Isolation and Initial Characterization of Constitutive Nitrate Reductase-Deficient Mutants NR328 and NR345 of Soybean (Glycine max)¹

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ABSTRACT

Two nitrate reductase deficient mutants of soybean (Glycine max [L.] Merr. cv Bragg) were isolated from approximately 10,000 M₂ seedlings, using a direct enzymic assay in microtiter plates. Stable inheritance of NR345 and NR328 phenotypes has been demonstrated through to the M₅ generation. Both mutants were affected in constitutive nitrate reductase activity. Assayable activities of cNR in nitrate-free grown seedlings was about 3 to 4% of the control for NR345 and 14 to 16% of the control for NR328. Both mutants expressed inducible NR during early plant development and were sensitive to nitrate and urea inhibition of nodulation. These new mutants will allow an extension of the characterization of nitrate reductases and their function in soybean. Preliminary evidence indicates that NR345 is similar to the previously isolated mutant nr₁, while NR328 is different.

Generally, NR² activity is only present when nitrate is present in the growth medium (2), but soybean is an exception to this generality and Harper (10) showed that leaf tissue from inoculated plants grown without combined nitrogen expressed NR activity. Similarly, it was demonstrated that leaf tissue from urea-grown soybean plants had NR activity (18). Since this activity was present in the absence of nitrate, it was termed constitutive or noninducible NR (12). cNR activity is not expressed in soybean roots (15, 21) or soybean cell cultures (22), and NR activity in these tissues is dependent on nitrate being present. Thus, in addition to cNR, there is also iNR activity in soybean. Multiple soybean NR activities have also been distinguished biochemically. Using column chromatography, Jolly et al. (14) and Campbell (3) separated two NR activities from young unifoliolate leaves of nitrate-grown soybean seedlings. The two activities differed in affinities for nitrate and for the reduced pyridine nucleotides NADH and NADPH. These NR activities were also identified in cotyledons of nitrate-grown seedlings (24). Genetic evidence for the presence of multiple NR activities in soybean has been reported by Nelson et al. (21). They isolated three allelic chlorate-resistant mutants (LNR-2, LNR-3, and LNR-4) (26) that lacked cNR, but expressed iNR in root and leaf tissue. Subsequent studies on the parent cultivar Williams and the cNR mutant nr₁ (formerly LNR-2) led to the identification of three biochemically distinct nitrate reductases in soybean, and to the correlation of these activities with cNR and iNR (27). Urea-grown wild-type plants contained two cNRs designated c₁NR and c₂NR. the c₁NR used both NADPH and NADH, whereas c₂NR used only NADH as an electron donor. Only one iNR was identified in nitrate-grown nr₁ plants (that lacked cNR). Like c₁NR, iNR used only NADH as an electron donor (27).

Efficient procedures for assaying NR (16) and the availability of chlorate as a positive selection agent (8, 13) have aided the isolation of nitrate metabolism mutants in several higher plant species (for review see Refs. 12 and 17). In legumes, NR-deficient mutants have been isolated in pea (9, 16) as well as in soybean (21). Such legume mutants are potentially useful for studying nitrate inhibition of nodulation and nitrogen fixation (4–7). In this paper, we describe the isolation and preliminary characterization of two independent NR-deficient mutants of soybean.

MATERIALS AND METHODS

Plant Material. M₂ families of cultivar Bragg were screened for cNR mutants. The families were derived from EMS mutagenesis, which we described in previous publications (5, 7). For comparison, Williams and nr₁ (formerly LNR-2) (21) were included in some experiments. Seeds of nr₁ were obtained from Dr. S. Ryan (CSIRO, Canberra).

Plant Culture. All experiments were conducted in the glasshouse. Glasshouse temperatures were kept between 14 and 30°C and incandescent bulbs extended the photoperiod to 16 h. M₂ seeds were planted 1 cm below the surface in sand trays (63 cm long, 23 cm wide, and 6.5 cm deep). The trays were inoculated with Rhizobium japonicum strain CB1809 (= USDA136), saturated with N-free nutrient solution (6, 7) twice a week and received tap water every other day. Ten to 16 d after planting, unifoliolate leaves were screened for cNR activity (see below). M₂ variants were saved to produce M₃ progeny which were screened for cNR activity as described below. The stability of the mutant character was also verified in M₄ and M₅ seedlings, except that these plants were cultured in 15 cm diameter pots of vermiculite. M₄ plants were also characterized for in vivo NR activity after culture on nitrate, and for nodulation on various nitrogen sources. In these experiments, plants were inoculated with R. japonicum strain CB1809 and cultured in 15 cm diameter pots of river sand (2 plants per pot). The pots were watered daily with 600 ml of nutrient solution. KNO₃ (2.5 mm) and urea (2.5 mm, i.e. 5 mM N) were added to the nutrient solution as required.

