Soybean Mutants Lacking Constitutive Nitrate Reductase Activity

II. NITROGEN ASSIMILATION, CHLORATE RESISTANCE, AND INHERITANCE

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ABSTRACT

Nitrogen assimilation in three nitrate reductase (NR) mutants of soybean (Glycine max L. Merr. cv. Williams) was studied in the growth chamber and in the field. These mutants, LNR-2, LNR-3, and LNR-4, lack the non-N03- inducible or constitutive fraction of leaf NR activity found in wild-type plants, but this had no effect on the concentration of nitrogen accumulated when grown on N03- in the growth chamber. Dry weight accumulation of two of the mutants (LNR-3 and LNR-4) was decreased relative to LNR-2 and wild type. In the field, LNR-2 had dry weights and nitrogen concentrations similar to the wild type at 34 and 61 days after planting, and at maturity. Acetylene reduction activities were also similar at 61 days.

Urea-grown LNR-2 seedlings lack both inducible and constitutive NR activity, and were resistant to four days of treatment with 0.5 mM ClO3-. Urea-grown wild-type seedlings, having only constitutive NR activity, developed ClO3- toxicity symptoms and suffered decreases in unifoliate leaf NR activity and chlorophyll concentration. This suggests that (a) the reduction of ClO3- to ClO2- by NR is the major cause of ClO3- toxicity in soybeans and (b) the constitutive NR is active in situ.

Segregation of the F2 of reciprocal crosses between the wild type and the mutants indicated that absence of constitutive NR activity was controlled by a single recessive nuclear gene. Evolution of NO3--gas was also absent in these mutants, and this was found to be inherited jointly with constitutive NR activity: in 346 segregants, no recombinants were found. Allelism tests between LNR-2 and LNR-3, and LNR-2 and LNR-4, indicated that the constitutive NR mutation was at the same locus in each mutant.

NR2 mutants have proven to be useful tools in the study of N03- assimilation in fungi (3). More recently, NR mutants have been isolated in several species of higher plants, including Arabidopsis thaliana (21), pea (5, 24), barley (22, 25), and soybean (19).

In the A. thaliana mutant, B25 (1), and the pea mutants, E1, A300, A317, and A334 (5, 24), the reduction of N03- is impaired by NR deficiency, and NO3- accumulates in the tissue. Due to the shortage of reduced N or to toxicity of the accumulated NO3-, the B25 and E1 plants grow poorly or die when N03- is the sole N source (1, 5). However, in the barley mutants Az 12 and Az 13, assimilation of NO3- is impaired despite an apparent 90% decrease in the in vivo level of NR activity (23). Similarly, the soybean mutant LNR-2 appears to grow normally on N03- despite its lack of constitutive NR activity (19). However, this mutant still has inducible NR activity.

The response of NR mutants to ClO3- has also produced some conflicting results. Direct evidence that the primary toxic effect of ClO3- is due to its reduction by NR to ClO2- has been difficult to establish (8). However, the principle that NR-deficient organisms should be resistant to ClO3- has been successfully used to isolate NR mutants in many organisms although not all NR-deficient organisms are tolerant to ClO3-. The barley NR mutants which were selected by NR assay, are still ClO3- sensitive (23), and tobacco cell lines which lack NR activity exhibit differential ClO3- resistance (16). Therefore, it has been suggested that ClO3- can have a toxic effect above that due to its reduction by NR (12, 16). Conversely, not all ClO3--resistant organisms are NR deficient. Both A. thaliana plants (21) and Rosa damascena cells (17) have been isolated which apparently have an NR-independent mechanism for ClO3- resistance.

The soybean NR mutants also lack the ability to evolve NO3- gases from the in vivo NR assay, a phenomenon exhibited by wild-type soybeans (19). Although it is not known whether evolution of NO3- from NO3- is enzymic (7) or due to a reaction with a metabolite (14), a two-step process from NO3- to NO3- to NO3- is implicated. Inasmuch as mutagenesis provided the variation in the material screened for the soybean mutants, a single gene (possibly regulatory) for the control of both constitutive NR activity and NO3- evolution seemed possible.

The present study further characterizes the soybean NR mutants previously described (19). Specifically, the objectives were to (a) determine the effect of the absence of constitutive NR on NO3- assimilation, (b) investigate the response of the mutants to ClO3-, and (c) determine the inheritance of the NR mutations and the genetic relationship between constitutive NR activity and NO3- evolution.

