Root Isoflavonoid Response to Grafting between Wild-Type and Nodulation-Mutant Soybean Plants

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ABSTRACT
It was previously reported that the hypernodulating soybean (Glycine max [L.] Merr.) mutants, derived from the cultivar Williams, had higher root concentration of isoflavonoid compounds (daidzein, genistein, and coumestrol) than did Williams at 9 to 12 days after inoculation with Bradyrhizobium japonicum. These compounds are known inducers of nod genes in B. japonicum and may be involved in subsequent nodule development. The current study involved reciprocal grafts between NOD1-3 (hypernodulating mutant) and Williams. Isoflavonoid and coumestrol content was determined in roots and shoots of both genotypes at 7 days after grafting. The shoot and root isoflavonoid concentration and content was more than twofold when the shoot genotype was NOD1-3. When grafted, NOD1-3 shoots induced hypernodulation on roots of both Williams and NOD1-3, while Williams shoots induced normal nodule formation on both root genotypes. This shoot control of isoflavonoid content may be caused by differential root isoflavonoid levels, which are regulated by the shoot. In contrast, the nonnodulating characteristic of the NNS mutant was strictly root controlled, based on reciprocal grafts. Delayed inoculation (7 days after planting) resulted in greater nodule numbers on both NOD1-3 and Williams, compared with a seed inoculation treatment. The nodule formation pattern of grafted plants was independent of whether the shoot portion was derived from inoculated seed or uninoculated seed, when grafted at day 7 onto seedling roots derived from uninoculated seed. This observation, coupled with the fact that no difference existed in nodule number of NOD1-3 and Williams until after 9 days from seed inoculation, indicated that isoflavonoids play a role in differential nodulation of the hypernodulating mutant and the wild type, the effect is on advanced stages of nodule ontogeny, possibly related to autoregulation, rather than on initial infection stages.

The formation of nitrogen-fixing nodules on soybean (Glycine max [L.] Merr.) roots occurs in response to infection by Bradyrhizobium japonicum. After bacterial infection, the nodule formation of a normal soybean plant is restricted by a plant process called autoregulation, in which the nodule formation on one part of the root systemically suppresses subsequent nodule formation in other root regions (2, 17, 21). Supernodulating and hypernodulating mutants that appear to have altered this autoregulatory control of nodule formation have recently been derived from Bragg and Williams parents (4, 12). These mutants have greater nodule numbers, nodule dry weights, and nitrogenase activities than their respective wild types. In addition, nonnodulating soybean mutants have also been isolated by Carroll et al. (5) and Harper (15) from Bragg and Williams, respectively.

Based on grafting studies (9–11), the supernodulation and hypernodulation phenotypes derived from wild-type Bragg were reported to be shoot controlled, while the nonnodulation phenotype was root controlled. It was proposed that a shoot-derived inhibitor(s) was involved in autoregulation of nodulation (14). Gresshoff et al. (13) reported that feeding methanol/water extracts from the inoculated Bragg wild-type to nts382 supernodulating mutant plants suppressed nodulation by 60 to 80%, but extracts from uninoculated wild-type or nts382 plants were not inhibitory to nodulation.

The isoflavones, daidzein and genistein, have been isolated and identified as major inducers of nod genes in B. japonicum (18). In addition, coumestrol and daidzein have also been shown to promote the growth of B. japonicum (8). Our recent studies (6, 7) have shown that the Williams-derived hypernodulating mutants (NOD1–3 and NOD2–4) accumulate higher concentrations of isoflavonoid compounds (daidzein, genistein, and coumestrol) than does the parent at 9 to 12 d after inoculation with B. japonicum. There were, however, no significant differences in isoflavonoid concentrations among lines when plants were not inoculated.

In this study, root isoflavonoid concentrations and nodulation characteristics from reciprocal self grafts between the Williams parent and hypernodulating and non-nodulating mutants were compared. The objective was to determine if shoot control of hypernodulation was possibly related to root isoflavonoid levels.