In Vivo cNR Screen. One unfoliolate leaf disc (4.5 mm diameter) from each M₃ seedling was screened for cNR activity in a micro-well of a micro-test plate (96F with lid, Nunc [Inter Med], Denmark). Each micro-test plate contained 96 wells. Prior

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² Abbreviations: NR, nitrate reductase; cNR, constitutive nitrate reductase; iNR, inducible nitrate reductase; EMS, ethyl methanesulfonate.
to sampling the leaf discs, 130 μl of NR assay solution (containing 50 mm KNO₃) (23) was dispensed into each well using an eight-tip micro-pipette (50–200 μl, serial no. 10379, Titertek). Leaf discs were removed from seedlings and placed in the assay solution and the plates were then incubated at 28°C for 3 to 4 h. Subsequently, the activity was detected by adding 130 μl of a 1:1 solution of 1% sulfanilamide in 3 N HCl and 0.02% N-napthyl-ethylene diamine HCl in distilled H₂O to each well. This solution turns pink in the presence of nitrite (28), and therefore decreased pink coloration in the micro-well assay indicated decreased cNR activity. The degree of pink coloration is seen as shading around the leaf discs in Figure 1. This screening method was very rapid and inexpensive, and 600 seedlings could be easily screened by one person in a day.

**In vivo NR Assay.** *In vivo* NR activity was determined by a modification of the assay described by Nicholas et al. (23). Six leaf discs (4.5 mm diameter) were sampled from unifoliate leaves and placed in chilled test tubes containing 5 ml of NR assay solution. The assay solution was the same as that used by Nicholas et al. (23) and contained either 0 or 50 mm KNO₃. The tubes were incubated in the dark in a 30°C water bath and samples were removed over a 50 min incubation period for nitrite determination (28). The data were expressed as nmol NO₂⁻ produced·leaf disc⁻¹·h⁻¹ or μmol NO₂⁻·g fresh weight⁻¹·h⁻¹. Each leaf disc weighed approximately 2.7 mg (fresh weight).

**Statistics.** Data were analyzed by analysis of variance, using the general statistical program Genstat (1).

### RESULTS

**Selection and Stability of cNR Mutants NR328 and NR345.**

Approximately 10,000 seedlings were screened for cNR activity (Table I). These plants were derived from 1428 M₂ families; 868 families came from EMS-population 1 and the remaining 560 families were from EMS-population 2. Ten seeds per M₂ family of population 1 were planted, whereas only five seeds were planted for each M₂ family in population 2, and approximately 7,550 and 2,450 M₂ plants were screened in population 1 and population 2, respectively. Thus, about three times as many M₂ plants were screened in population 1 than in population 2 (Table I).

Several M₂ families apparently segregated for decreased cNR activity, but only two M₂ variants produced M₃ progeny that had little or no cNR activity. The two confirmed cNR-deficient mutants were derived from M₂ families 328 and 345, which belonged to population 2 (Table I). In each of these families, one out of the five plants screened was cNR-deficient. These variant plants were designated NR328 and NR345. All three M₂ plants derived from NR328 had decreased cNR activity. Similarly, NR345 was pure-breeding and all 12 M₃ progeny had decreased cNR activity (Fig. 1). Bragg had considerable cNR activity as indicated by the deep shading in micro-wells containing a Bragg leaf disc. In contrast, micro-wells with an nr, NR328, or NR345 leaf disc had almost no color (Fig. 1), indicating that these lines had decreased cNR activity. *In vivo* NR assays were also conducted on unifoliate leaf tissue from these plants and the results are listed in Table II. NR328 had cNR activity significantly less than Bragg, but significantly more than either NR345 or nr, Activity in NR328 was 16.2% that of Bragg, whereas NR345 and nr, had negligible activity compared to Bragg.

**In vivo cNR activity was also determined in unifoliate leaves of M₃ progeny, and the results obtained confirmed the M₂ data (data not shown). Mutants NR345 and nr, had very low activity compared to the respective parent cultivars Bragg and Williams, whereas NR328 had 14.1% of the wild-type activity. The stability of the mutants was also verified in the M₃ generation (data not shown).**

**In Vivo Activity in Nitrate-Grown Plants.** Bragg, NR328, and NR345 plants were grown on nitrate and assayed for NR activity 21 d after planting. Both of the cNR mutants expressed iNR activity (Table III). When NR activity was dependent on endogenous nitrate in the leaf tissue (minus NO₃⁻ assay), activity was the same in Bragg, NR328, and NR345. However, with 50 mm KNO₃ added to the assay solution, Bragg NR activity was significantly higher than the activity in either NR328 or NR345 (Table III).