MATERIALS AND METHODS

Plant Growth. Growth Chamber. Seed of wild-type soybean (Glycine max L. Merr. cv. Williams), mutant lines LNR-2, LNR-3, and LNR-4 (19), F2 of mutant x wild-type crosses, and F3 lines of LNR-2 x wild type were germinated in a growth chamber in sand subirrigated with deionized H2O. At 7 DAP, seedlings were transplanted to 2 L black plastic pots (six plants per pot) containing, at full strength (X), a basic nutrient solution of 1.0 mM MgSO4, 18.0

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3 Abbreviations: NR, nitrate reductase; LNR, low nitrate reductase; NO3-3, NO and NO3 collectively; DAP, days after planting.
NR MUTANTS: N ASSIMILATION AND INHERITANCE

µM Fe, 25.0 µM KCl, 12.5 µM H₂BO₃, 3.0 µM MnSO₄, 1.0 µM ZnSO₄, 2.5 µM CuSO₄, and 0.0075 µM (NH₄)₂MoO₄. Remaining nutrients were added with NO₃⁻ (2.5 mM Ca(NO₃)₂, 2.5 mM KNO₃, and 0.5 mM K-phosphate buffer, pH 6.5) or with urea (3.75 mM urea, 2.5 mM CaCl₂, 1.25 mM K₂SO₄, and 0.25 mM K-phosphate, pH 6.5). Nutrient solutions were continuously aerated and the pH of urea solutions was maintained with ion-exchange columns (10). Plants were provided with 14 h/28°C light periods and 10 h/18°C dark periods during germination and growth. RH was maintained at about 50%. Light flux density (PAR), supplied by fluorescent and incandescent lamps, was 600 µE m⁻² s⁻¹.

For the N assimilation experiment, LNR-4 seeds were planted 2 d earlier than LNR-2, LNR-3, and wild-type seed; germination and initial growth of LNR-4 was slower than for other plant types. Four pots of each genotype were randomly placed in each of two growth chambers. Initial nutrient concentrations were 2X, and these were replaced with 1X strength at 10 and 13 DAP. Plants were harvested at 16 DAP. For each genotype, one plant was harvested from random pots from each genotype. These plants were composited to give one sample. This was repeated to give three replications. Plants were harvested at 2 d earlier (just before the first trifoliolate leaf), stems plus petioles, and leaves. Young leaves which had not unfurled were included in the stem fraction. Dried plant material (60°C for 72 h) was weighed and analyzed for total-N and NO₃⁻-N content.

For the ClO₃⁻ experiment, wild-type and LNR-2 seedlings were transplanted at 5 DAP. Three replications of the eight treatment combinations of genotype, N-source, and presence or absence of ClO₃⁻ were completely randomized among 24 pots. Nutrient strength was 1/2X and ClO₃⁻ was supplied as 0.5 mM KClO₃ added at transplanting. All pots contained ion-exchange columns. At 9 DAP when unifoliate leaves were fully expanded, three plants per pot were composited and partitioned into unifoliate leaf and remainder of the plant, for dry matter determination. The unifoliate leaves of the remaining three plants per pot were bulked for NR assay and determination of Chl content. Residual leaf material (minus midribs) was used to determine a dry matter conversion factor. Results were analyzed in an analysis of variance.

For the genetic experiments, seedlings were transplanted into 1X urea nutrient solution. Each pot contained six individual F₂ seedlings from two F₂ families of three seedlings each. Nine plants were tested in each of 16 randomly chosen F₂ families. The nutrient solution was changed at 12 DAP and the first trifoliolate leaf of individual plants was assayed for NR activity and NO₃⁻ evolution at 14 DAP.

Field. Wild-type and LNR-2 seeds were sown in the field in a randomized complete block design with five replicates. At 34 and 61 DAP, four plants per plot were harvested at the unifoliate leaf node and partitioned into leaf and stem plus petiole fractions. Roots were also harvested at 61 DAP for acetylene reduction assay. At maturity (122 DAP), eight plants per plot were harvested and threshed. Clean seed (8% moisture) and dried stem and pods were weighed and analyzed for total-N and NO₃⁻-N concentrations. Results were analyzed in an analysis of variance.