MATERIALS AND METHODS
Plant Culture and Grafting
Three soybean (Glycine max [L.] Merr.) lines were evaluated, including the parent line (Williams), a hypernodulating mutant (NOD1–3), and a nonnodulating mutant (NN5). Both mutants were previously selected from the cv Williams (12, 15). Seeds were surface-sterilized with 5% (v/v) Clorox2 plus

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one drop of Tween 20 for 10 min and rinsed with deionized distilled water. Seeds were planted in sterilized sand in perforated trays and watered from the bottom with distilled water. Seeds were germinated and subsequently grown in growth chambers programmed for 14-h photoperiods at 650 μmol photons m⁻² s⁻¹ at 29°C and 10-h dark periods at 20°C. At 7 d after planting, depending on experiment, seedlings were either transplanted to nutrient solution or were subjected to a grafting treatment and transplanted 4 d later. Bradyrhizobium japonicum strain USDA 110 was used to inoculate either seeds or seedling roots as indicated in the Table and Figure legends. Inoculum was grown in liquid yeast extract-mannitol-glucanate medium (2) and diluted with sterilized deionized water to 10⁵ cells/mL. An uninoculated control was included with some studies as indicated.

For studies involving grafting, grafts were made on 7-d-old seedlings in the sand trays used for germination. Wedge-shaped grafts with cotyledons left on the scion were used, essentially as described by Bezdicek et al. (1). Grafts were held in place by ≈1-cm sections of plastic drinking straws (2.5 mm i.d.) which had been split on one side to allow for expansion. Use of the smaller straw eliminated the need for any band as described (1). Each tray of grafted plants was covered by a transparent plastic lid embedded into the moist sand to maintain high humidity around the seedlings. Grafted seedlings were grown for 2 d in the dark followed by 2 d in diurnal light/dark before transplanting.

Both intact and grafted seedlings were transplanted to 10-L polypropylene trays containing a modified minus N Hoagland nutrient solution as previously described (12). Seedlings were suspended through holes in Styrofoam lids placed over the trays. The solution pH was maintained at 6.5 ± 0.5 with ion exchange resin columns (16). Nutrient solutions were completely changed at 7-d intervals after transplanting.

HPLC Analysis of Isoflavonoid Compounds

Grafted plants were analyzed for isoflavonoid compounds by HPLC as previously reported (7). Samples were taken at 15 and 20 d after grafting and inoculation, with four and three replicates, respectively. Replicates consisted of two plants at 15 d and one plant at 20 d.

C₂H₂ Reduction Assay and Nodule Observations

Twenty-five days after inoculation, plants were harvested for in vivo C₂H₂ reduction assay as described earlier (12), except that a single nodulated root was incubated in 250-mL gas-tight jars with 10% (v/v) C₂H₂. Four single-plant replicates of each treatment were evaluated. The jars were sealed and 25 mL of C₂H₂ was injected. After the assay, nodules were removed from the roots and counted. Plant roots and nodules were then dried for dry matter determinations. The ontogeny of nodule development was followed in two separate studies: one involving grafted plants and another comparing seed inoculation with delayed inoculation. Observation of nodule development was made with a light microscope (Bausch & Lomb Inc., Rochester, NY) at 16 × magnification.

Statistical Analysis

Analysis of variance was performed for most experiments and treatment means were compared using Fisher’s LSD when significant F tests occurred. Due to unequal sample variance when comparing nodule numbers of hypernodulating, normal nodulating, and nonnODULEtating lines, data are presented as means ± SD.

RESULTS

Nodulation and Growth Characteristics of Grafted Plants

Data of Table I show the nodulation characteristics of reciprocal- and self-grafted plants between the NOD1–3 hypernodulating mutant and the Williams wild type at 25 d after inoculation. NOD1–3 shoots induced hypernodulation when grafted onto roots of Williams and NOD1–3. In contrast, the Williams wild-type shoots gave wild-type nodulation patterns on roots of both soybean lines. Grafted NOD1–3 shoots induced four times as many nodules as Williams shoots, irrespective of root genotype. Nodule mass and C₂H₂