**Plant Fresh Weight Accumulation in N₂-dependent and Nitrate-grown Plants.** Table IV shows plant fresh weights of Bragg, NR328, and NR345 plants after 7 weeks culture on N-free or KNO₃-supplemented nutrients. Two-way analysis of variance showed that the effect of nitrate was highly significant (calculated *F* = 105.8 > *F*₉,₃₉ [0.05] = 4.17), but that the genotypes were not significantly different and that they did not respond differently to 2.5 mm nitrate being supplied.

**Inhibition of Nodulation by Combined Nitrogen.** A preliminary experiment was conducted to ascertain the inhibitory effect of 2.5 mm nitrate or urea on nodulation of the parent cultivars and the cNR mutants. As previously described for this system (6), urea was more inhibitory than was nitrate. The absolute amount of nodulation per plant was not decreased by treatment with 2.5 mm nitrate (data not shown), but these plants were considerably larger than N₂-dependent plants (Table IV) and the nitrate treatment did cause significant inhibition when nodulation was expressed per g plant fresh weight (Table V). Two-way analysis of variance indicated that the effect of combined nitrogen on nodule number·g⁻¹ plant fresh weight was highly significant (calculated *F* = 135.4 > *F*₉,₃₉ [0.05] = 3.15). Genotypic differences in this parameter were marginally significant (calculated *F* = 2.87 > *F*₉,₃₉ [0.05] = 2.53). However, there was no significant interaction between genotype and nitrogen source (calculated *F* = 0.95 > *F*₉,₃₉ [0.05] = 2.10), indicating that nodule number in the different genotypes was similarly affected by 2.5 mm nitrate or urea. Essentially the same trend was observed for mg nodule fresh weight·g⁻¹ plant fresh weight (Table V). The effect of nitrogen source was highly significant (calculated *F* = 475.9 > *F*₉,₃₉ [0.05] = 3.15). There was also a significant genotype effect (calculated *F* = 10.99 > *F*₉,₃₉ [0.05] = 2.53) and nodule fresh weight per plant biomass was consistently lower in NR328 than in Bragg (Table V). However, the genotypes did not differ in

### Table I. Frequencies of Chi-deficient Variants, nts, and cNR Mutants in Two Mutagenized Populations of Soybean

<table>
<thead>
<tr>
<th>Population</th>
<th>Frequency of Chi-deficient Variants in the M₂</th>
<th>Frequency of nts Mutants in the M₂</th>
<th>No. of M₂ Families Screened for cNR</th>
<th>Approx. No. of M₂ Individuals Screened for cNR</th>
<th>No. of cNR Mutants Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS-1</td>
<td>0.9 × 10⁻²</td>
<td>3.6 × 10⁻⁴</td>
<td>868</td>
<td>7,550</td>
<td>0</td>
</tr>
<tr>
<td>EMS-2</td>
<td>2.8 × 10⁻²</td>
<td>1.4 × 10⁻³</td>
<td>560</td>
<td>2,450</td>
<td>2</td>
</tr>
</tbody>
</table>

* Cited from Carroll et al. (5).
  c Total number of M₂ families screened for cNR activity.
  d Approximate number of M₂ plants screened for cNR activity.
  e Number of mutants recovered from the cNR screen.
FIG. 1. Rapid screening cNR assay on Bragg, nr1, and M3 progeny of NR328 and NR345. For assay procedures see "Materials and Methods." Each micro-well contained a unifoliolate leaf disc from a 14-d-old plant that had been cultured on N-free nutrients. The degree of shading around the leaf disc reflects the amount of nitrite in each micro-well. Row 1, Bragg; row 2, nr1 (formerly LNR-2); row 3, NR328; row 4, NR345; and rows 5 to 8, Bragg.

Table II. In vivo cNR Activity of Bragg, nr1, and M3 Progeny of NR328 and NR345

Unifoliolate leaves were assayed (+ NO3 assay) 16 d after planting and culture in sand trays. The trays were watered with N-free nutrient solution as described in "Materials and Methods." Each entry in the table is the mean of three or four assays on leaf tissue from separate plants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NR Activity</th>
<th>log (NR Activity)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol NO3⁻·disc⁻¹·h⁻¹</td>
<td></td>
</tr>
<tr>
<td>Bragg</td>
<td>15.7</td>
<td>2.74</td>
</tr>
<tr>
<td>NR328</td>
<td>2.6</td>
<td>0.90</td>
</tr>
<tr>
<td>NR345</td>
<td>0.6</td>
<td>-0.88</td>
</tr>
<tr>
<td>nr1</td>
<td>0.5</td>
<td>-0.60</td>
</tr>
</tbody>
</table>

* Raw data required log transformation to satisfy assumptions for analysis of variance. The LSD0.05 (based on transformed data) was 0.48.

