Nodulation and Growth of a Supernodulating Soybean Mutant SS2-2 Symbiotically Associated with Bradyrhizobium japonicum

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INTRODUCTION

A symbiosis between the soil bacteria and leguminous plants is characterized by a specific multi step signal exchange (Israel et al. 1986). The nodulation of soybean plants is a developmentally complex process requiring interaction between Bradyrhizobium and the host plants (Sutton 1983). Successful development of nitrogen-fixing nodules, including supernodulating soybean requires on-going communication between the plant host and the endosymbiotic Rhizobium (Green and Emerich 1999).

In soybean, nodulation mutants have been isolated either from normal soybean population or by chemical or physical mutagenesis (Carroll et al. 1985a; Gremaud and Harper 1989). Three groups of scientists have independently isolated supernodulating or hypernodulating soybean mutants from cultivars Bragg (Carroll et al. 1985a), Williams (Gremaud and Harper 1989), and Enrei (Akao and Kouchi 1992). All these mutants were capable of producing several-fold more nodules than their parent lines in the presence of nitrate. These supernodulating soybean mutants may not only be unique biological materials for a study on host plant factors, which could be involved in the process of nodulation, but also be a useful germplasm in a breeding program on improvement of nodulation and nitrogen fixation (Lee et al. 1997).

The super- and hypernodulating soybean mutants formed considerably more nodules than their wild type, and an extensive region of the roots was covered with nodules (Akao and Kouchi 1992). Several soybean mutants generated from cv. Bragg (Carroll et al. 1985b) displayed N-fixation and produced nodule 10 times more than their wild type (Carroll et al. 1985b). Evaluation of the supernodulating soybean mutants in the field has been reported in previous studies, involving mutants of soybean cultivars Bragg and Williams (Herridge and Rose 1994). It was revealed that intermediate supernodulators could grow as vigorously as their parent cultivars. However, the yield of extreme supernodulators (nts382, nts1007, mutants of Bragg) and intermediate supernodulators (nts1116) have been consistently low (Song et al. 1995). Supernodulating soybean mutants also showed higher activities in the N fertilized than commercial cultivars Bragg, Centaur, and Manark (Song et al. 1995). It has been suggested that these were due to other mutations.

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unrelated to noduleation (Mathews et al. 1989). In series of split-root and grafting experiment, the auto-regulatory response was found to be systemic (Olsson et al. 1989), requiring the action of the shoot (Delves et al. 1986).

The yield of supernodulating mutant SS2-2, generated from Sinpaldalkong 2 that was mutagenized by EMS (Lee et al. 1997), was not changed significantly due to nitrogen supply. Furthermore, non-inoculated plants of mutants, SS2-2 and nts382 showed greater nodules number and nodule mass, as well as greater ARA than their wild types, regardless of the level of exogenous nitrogen supply (Ha et al. 1999). Investigation of nitrogen fixation and agronomical traits of SS2-2 in association with Rhizobium has not been reported. This paper therefore, reported a study on a supernodulating soybean SS2-2 that was compared with its wild type in growth and nodulation in response to Bradyrhizobium japonicum.

MATERIALS AND METHODS

Plant Materials and Growth Condition for Bacteria

*B. japonicum* USDA 110 (obtained from the USDA *Rhizobium* culture collection, Beltsville, Md., USA) was used as inoculum in this study. Bacterial cells were grown in yeast extract mannitol/YEM medium which consists of K_{2}HPO_{4} 0.05%, MgSO_{4} 7H_{2}O 0.02%, NaCl 0.01%, mannitol 1%, and yeast extract 0.04% with a pH adjusted to 6.8 (Vincent 1970). The bacterial cultures were incubated in a shaking incubator at 200 rpm, 30°C, for 6 days. In this study, three soybean genotypes, a supernodulating mutant SS2-2, its wild type Sinpaldalkong 2, and a control genotype Jangyeobkong were used for investigation.

Characterization of Nodulation and Growth

Surface sterilized soybean seeds were sown in plastic pots filled with 1 : 1 sand and soil mixture, then inoculated with *B. japonicum* USDA 110 (10^{8} CFU/ml). A 3 x 2 factorial treatment combinations of three soybean genotypes and two inoculation levels (inoculated and non-inoculated treatment) were laid out in a completely randomized design with 3 replications. The plants were kept in a greenhouse and nitrogen-free Jensen’s reagent (Fang and Hirsch 1998) was applied twice a week. The dry weight of each plant part (shoot, root, and nodule), nodule number on the root system, and acetylene reduction activity (ARA) representing the nitrogen fixation were measured at 28 and 47 days after planting (DAP) respectively, while plant height and yield were measured around R3 stage (82 DAP).