<table>
<thead>
<tr>
<th>Goldenrod</th>
<th>Williams/Williams</th>
<th>196</th>
<th>56</th>
<th>22.0</th>
<th>0.375 (0.319)</th>
<th>0.617</th>
</tr>
</thead>
<tbody>
<tr>
<td>Williams/NOD1-3</td>
<td>187</td>
<td>47</td>
<td>5.11</td>
<td>0.333 (0.286)</td>
<td>0.816</td>
<td></td>
</tr>
<tr>
<td>NOD1-3/Williams</td>
<td>772</td>
<td>104</td>
<td>11.19</td>
<td>0.251 (0.147)</td>
<td>0.312</td>
<td></td>
</tr>
<tr>
<td>NOD1-3/NOD1-3</td>
<td>752</td>
<td>108</td>
<td>11.64</td>
<td>0.236 (0.128)</td>
<td>0.390</td>
<td></td>
</tr>
<tr>
<td>LSD0.05</td>
<td>165</td>
<td>24</td>
<td>2.03</td>
<td>0.065 (0.046)</td>
<td>0.098</td>
<td></td>
</tr>
</tbody>
</table>
reduction activities were also enhanced by the NOD1-3 shoot when grafted to either NOD1-3 or Williams roots, compared with the situation in which Williams was used as the shoot source for grafting. Williams shoots induced greater shoot and root growth than did NOD1-3 shoots. The root genotype had no effect on growth of either root or shoot when a common shoot was grafted.

Microscopic observation of soybean nodulation showed that nodule numbers continued to increase throughout the 24-d growth period after inoculation with all grafting treatments (Fig. 1). Through 9 d after inoculation, no significant difference in nodule number among grafting treatments was measurable. After that time, however, grafts involving NOD1-3 shoots had significantly greater nodule numbers than those involving Williams shoots, regardless of whether the grafted root was from Williams or NOD1-3. This difference increased with time. Greater nodule numbers were detected in all graft combinations upon microscopic observation (Fig. 1) than when physically removed and counted (Table I), due to inability to remove the very small nodules.

Isoflavonoid Analysis of Root Extracts from Grafted Plants

When hypernodulating mutant NOD1-3 shoots were grafted onto roots of either Williams or NOD1-3, higher root isoflavonoid concentrations (and contents, data not shown) were observed at 15 d after inoculation, compared with grafts involving Williams shoots (Fig. 2). Self-grafts of NOD1-3 had even higher isoflavonoid concentrations than did reciprocal grafts of hypernodulating mutant NOD1-3 shoots onto Williams roots. However, there was no significant difference in root isoflavonoid concentration between self and reciprocal grafts involving Williams shoots. A repeat evaluation (data not shown) of root isoflavonoids at 20 d after inoculation provided confirmation of the pattern and level of isoflavonoids presented in Figure 2.

Shoot versus Root Control of Nodulation

The nodulation pattern of grafted plants was independent of whether the shoot portion was derived from inoculated seed or noninoculated seed, when grafted at day 7 onto seedling roots derived from inoculated seed (Table II). This finding was true across all combinations involving Williams, NOD1-3, and NN5. Seven-day-delayed inoculation (Table I) resulted in considerably greater numbers of nodules per plant than did seed inoculation (Table II). The shoot control of hypernodulation and normal nodulation noted for seedling-inoculated plants (Table I) was confirmed with grafts of seed-inoculated plants (Table II). In contrast to the hypernodulation phenotype, the inability of the NN5 nonnodulating mutant to form nodules was strictly determined by the root (Table II). Regardless of whether the grafted shoot was that of Williams or NOD1-3, the NN5 roots failed to form nodules. Grafting nonnodulating mutant NN5 shoots onto both Williams and NOD1-3 roots resulted in similar nodule numbers to that observed when Williams served as the shoot source.

Delayed inoculation Effect on Nodulation

Seven-day-delayed inoculation markedly increased nodule numbers of both NOD1-3 and Williams, compared with the seed-inoculation treatment (Fig. 3A). The 7-d-delayed inoculation treatment induced nodulation mainly (about 90% of total nodules) on the branched lateral roots of both soybean lines (Fig. 3B). In contrast, seed-inoculated plants formed nodules primarily on the tap root at the early observation times but the percentage of nodules on the tap root decreased

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**Figure 1.** Changes in nodulation of reciprocal- and self-grafted plants between NOD1-3 and the Williams parent. Nodules were counted with a light microscope (×16) at 3-d intervals through a 24-d growth period after grafting and inoculation. Other details as in Table I legend. Values represent means of three replicates for each grafting treatment within a sampling time and vertical bars are LSD0.05. n.s. denotes nonsignificant differences among treatments at the 0.05 level.

