nodulating cultivar over a wide range of CO₂ concentration and light intensity (Fig. 1-3), reflecting the higher N, chlorophyll and Rubisco contents per leaf area (Fig. 4, 5). It is notable that the larger amount of photosynthesis-related N compounds in a leaf was not due to the increased allocation of leaf N to these compounds; the ratio of these compounds to total leaf N was relatively low in Sakukei 4 (Fig. 4, 5). Therefore, the high photosynthetic capability of Sakukei 4 is ascribed to the high chlorophyll and...
When CER was measured on different days at the same stage, the CER under the same CO₂ concentration or light intensity varied with the day (Fig. 1 and 3). What caused the difference? It is well documented that the CER in soybean leaf is affected by the environmental conditions at the time of measurement and/or during the development of the leaf (Shibles et al., 1987). To analyze the environmental effects on CER values, we calculated the correlation coefficients between CER and the environmental parameters (daily average temperature, duration of sunshine and rainfall for different periods prior to the CER measurement) (Table 2). A positive significant correlation was observed only between CER and duration of sunshine at the day of measurement. Thus, it appears that the daily change of CER in the leaf depends on the duration of sunshine at the day of measurement. It was predicted that environmental conditions before the day of measurement might affect the photosynthetic capacity of a leaf, but this was not the case in the present experiment.

Fig. 7 shows the relationship between leaf N content and CER based on the data obtained in Exp. 1-3. Sakukei 4 seems to have higher leaf N content per leaf area and tended to have higher CER than the other two genotypes. By contrast, N content and CER of En1282 were lower than those of the other two genotypes, and those of Enrei were intermediate. Interestingly, Sakukei 4 appeared to have a higher CER per leaf N content than Enrei (Exp. 1). This phenomenon was also observed in our previous study.

Table 2. Correlation coefficients between leaf CO₂ exchange rate (CER) and climatic parameters obtained for different periods prior to CER measurement.

| Genotype  | 10 days prior to measurement | 3 days prior to measurement | The day before measurement | The day of measurement |
|-----------|-----------------------------|-----------------------------|---------------------------|------------------------|
|           | Temp. Light Rain            | Temp. Light Rain            | Temp. Light Rain          | Temp. Light Rain       |
| Sakukei 4 | 0.23 0.43 −0.23             | −0.03 0.28 −0.21            | −0.29 −0.30 −0.32         | 0.07 0.59* −0.16       |
| Enrei     | 0.17 0.41 −0.17             | −0.03 0.28 −0.31            | −0.48* −0.22 −0.57*       | −0.13 0.50* −0.02      |
| En1282    | 0.03 0.27 0.38              | 0.15 0.24 −0.20             | −0.09 −0.28 −0.43         | 0.33 0.25 0.27         |

Temp.: Daily average temperature, Light: Duration of sunshine, Rain: Precipitation. *: Significant at 5% level.
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Further study is needed to elucidate whether the high photosynthetic efficiency of Sakukei 4 is attributed only to the increased amount of chlorophyll and Rubisco contents per leaf area, or to an additive increase of the other N compounds in a leaf.

SLW in Sakukei 4 did not exceed that in Enrei and En1282 (Fig. 6). The positive correlation between CER per leaf area and SLW was commonly observed among normally nodulating cultivars (Sinclair, 2004). SLW is likely to be greater in a sun leaf than in a shade leaf, because the sun leaf had more layers in palisade tissue and a thicker mesophyll than the shade leaf (Terashima et al., 2001). Despite the smaller SLW, however, the leaf of Sakukei 4 had a higher CER as the sun leaf did.

It is well known that the leaf area increases with increasing dosage of N. The leaf area tended to be lower in Sakukei 4 than in Enrei, although the leaf N content per leaf area was higher in Sakukei 4 (Fig. 4, 6). In our previous studies, we indicated that the inferior capacity of leaf expansion per plant in Sakukei 4 restricted its dry-matter production, despite its superior photosynthetic ability (Maekawa et al., 2003; Matsunami et al., 2004). The low capacity of leaf expansion in Sakukei 4 was attributed to the small size of individual leaf in the present study. It is commonly considered that plants having small SLW have large SLA, the reciprocal of SLW, and large leaf area, but Sakukei 4 did not bear this relationship.

It is assumed that the plant community displays a high photosynthetic capability when the plants possess smaller leaves relative to N content per leaf area, because this type of plants reduces mutual shading, allowing the lower leaves to receive more solar radiation than the plants with larger leaves. Therefore, it is estimated that the supernodulating cultivar Sakukei 4 that has small leaf area and high CER has a potential to produce superior dry matter and yield under dense planting condition. This possibility is currently being examined in our laboratory.

In conclusion, the present study clarified that the high CER of supernodulating genotype Sakukei 4 was not attributed to high rate of allocation of leaf N to the photosynthesis-related N compounds (chlorophyll and Rubisco) but to a large amount of the N compounds per leaf area, due to increased amount of total N per leaf area. It was also clarified that the photosynthetic capability per leaf area of Sakukei 4 tended to exceed that of Enrei under various CO₂ concentrations and light intensities. However, it is not clear whether these characteristics of Sakukei 4 can alleviate the self-destructive translocation of N at the pod-expansion stage or seed-filling stage. Further studies are needed to address this issue.

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