Analysis of Acetylene Reduction Activity (ARA)

Acetylene reduction activity was measured on removed root from the whole plant which was placed in a 1 liter jar, and sealed with a lid containing a serological stopper. Using a 50-cc syringe, a 50-cc aliquot of air was removed from the jar, and the same amount of C_{2}H_{2} was then injected into the sample jar. The root system was allowed to remain in the jar with C_{2}H_{2} for 30 min, after a 10-cc aliquot was withdrawn from the jar, and the aliquot was injected into a 10-cc vacutainer tube. From this 10-cc tube, 0.5-cc aliquot was later drawn for gas chromatography/GC (Model DS 6200) analysis of C_{2}H_{2} (Denison et al. 1983). ARA was determined by measurement of C_{2}H_{2} reduction activity per plant, while specific ARA based on the C_{2}H_{2} reduction activity per nodule dry weight.

Statistical Analysis

Parameters of growth and nodulation characters were measured as quantitative variations. The phenotypic data were analyzed using SAS procedure (Goodnight 1982), and LSD test was applied to evaluate the variance.

RESULTS AND DISCUSSION

Characterization of Nodulation and Growth

The supernodulating soybean mutant SS2-2 had smaller nodule size but greater nodule number than those of the wild type. The nodules on the root system of Sinpaldalkong 2 were clustered near the root crown (Figure 1). There was significant effect of soybean genotype but no significant difference of soybean-*Rhizobium* interaction effect on number and dry weight of nodule per plant. Inoculation of *B. japonicum* increased number and dry weight of nodules significantly at 28 and 47 DAP (Figure 2). Under given condition, 47-day-old SS2-2 plants had considerably higher both nodule number, about 20 times and nodule dry weight than the wild type and the control genotype.

Results of this study were consistent with the conclusion of Hansen et al. (1992) that the supernodulators were producing relatively more nodular tissue than normally-nodulating types (non-mutated plant) in the absence of soil nitrogen. Nodulation behaviour in SS2-2 was similar with that of a pea mutant (*Pisum sativum*) (Jacobsen and Feenstra 1984) and a lupine hypernodulating mutant (*Lupinus albus*) L-62 (Burity et al. 1999). It was shown that without nitrogen supply, the mutant presented better nodulation in association with *Rhizobium*. Addition-
ally, *B. japonicum* was effective to induce nodulation and increase nitrogen fixation (Israel et al. 1986).

There was no significant effect of soybean genotype on nodule dry weight at 28 and shoot dry weight at 47 DAP, but significant difference measured at 47 DAP on root dry weight (Table 1). *B. japonicum* induction produced healthier and more vigorous plants and stimulated growth of SS2-2, Sinpaldalkong 2 and Jangyeobkong better than in non-inoculated treatments. In this study, the dry weights of root and shoot were lower than those in infertile soil (clay/river sand/horticultural bed = 6 : 3 : 1) conducted by Ha and Lee (2001). Regardless of the genotype, SS2-2 growth was consistently lower than that of its wild type. According to shoot/root ratio, it was clear that SS2-2 had smaller roots and shoots with greater nodules. However, shoot growth of the wild type and the mutant were identical under symbiotic condition, while the root growths were still lagged off behind (Table 1). Small size and short root of SS2-2 were predicted as the reduction of plant growth that had also been observed in other supernodulating or hyper-nodulating mutants of soybean (Carroll et al. 1985a; Day et al. 1986) and other legumes (Sheng and Harper 1997). The short root phenotypes of other legume
mutants had also been documented, such as in a Lotus japonicus hypernodulating mutant harl-I (de Bruijn et al. 1998) and a pea hypernodulating mutant nod3 (Jacobsen and Feenstra 1984). Thus, it was suggested that the short root length in the supernodulating mutant SS2-2 was likely to be due to a decrease or modification in root meristem activities (Wopereis et al. 2000), because of a defect of essential component of the regulatory mechanism responsible for maintaining the root growth.

B. japonicum increased the total dry weight (shoot, root, nodule) significantly compared to that of the control (Table 1). For this reason, the increase in total dry weights on rhizobia application in all soybean genotypes were more pronounced. Regardless of the Bradyrhizobium inoculation, during the plant growth period, the total dry weights of SS2-2 climbed to levels which were not significantly different from those of the wild type and control genotypes. Total plant dry weight in SS2-2 was not different from that of the normally-nodulating soybean. According to the root and shoot growth of the supernodulating mutant, it was suggested that the development of the soybean-Bradyrhizobium symbiosis was under the control of both shoot and root factors and these factors interact each other (Delves et al. 1986). Furthermore, rapid regulatory response in soybean was reproducible elicited by Rhizobium japonicum and autoregulatory control mechanism (Van Workum et al. 1998).