**Figure 2.** Isoflavonoid concentration in root extracts from reciprocal- and self-grafted plants between NOD1-3 and the Williams parent. Plant roots were sampled for HPLC analysis of isoflavonoid compounds at 15 d after grafting and inoculation. Values represent means of four replicates for each grafting treatment. Means with the same letter are not significantly different among grafting treatments within an isoflavonoid compound at the 0.05 level using an LSD test.
Table II. Nodulation Characteristics of Reciprocal- and Self-Grafted Plants among Williams, NOD1-3, and NN5 with Inoculation Treatments

Seeds were inoculated with *B. japonicum* prior to planting, with the exception of those seeds giving rise to shoot portions designated below as "noninoculated." Germination, grafting, and subsequent growth conditions were as described in Table I. Plants were harvested for nodule measurement at 26 d after inoculation and germination. Values expressed are as means ± SD (n = 3 or 4).

<table>
<thead>
<tr>
<th>Graft (Shoot/Root)</th>
<th>Nodule No.</th>
<th>Nodule Dry Wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated/inoculated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Williams/Williams</td>
<td>33 ± 13</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>Williams/NOD1-3</td>
<td>36 ± 6</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>Williams/NN5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NOD1-3/Williams</td>
<td>242 ± 30</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>NOD1-3/NOD1-3</td>
<td>203 ± 18</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>NN5/Williams</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NN5/NOD1-3</td>
<td>34 ± 10</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>NN5/Williams</td>
<td>53 ± 9</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>NN5/NN5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Noninoculated/inoculated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Williams/Williams</td>
<td>53 ± 10</td>
<td>34 ± 11</td>
</tr>
<tr>
<td>Williams/NOD1-3</td>
<td>37 ± 3</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>Williams/NN5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NOD1-3/Williams</td>
<td>255 ± 29</td>
<td>94 ± 17</td>
</tr>
<tr>
<td>NOD1-3/NOD1-3</td>
<td>194 ± 25</td>
<td>69 ± 22</td>
</tr>
<tr>
<td>NOD1-3/NN5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>NN5/Williams</td>
<td>37 ± 5</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>NN5/NOD1-3</td>
<td>42 ± 12</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>NN5/NN5</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

DISCUSSION

The current grafting study showed that shoots from the Williams wild type induced wild-type nodulation, while shoots from the NOD1-3 hypernodulating mutant induced hypernodulation on roots of both lines (Table I, II, Fig. 1). This finding confirms the conclusion made in previous grafting studies (9-11) that the supernodulation and hypernodulation mutants derived from Bragg are controlled through the action of the shoot. Our recent studies (6, 7) demonstrated that the hypernodulating mutants from Williams accumulated higher concentrations of isoflavonoid compounds (daidzein, genistein, and coumestrol) than did the Williams parent when inoculated with *B. japonicum*. The current study extended this work by determining root isoflavonoid concentrations and contents from reciprocal and self grafts between NOD1-3 and the Williams parent. Isoflavonoid analyses from root extracts of grafted plants showed that NOD1-3 shoots had markedly higher root isoflavonoid concentrations (Fig. 2) and contents (data not shown) in roots of both lines compared with Williams shoots. These results are consistent with the hypothesis that root isoflavonoid levels may be related to differential nodulation expression by a mechanism that is unrelated to the previously reported involvement of isoflavonoids on nod gene induction (18). Measuring isoflavonoid levels in root exudates did not appear pertinent to this study, because the responses noted appear to be postinfection. This conclusion is consistent with the observation that Bragg and its supernodulating *nts382* mutant were similar in terms of

![Figure 3. Effect of delayed inoculation on (A) nodule numbers and (B) percentage of nodules on the tap roots of NOD1-3 and the Williams parent. Either seeds or 7-d-old seedlings were inoculated with *B. japonicum*. After 7-d growth in sand, plants were transplanted to 18-L trays containing nutrient solution. Seedling-inoculated plants were transplanted 1 h after inoculation. Plant roots were harvested and separated into top roots and lateral roots. Nodules were counted with a light microscope (×16) at 3-d intervals through a 21-d growth period after inoculation. Values represent means of three replicates for each treatment within a sampling time and vertical bars are LSD; n.s. denotes nonsignificant differences among treatments at the 0.05 level.](https://www.plantphysiol.org/doi/abs/10.1104/pp.96.4.1280)
number of infection events but differed in number of infection events that subsequently progressed to form a nodule (19).