Assays. Nitrate Reductase. For the N assimilation, ClO₃⁻, and field experiments, in vivo NR activity was assayed as described previously (20). For the genetic experiments, the method was modified to enable rapid qualitative evaluation of the presence or absence of NR activity. A single 12-mm diameter leaf disc was cut and lightly jammed in the bottom of a 12- x 100-mm test tube sitting in ice. A 2-ml aliquot of incubation medium (0.1 M K-phosphate [pH 7.5], 50 mM KNO₃, 1% [v/v] propanol) was added and the tubes were vacuum infiltrated twice for 1 min, any discs floating to the surface being resubmerged between and following the infiltration steps. Tubes were then incubated at 30°C for 15 min. The reaction was stopped and color developed in one step by adding 3 ml of color reagent (4.5 g sulfanilic acid, 170 ml HCl, 0.09 g 1-naphthylethylene-diamine diHCl/liter). Tubes were scored for presence of color after 20 min.

NO₃⁻ Evolution. The presence of NO₃⁻ evolution was determined using the system described by Harper (7) except that single discs were prepared as described above, and each small tube was inserted into one of the 25- x 150-mm foil-covered tubes containing 5 ml water for rapid temperature equilibration. The incubation medium was the same as for the NR assay, and the assay was run for 20 min using a single aliquot of trapping solution. Samples which gave no NO₃⁻ were immediately evaluated for response to NO₂⁻ by replacing the incubation medium with 2 ml of fresh medium [10 mM KNO₃, 0.1 mM K-phosphate [pH 7.5], 1% [v/v] propanol], vacuum infiltrating as before, and reassaying for NO₃⁻ evolution.

Total-N, NO₃⁻-N, and Reduced-N. Total-N was determined by the method of Nelson and Sommers (18). Nitrate was extracted in water at 60°C for 90 min and then determined using the E. coli method (15). Reduced-N was calculated as total-N minus NO₃⁻-N.

Chl. Tissue was ground in absolute methanol in a mortar and pestle. After filtration (Whatman No. 1 qualitative), an aliquot was diluted 5- or 10-fold with absolute methanol and A determined at 650 and 665 nm. Chl concentration was calculated according to Holden (11).

Acetylene Reduction. Acetylene reduction was analyzed by GC (9).

RESULTS

N Assimilation. The NR mutants (LNR-2, LNR-3, and LNR-4) maintained total N concentrations similar to the wild type when grown on NO₃⁻ in the growth chamber (Fig. 1). There was little difference between the lines in NO₃⁻ concentration. LNR-3 accumulated more reduced-N in the stems plus petioles fraction, but this did not result in a significantly greater accumulation in the whole plant (data not shown). Dry matter accumulation was the same for LNR-2 and the wild type but was decreased in all plant parts for LNR-3 and LNR-4 (Fig. 1).

In the field, LNR-2 again maintained N concentrations (total-N and NO₃⁻-N) and dry weights similar to the wild type (Table I). Acetylene reduction activity at 61 DAP was also similar (25.1 and 20.9 µmol C₅H₄ plant⁻¹ h⁻¹ for the wild type and LNR-2, respectively, not statistically different).

Chlorate. NR activities, Chl concentrations, and dry weights of unifoliate leaves were decreased by ClO₃⁻ in all treatments having measurable NR activity in the untreated plants (Fig. 2). Urea-grown LNR-2 plants, which had no unifoliate leaf NR activity, did not develop the typical ClO₃⁻ toxicity symptoms observed in the other ClO₃⁻-treated plants. Over the 4 d of the ClO₃⁻ treatment, the effects of ClO₃⁻ were restricted to the unifoliate leaf since the dry weight of the remainder of the plant was the same in all treatments. The constitutive NR activity, characterized by wild-type plants grown on urea, and the inductive activity, characterized by LNR-2 plants grown on NO₃⁻, were both inhibited by ClO₃⁻ treatment (Fig. 2).

Genetics. Segregation of the F₂ of crosses between the wild type and the mutants indicated that, in each case, absence of constitutive NR activity and NO₃⁻ evolution was controlled by a single recessive nuclear gene (Table II). A total of 346 segregants was tested for presence of constitutive NR activity and NO₃⁻ evolution. In those segregants lacking NR activity, NO₃⁻ was supplied...
evolution had on dependent plants without recessive double of activity was a nine four lines had both factors a wild-type activity, no other groups could be distinguished. The ability of exogenous NO$_3^-$ to act as a substrate for NO$_3$ evolution had previously been verified on urea-grown wild-type plants without NO$_3$ present in the assay (data not shown). NR activity was quantified in individual F$_2$ plants to determine the degree of dominance of the constitutive NR gene. Apart from the double recessive individuals which totally lacked constitutive NR activity, no other segregation groups could be distinguished. Joint inheritance of constitutive NR activity and NO$_3$ evolution as a single dominant nuclear gene was confirmed in 16 F$_3$ lines of a wild-type $\times$ LNR-2 cross. Three lines were totally absent in both factors, four lines had both factors present in every plant, and the remaining nine lines were still segregating ($X^2 = 0.38, 0.75 < P < 0.90$ for a 1:2:1 expected ratio). Allelism tests between LNR-2 and LNR-3, and LNR-2 and LNR-4, indicated that all the mutations were at the same locus. Analyses of 10 F$_2$ seedlings of LNR-2 $\times$ LNR-3, and 10 F$_2$ seedlings of LNR-2 $\times$ LNR-4 showed that all lacked both constitutive NR activity and NO$_3$ evolution.