Table III. In vivo NR Activity of Nitrate-grown Bragg and M3 Progeny of NR328 and NR345

Unifoliolate leaves were assayed 21 d after planting and culture in pots of river sand. The pots were inoculated with R. japonicum strain CB1809 and watered daily with 2.5 mM KNO3-supplemented nutrient solution. Leaf tissue was assayed with and without 50 mM KNO3 in the assay solution. Each entry in the table is the mean of six to eight plants.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NR Activity</th>
<th>Minus NO3⁻ assay*</th>
<th>Plus NO3⁻ assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol NO3⁻·disc⁻¹·h⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bragg</td>
<td>9.1</td>
<td>18.2</td>
<td></td>
</tr>
<tr>
<td>NR328</td>
<td>8.9</td>
<td>14.1</td>
<td></td>
</tr>
<tr>
<td>NR345</td>
<td>8.9</td>
<td>11.8</td>
<td></td>
</tr>
</tbody>
</table>

* No significant difference between genotypes. b F-statistic was significant and LSD0.05 = 3.7.

sensitivity to combined nitrogen (calculated $F_{interaction} = 1.74 < F_{0.05} [0.05] = 2.15$). Urea-grown plants in these experiments had considerable NR activity without nitrate being added to the assay solution (data not shown). Evidently, in this sand culture, considerable nitrification of urea had occurred. Nevertheless, it is evident that cNR-deficiency did not confer nitrate-tolerant nodulation in nr1, NR328, or NR345.

Table IV. Effect of Nitrate on Plant Fresh weight Accumulation in Bragg and M3 Progeny of NR328 and NR345

Plants were inoculated with R. japonicum strain CB1809, cultured in sand pots as described in "Materials and Methods" and harvested 7 weeks after planting. Each entry in the table is the mean of six plants. Two-way analysis of variance on the data indicated that the effect of nitrate supplementation was significant, but that there was no difference between the genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plant Fresh Weight</th>
<th>g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bragg</td>
<td>16.2</td>
<td>45.7</td>
</tr>
<tr>
<td>NR328</td>
<td>8.2</td>
<td>43.4</td>
</tr>
<tr>
<td>NR345</td>
<td>11.6</td>
<td>34.7</td>
</tr>
</tbody>
</table>

DISCUSSION

The results presented here show that it is possible to screen successfully for soybean mutants in a specific enzyme activity. Two cNR mutants, NR328 and NR345, were isolated using an in vivo NR screen. An in vivo assay procedure has been previously used to isolate NR mutants in barley (29) and pea (16). In contrast to the approach used to identify NR328 and NR345, cNR soybean mutant nr1 (formerly LNR-2) was isolated using chlorate as a positive selection agent (21). The screening procedure described here could also be useful in screening remutagenized cNR-deficient lines for nitrate uptake and nIR mutants. The direct assay for NR activity may have advantages over testing for chlorate resistance, in view of possible secondary effects of chlorate (8).

NR328 and NR345 were recovered from separate M3 families. Both these families segregated for the mutant character, indicating that the mutation arose from the mutagenesis treatment and also that NR328 and NR345 were the result of separate mutation events. The frequency of ns (nitrate-tolerant symbiotic mutants), cNR, and Chl-deficient mutants was consistently higher in population 2 than in population 1 (Table I). M2 selections NR328 and NR345 were pure breeding and homozygous for the respective mutations and the stability of the mutant characters have been demonstrated through to the M5 generation. NR345 and nr1 had negligible cNR activity, whereas NR328 had approximately 15% of the wild-type activity (Table II). Constitutive NR-deficient mutant nr1 lacks both c,NR and c2 NR activity that are present in parent cultivar Williams (27). Studies on wild-type Bragg grown in the absence of nitrate have shown that this cultivar expresses c2 NR and c2 NR activity (19). It therefore appears that NR345 described here also lacks c1 NR and c2 NR activity. However, it is plausible that NR328 lacks only one of the cNR activities or, alternatively, this mutant may be leaky and have decreased amounts of both cNR activities. Allelism tests are being conducted on nr1, NR328, and NR345. Crosses have also been made between Bragg and the mutants to determine the nature of inheritance of the NR328 and NR345 characters.