In contrast to shoot control of hypernodulation, the nonnodulating mutant NN5 roots failed to nodulate, regardless of whether the shoot used in grafting was from NN5, Williams, or NOD1–3 (Table II). Thus, the inability of the NN5 mutant to form nodules is strictly controlled by the root, not the shoot. This also confirms the conclusion of Delves et al. (11) that nonnodulation is root controlled in mutant nod49, regardless of whether the grafted shoot was that of nts382 or the Bragg parent. It is known from analysis for nod gene inducibility in *B. japonicum* that seedling extracts or exudates from nonnodulating, supernodulating, and the parent (Bragg) lines have similar competence in the signal (20, 22). Nonnodulating mutants are either insensitive to the recognition of the plant signal compounds or unable to convert the cell division stimulus into an actual infection after response to the signal (14). Our previous result (6) also showed that, even though the inoculated NN5 nonnodulating mutant had higher isoflavonoid concentration than did the Williams parent, it did not develop any nodules, nor did it respond to the addition of isoflavonoids to the growth medium. Thus, the nonnodulation characteristic is not related to isoflavonoids (6).

Shoot control of autoregulation of nodulation may involve the presence of shoot-derived inhibitor(s) as well as activator(s). It was reported that injection of methanol/water extracts from the inoculated Bragg wild type (but not uninoculated wild type or either uninoculated or inoculated nts382) suppressed nodulation of the nts382 supernodulating mutant (13). Gresshoff et al. (14) proposed that the suppression activity of nodulation is correlated with an alteration of ABA concentration in the shoot. ABA remained at a preinoculated level in both inoculated supernodulating mutants and uninoculated wild-type plants, but it increased in uninoculated wild-type plants in the shoot after 2 to 3 d, reaching a plateau by day 7 or 8 (14). The current study evaluated the grafting effect of shoots from seed-inoculated or uninoculated plants, which might have different levels of shoot inhibitor(s), on nodulation of seed-inoculated root stocks. The lack of any difference in nodulation pattern of seed-inoculated roots when grafted on day 7 to shoots derived from seedlings from inoculated and uninoculated seed (Table II) indicates that inoculation may not significantly alter the levels of possible shoot inhibitor(s) through day 7. In addition, no significant difference in nodule numbers between NOD1–3 and Williams was detected until after 9 d following seed inoculation (Fig. 3A). These results indirectly support the previous conclusions (3, 19) that subsequent nodule formation is suppressed primarily at the stage of nodule emergence rather than at the stage of initial infection.

Inoculation of 7-d-old grafted plants resulted in a marked increase of nodulation in all grafting treatments of both NOD1–3 and Williams compared with grafting of seed-inoculated plants (Tables I and II). This was likely due to more potential infection sites becoming simultaneously infected on 7-d-old seedlings as opposed to stronger autoregulatory control exercised in seed-inoculated plants. The same observation was made on ungrafted plants (Fig. 3A). This may be causally related to higher root isoflavonoid concentrations in the roots of the older plants during the period of nodule initiation and nodule development. Our previous results (6) showed that isoflavonoid levels increased with age of the roots of uninoculated soybean plants as well as of inoculated plants. Delayed inoculation induced nodule formation mostly on the branched lateral roots, whereas seed inoculation induced nodulation initially on the tap roots with increasing nodule numbers on the lateral roots and in the developing root zone of the tap roots with time (Fig. 3B). This again relates to the potential infectable sites at the time of inoculation. Nodules of delayed-inoculated plants were almost completely eliminated in the upper zone of the tap roots (data not shown). This is consistent with the conclusion of Bhuvaneswari et al. (2) that, when inoculations were delayed for intervals of 1 to 4 h after marking the positions of the root tips, the nodulation above the root tip marks was progressively decreased.

In conclusion, the results shown here support the previous conclusion (6) that isoflavonoids may be involved in differential nodulation expression between hypernodulated and wild-type soybean lines at the advanced stage of nodule ontogeny rather than at the stage of initial infection. The similarity of response to grafting for the hypernodulating and nonnodulating mutants of Williams reported here and for mutants of Bragg reported previously (11) may indicate that the selected mutants involve similar genetic defects.

**LITERATURE CITED**


