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Soybean Mutants Lacking Constitutive Nitrate Reductase Activity[1]

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-NO_3^--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 NO_3^- 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 ClO_3^-. Urea-grown wild-type seedlings, having only constitutive NR activity, developed ClO_3^- toxicity symptoms and suffered decreases in unifoliolate leaf NR activity and chlorophyll concentration. This suggests that (a) the reduction of ClO_3^- to ClO_2^- by NR is the major cause of ClO_3^- toxicity in soybeans and (b) the constitutive NR is active *in situ*.

Segregation of the F_2 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 $NO_{(x)}$ 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.

NR[3] mutants have proven to be useful tools in the study of NO_3^- 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, E_1, A300, A317, and A334 (5, 24), the reduction of NO_3^- is impaired by NR deficiency, and NO_3^- accumulates in the tissue. Due to the shortage of reduced N or to toxicity of the accumulated NO_3^-, the B25 and E_1 plants grow poorly or die when NO_3^- is the sole N source (1, 5). However, in the barley mutants Az 12 and Az 13, assimilation of NO_3^- is unimpaired 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 NO_3^- despite its lack of constitutive NR activity (19). However, this mutant still has inducible NR activity.

The response of NR mutants to ClO_3^- has also produced some conflicting results. Direct evidence that the primary toxic effect of ClO_3^- is due to its reduction by NR to ClO_2^- has been difficult to establish (8). However, the principle that NR-deficient organisms should be resistant to ClO_3^- has been successfully used to isolate NR mutants in many organisms although not all NR-deficient organisms are tolerant to ClO_3^-. The barley NR mutants which were selected by NR assay, are still ClO_3^- sensitive (23), and tobacco cell lines which lack NR activity exhibit differential ClO_3^- resistance (16). Therefore, it has been suggested that ClO_3^- can have a toxic effect above that due to its reduction by NR (12, 16). Conversely, not all ClO_3^--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 ClO_3^- resistance.

The soybean NR mutants also lack the ability to evolve $NO_{(x)}$ gases from the *in vivo* NR assay, a phenomenon exhibited by wild-type soybeans (19). Although it is not known whether evolution of $NO_{(x)}$ from NO_2^- is enzymic (7) or due to a reaction with a metabolite (14), a two-step process from NO_3^- to NO_2^- to $NO_{(x)}$ 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 $NO_{(x)}$ 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 NO_3^- assimilation, (b) investigate the response of the mutants to ClO_3^-, and (c) determine the inheritance of the NR mutations and the genetic relationship between constitutive NR activity and $NO_{(x)}$ 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), F_2 of mutant x wild-type crosses, and F_3 lines of LNR-2 × wild type were germinated in a growth chamber in sand subirrigated with deionized H_2O. 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 $MgSO_4$, 18.0

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[3] Abbreviations: NR, nitrate reductase; LNR, low nitrate reductase; $NO_{(x)}$, NO and NO_2 collectively; DAP, days after planting.

μM Fe, 25.0 μM KCl, 12.5 μM H_3BO_3, 3.0 μM $MnSO_4$, 1.0 μM $ZnSO_4$, 2.5 μM $CuSO_4$, and 0.0075 μM $(NH_4)_6Mo_7O_{24}$. Remaining nutrients were added with NO_3^- (2.5 mM $Ca[NO_3]_2$, 2.5 mM KNO_3, and 0.5 mM K-phosphate buffer, pH 6.5) or with urea (3.75 mM urea, 2.5 mM $CaCl_2$, 1.25 mM K_2SO_4, 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 at random from each pot and these eight plants were composited to give one sample. This was repeated to give three replications. Plants were separated into roots (cut just below cotyledonary node), 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 NO_3^--N content.

For the ClO_3^- 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 ClO_3^- were completely randomized among 24 pots. Nutrient strength was 1/2X and ClO_3^- was supplied as 0.5 mM $KClO_3$, added at transplanting. All pots contained ion-exchange columns. At 9 DAP when unifoliolate leaves were fully expanded, three plants per pot were composited and partitioned into unifoliolate leaf and remainder of the plant, for dry matter determination. The unifoliolate 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 F_2 seedlings or two F_3 families of three seedlings each. Nine plants were tested in each of 16 randomly chosen F_3 families. The nutrient solution was changed at 12 DAP and the first trifoliolate leaf of individual plants was assayed for NR activity and $NO_{(x)}$ 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 unifoliolate 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 NO_3^--N concentrations. Results were analyzed in an analysis of variance.

Assays. *Nitrate Reductase.* For the N assimilation, ClO_3^-, 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- × 100-mm test tube sitting in ice. A 2-ml aliquot of incubation medium (0.1 M K-phosphate [pH 7.5], 50 mM KNO_3, 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 *n*-1-naphthylethylene-diamine diHCl/liter). Tubes were scored for presence of color after 20 min.