Nitrogen fixation was observed by determination of ARA per plant. Based on the nodule dry weight, specific ARA was obtained from the ARA. The more nodule dry weight of the plant, the higher specific ARA would be. Under symbiotical growth, there was significant difference on ARA at 28 and 47 DAP between mutant SS2-2 and its wild type (Table 1). SS2-2 gave a higher ARA than the wild type and control genotype, positively correlated with the nodule dry weight. B. japonicum caused the increase of ARA at 47 DAP, in part because of the nodule development. However, as the symbiosis progressed, the lowest specific ARA was observed on supernodulating soybean mutant. The specific ARA was important to be determined to know the ability of nitrogen fixation on soybean plant. Moreover, leghaemoglobin content in the nodule would be able to be detected qualitatively.

Even though nitrogen fixation rate (ARA) of supernodulating mutant was higher than that of the wild type, specific ARA in nodules of the supernodulating plants was reduced. Such differences in the
ARA could be as results of differences in the ratio of bacteroidal in the nodules or differences in the nitrogenase activity per unit of bacteroid (Israel et al. 1986). Whereas, ARA attributable to these nodule would steadily declined at later stage of growth, not only because of nodule senescence but also because of the steady increase in nodule dry weight. Therefore, this apparent discrepancy might be also related to nutritional status of the host plants (Hattory and Johnson 1984), even though contribution of indigenous Rhizobium could be considered, however it was not strong enough to increase nitrogen fixation at the late stage of growth. Regardless of Bradyrhizobium inoculation, supermodulating soybeans showed less tolerant to effects of nitrogen (Song et al. 1995). Day et al. (1989) showed that lower specific ARA on supermodulating mutant that represented specific nitrogenase activity, was mainly due to the reduction in amount of symbiotic tissue which was characterized by smaller nodule cells, fewer bacteroids per peribacteroid membrane vesicle, and lower haem content per nodule. Consequently, although nodule emergence and subsequent nodule growth were specific parameters, ARA was not coordinately regulated with this nodule development in the mutant.

Regardless of the soybean genotypes, the lower soil fertility (non-inoculated) resulted in the lower plant height as well as pod and seed numbers per plant, and plant yield. There were significant effects of the soybean genotypes and the inoculation levels on the plant height, pod and seed number per plant, and plant yield (Table 2). It was clear that plant height of SS2-2 was shorter than the wild type under symbiotical growth. The mutants gave less plant yield than those of the wild types.

Low yield of SS2-2 was consistent with previous results of other supermodulating mutants (Maloney and Oplinger 1997). Apparently, under symbiotical growth, both SS2-2 and Sinpaldalkong 2 gave better yield than non-inoculated plants. The ability of the supermodulating mutant SS2-2 to develop more root nodules and to have greater nitrogen fixation did not result in increased grain yield compared with its normally-nodulating parent. This result was supported previous studies (Song et al. 1995), in which grain yield of the wild type soybean plant was significantly higher than that of supermodulating mutant. The greater yield in normally-nodulating soybean seemed to be associated with the photoassimilates. The supermodulating soybean mutant needed more leaf photoassimilates from the top for nodule development and maintenance. This caused the reduced yield of the supermodulating soybean mutant when compared to the wild types. Based on these results, SS2-2 might be able to maintain a fairly high total dry weight at low level of nitrogen even in the absence of exogenous nitrogen.

### Table 2. Yield and yield components of three genotypes of soybean as affected by inoculation of B. japonicum at 82 DAP.