Both NR328 and NR345 expressed nIR activity (Table III). When NR activity was dependent on endogenous nitrate (minus NO3⁻ assay), unifoliolate leaves of nitrate-grown Bragg, NR328, and NR345 plants had the same NR activity. The addition of nitrate to the assay medium resulted in Bragg having significantly higher NR activity than NR328 and NR345. It is likely, therefore, that Bragg had more NR enzyme (presumably cNR) present in situ, and that substrate supply limited activity in the minus NO3⁻ assay. The presence of nIR in NR328 and NR345 was also reflected in the fact that nitrate stimulated plant fresh weight accumulation in nitrate-grown NR328 and NR345 plants. Thus, NR328 and NR345 are similar to nr1, in that nIR was expressed...
and nitrate utilization was evident. However, as well as being leaky, NR328 was also unique in that nitrate-grown plants developed necrotic lesions on the distal margin of mature leaves (D. Whitmore Smith, BJ Carroll, unpublished data). This did not occur in N2-dependent NR328 plants and thus the necrosis observed in nitrate-grown plants was probably due to nitrate accumulation. Necrosis of mature leaf margins was not evident in nitrate-grown Bragg, NR345, or nr1 plants and it is therefore possible that INR activity is impaired at some stages of development in NR328. These hypotheses are currently being tested.

Mutant nr1 has been extensively characterized. The mutant character is inherited as a Mendelian monogenic recessive (26), but it is not clear whether the mutation is located in a regulatory gene or in a structural gene required for both cNR and cNR activities (25). Mutant nr1 also lacks in vivo NO3 evolution normally present in the wild-type (26). The NO3 compound evolved during an in vivo NR assay (11) has since been identified as being predominantly acetaldehyde oxime (20). All F2 cNR-deficient segregates derived from Williams · nr1 crosses also lacked acetaldehyde oxime evolution, and all cNR-positive segregants (i.e. wild-types) evolved acetaldehyde oxime (26). Furthermore, soybean root tissue (21) and cell cultures (22) lack both cNR activity and in vivo acetaldehyde oxime evolution. Although these genetic and physiological studies indicated that in vivo acetaldehyde oxime evolution is associated with cNR activity, the nature of this association has not been further characterized. NR328 and NR345 may contribute to the knowledge on acetaldehyde oxime evolution and its relationship to cNR activity.

As reported by Ryan et al. (26), the nr1 mutation did not affect nitrate utilization or confer nitrate-tolerance to nitrogen fixation. The results presented here also showed that cNR-deficiency in NR328, NR345, and nr1 did not condition nitrate-tolerant nodulation in these lines. Both nodule number and nodule growth (Table V) in the mutants were as sensitive as the wild type to nitrate and urea inhibition. Although nitrogen fixation does not appear to be nitrate-tolerant in nr1 (26), this area needs to be investigated in NR328 and NR345. The cNR constitutes approximately 12.5 to 20% of total NR activity in soybean (12), but the physiological significance of this form of NR remains obscure. The fact that nr1 was selected on the basis of chlorate resistance implies that cNR reduces nitrate in planta (assuming nitrate and chlorate behave the same in this respect). However, cNR activity does not appear to be regulated by nitrate (21) or ammonia (19) and lack of cNR activity does not impede nitrate utilization (26). Further characterization of NR328 and NR345 may lead to a better understanding of the role of cNR in soybean.

Table V. Effect of KNO3 and Urea on Nodulation per Gram Plant Fresh weight in Bragg, Williams, nr1, and M4, Progeny of NR328 and NR345

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Nodule</th>
<th>Nodule</th>
<th>Number of plant fresh wt</th>
<th>mg g-1 of plant fresh wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N-free</td>
<td>2.5 mm KNO3</td>
<td>2.5 mm urea</td>
<td>2.5 mm KNO3</td>
</tr>
<tr>
<td>Bragg</td>
<td>6.5</td>
<td>2.9</td>
<td>0.9</td>
<td>69.4</td>
</tr>
<tr>
<td>NR328</td>
<td>5.1</td>
<td>2.5</td>
<td>0.7</td>
<td>53.8</td>
</tr>
<tr>
<td>NR345</td>
<td>10.1</td>
<td>2.7</td>
<td>0.7</td>
<td>87.2</td>
</tr>
<tr>
<td>Williams</td>
<td>5.1</td>
<td>2.2</td>
<td>0.9</td>
<td>53.1</td>
</tr>
<tr>
<td>nr1</td>
<td>5.3</td>
<td>1.7</td>
<td>0.9</td>
<td>66.4</td>
</tr>
</tbody>
</table>

*Raw data required log, and square root transformation, respectively, to satisfy assumptions for a two-way analysis of variance.

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