**DISCUSSION**

The absence of constitutive NR activity in the mutants, LNR-2, LNR-3, and LNR-4 (19), did not increase NO$_3^-$ -N or decrease reduced-N concentrations in the plants (Fig. 1). Absolute amounts of N assimilated were depressed in LNR-3 and LNR-4, but this was attributed to a decreased dry matter accumulation. Field results with LNR-2 confirmed that dry matter and N accumulation were unaffected in this mutant. In LNR-3 and LNR-4, the decrease in dry matter accumulation could have been due to pleotropic effects of their mutations in the constitutive NR gene, to deletion of a sequence of adjacent genes, or to additional independent mutations. Current evidence does not permit distinction between these possibilities.

The ability of these soybean mutants to maintain apparently normal N metabolism, despite lowered NR activity, is similar to the barley mutants of Warner and Kleinhofs (23). They suggested that the 10% residual level of NR in the barley mutants was insufficient to account for the NO$_3^-$ reduced, and that perhaps NO$_3^-$ was reduced by a NR with a different cofactor specificity, or by an enzyme other than the conventional NR. Recently, Dailey et al. (4) have shown a NAD(P)H-bispecific NR enzyme in the Az 12 barley mutant which differs from the NR enzyme in the wild type and may account for the residual NR activity in this mutant. Maintenance of normal N metabolism in the soybean mutants questions the role of constitutive NR in the plant. However, the presence of ClO$_3^-$ toxicity effects in urea-grown wild-type plants (Fig. 2) indicated that the constitutive NR does reduce

![Figure 1](https://www.plantphysiol.org)
The absence of ClO₃⁻ toxicity symptoms in the urea-grown mutant suggested that the major effect of ClO₃⁻ on soybeans was due to its reduction by NR to ClO₂⁻, at least with the concentration and treatment duration used. These results contrast with barley where the wild type was more sensitive to ClO₂⁻ when NR was not induced, and where mutants having 10% of normal NR activity were still sensitive to ClO₂⁻ (23). Although the response to ClO₂⁻ appears to be somewhat species dependent, ClO₂⁻ has been successfully used to identify NR mutants in soybeans (19), peas (5), and barley (22), and should continue to be effective in other species. However, based on the pattern of development of constitutive and inducible NR activity in soybeans (19), we believe that ClO₂⁻ cannot be used to isolate totally NR-deficient mutants in this species unless a double mutation is present, or unless a gene common to the functions of both NR enzymes can be affected.

The absence of constitutive NR was inherited as a single recessive nuclear gene. This is in common with most other plant NR mutations (1, 5, 13). The cause of the very close association of constitutive NR with NO₃⁻ evolution in soybeans cannot be determined at this stage. The biochemical nature of NO₃⁻ evolution is not known (7), or whether it is indeed an enzymic process (14). Nevertheless, the data presented here and in a previous paper (19) indicate a close genetic and biochemical relationship between NO₃⁻ evolution and constitutive NR activity. We cannot discount the possibility of two very closely linked genes, although no recombinants were found among 346 segregants. Deletions and chromosome rearrangements have been reported when gamma irradiation is used (2) and this was one of the mutants used to obtain our soybean mutants (19).

The LNR-2 mutant appeared normal with respect to nitrogen fixation measured by acetylene reduction. This contrasts with the E₁ NR mutant of pea in which inhibition of acetylene reduction by nitrate was less than in the wild type (6). However, the pea mutant had only 20% of the normal in vivo NR activity, and was impaired in N metabolism (5). The LNR-2 soybean mutant has 75% of normal canopy NR activity (19) and does not appear to be impaired in N metabolism.

We are currently remutagenizing seed of LNR-2 in an effort to isolate double NR mutants. Selection of revertants may also clarify whether constitutive NR activity and NO₃⁻ evolution in our mutants are controlled by one or two genes.

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