<table>
<thead>
<tr>
<th>Soybean genotype</th>
<th>Non-inoculated</th>
<th>Inoculated</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS2-2</td>
<td>34.0</td>
<td>38.3</td>
<td>36.1b</td>
</tr>
<tr>
<td>Sinpaldalkong 2</td>
<td>46.0</td>
<td>53.2</td>
<td>49.6a</td>
</tr>
<tr>
<td>Jangyeobkong</td>
<td>45.0</td>
<td>52.2</td>
<td>48.6a</td>
</tr>
<tr>
<td>Mean</td>
<td>41.7b</td>
<td>47.9a</td>
<td></td>
</tr>
<tr>
<td>Pod number per plant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS2-2</td>
<td>46.0</td>
<td>48.0</td>
<td>47.0b</td>
</tr>
<tr>
<td>Sinpaldalkong 2</td>
<td>74.0</td>
<td>78.0</td>
<td>76.0a</td>
</tr>
<tr>
<td>Jangyeobkong</td>
<td>73.0</td>
<td>78.0</td>
<td>75.5a</td>
</tr>
<tr>
<td>Mean</td>
<td>64.3b</td>
<td>69.3a</td>
<td></td>
</tr>
<tr>
<td>Seed number per plant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS2-2</td>
<td>67.0</td>
<td>77.0</td>
<td>72.0b</td>
</tr>
<tr>
<td>Sinpaldalkong 2</td>
<td>105.0</td>
<td>120.0</td>
<td>112.5a</td>
</tr>
<tr>
<td>Jangyeobkong</td>
<td>103.0</td>
<td>118.0</td>
<td>110.5a</td>
</tr>
<tr>
<td>Mean</td>
<td>91.7b</td>
<td>105.0a</td>
<td></td>
</tr>
<tr>
<td>Plant seed yield (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS2-2</td>
<td>11.0</td>
<td>14.0</td>
<td>12.5b</td>
</tr>
<tr>
<td>Sinpaldalkong 2</td>
<td>18.0</td>
<td>24.0</td>
<td>21.0a</td>
</tr>
<tr>
<td>Jangyeobkong</td>
<td>17.0</td>
<td>23.2</td>
<td>20.1a</td>
</tr>
<tr>
<td>Mean</td>
<td>15.3b</td>
<td>21.7a</td>
<td></td>
</tr>
</tbody>
</table>

Within categories, means (column or row) not followed by the same letter are significantly different at P 0.05 based on LSD.
**SS2-2-Bradyrhizobium japonicum Symbiosis**

SS2-2 less responded to *B. japonicum* induction in terms of plant growth and plant yield than its wild type (Table 3). Even though SS2-2 performed an effective symbiosis as the normally-nodulating soybean, it showed less response to *B. japonicum* induction in terms of nodulation, nitrogen fixation and nodulation characters (Lestari et al. 2005). Accordingly, although SS2-2 is a supernodulator, it is not a constitutive nodulator, since it still requires an inducer (*R. japonicum*) to be present. It was to be likely that some mechanisms were undergone in the supernodulating mutant. The nodulation in the supernodulating mutant suggested a mutational alteration of the autoregulation system, indicating closed relationship between nitrogen fixation and autoregulation of nodule development (Delves et al. 1986). Supernodulating mutant might maintain its growth by biologically fixed nitrogen (Delves et al. 1986). Clearly, SS2-2 was a mutant in the autoregulation pathway and was less sensitive to regulation by external conditions.

Based on the character of nodulation in SS2-2 especially nitrogen fixation which was represented by ARA observed in this study, supernodulating mutant that is potential for reducing uptake of soil N, would be more attractive for planting with “high density population” to increase the yield. Hansen et al. (1992) reported that although the large number of nodules of supernodulators were to be some degree parasitic on the host, they were potentially useful as N contributors in the cropping systems (Day et al. 1989) and might represent a source of N for the succeeding crops (Song et al. 1995). These results suggested that a major advantage of the supermodulation trait was in crop rotation, particularly for the subsequent cereal crop. Further more, the supermodulating soybean mutant SS2-2 would be useful for an environmentally-friendly agriculture.

**CONCLUSION**

Evaluation of symbiotic association between *B. japonicum* in a supermodulating soybean mutant SS2-2 compared with the wild type, Sinpaldalkong 2 and the control genotype, Jangyeobkong revealed distinct phenotypic characters among them. Soybean plant inoculated with *B. japonicum* increased number and dry weight of nodule significantly either at 28 or 47 DAP than non-inoculated plants. The inoculated SS2-2 plants produced nodules almost 20 times more than the wild type. An ARA was observed lower in normally-nodulating soybean than that of SS2-2, otherwise, specific ARA was observed the lowest on SS2-2. Inoculation with *B. japonicum* lead to the increase of ARA in 47 DAP, due in part to nodule development. SS2-2 had smaller plant and gave less yield, but revealed greater number of nodule and nitrogen fixation than its wild type, either grown symbiotically or non-symbiotically. Thus, SS2-2 less responded to *B. japonicum* induction in terms of nitrogen fixation, nodulation characters, growth and plant yield than its wild type. In another word, the interaction between *Rhizobium* and the supermodulating mutant SS2-2 was less symbiotically associated response than the normally-nodulating soybean. Clearly, SS2-2 was a mutant in the autoregulation pathway that was less sensitive to regulation by external conditions.

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