$NO_{(x)}$ Evolution. The presence of $NO_{(x)}$ 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- × 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 $NO_{(x)}$ were immediately evaluated for response to NO_2^- by replacing the incubation medium with 2 ml of fresh medium (10 mM KNO_2, 0.1 M K-phosphate [pH 7.5], 1% [v/v] propanol), vacuum infiltrating as before, and reassaying for $NO_{(x)}$ evolution.

Total-N, NO_3^--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 NO_3^--N.

Chl. Tissue was ground in absolute methanol in a mortar and pestle. After filtration (Whatman[4] 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 NO_3^- in the growth chamber (Fig. 1). There also was little difference between the lines in NO_3^- 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 NO_3^--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 C_2H_4 $plant^{-1}$ h^{-1} for the wild type and LNR-2, respectively, not statistically different).

Chlorate. NR activities, Chl concentrations, and dry weights of unifoliolate leaves were decreased by ClO_3^- in all treatments having measurable NR activity in the untreated plants (Fig. 2). Urea-grown LNR-2 plants, which had no unifoliolate leaf NR activity, did not develop the typical ClO_3^- toxicity symptoms observed in the other ClO_3^--treated plants. Over the 4 d of the ClO_3^- treatment, the effects of ClO_3^- were restricted to the unifoliolate 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 inducible activity, characterized by LNR-2 plants grown on NO_3^-, were both inhibited by ClO_3^- treatment (Fig. 2).

Genetics. Segregation of the F_2 of crosses between the wild type and the mutants indicated that, in each case, absence of constitutive NR activity and $NO_{(x)}$ 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 $NO_{(x)}$ evolution. In those segregants lacking NR activity, NO_2^- was supplied

[4] Mention of a trademark, vendor, or proprietory product does not constitute a guarantee or warranty of the vendor or product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other vendors or products that may also be suitable.

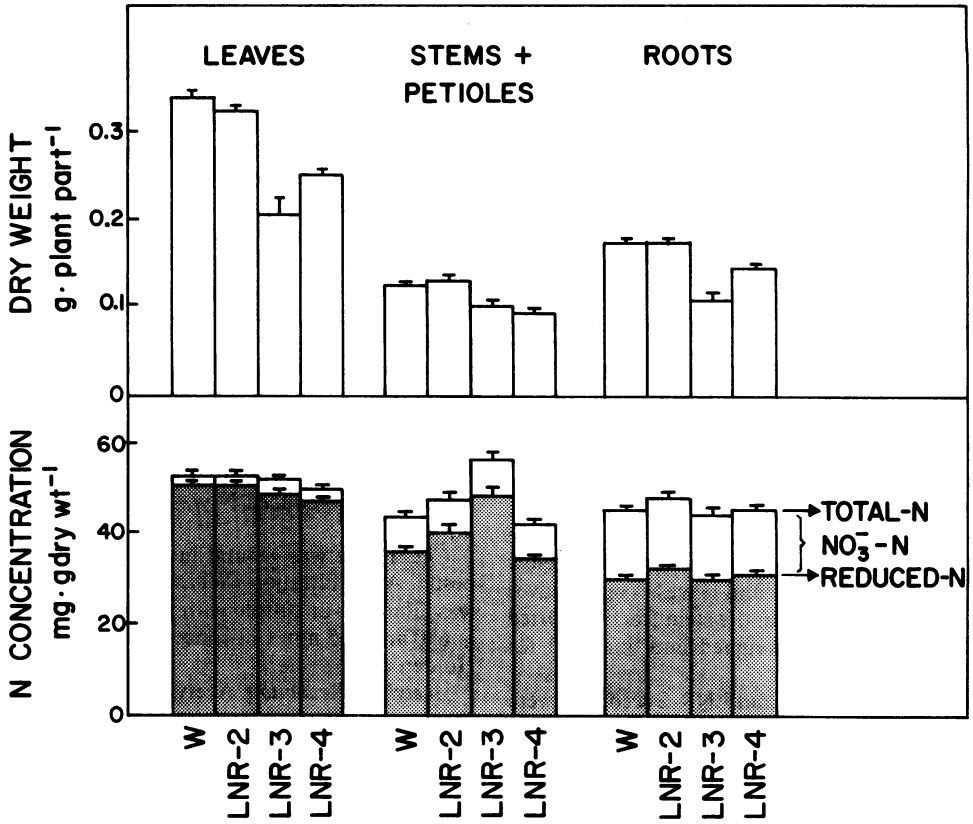

FIG. 1. Concentrations of total-N, NO_3^--N, and reduced-N, and dry weights of plant parts for wild-type (W) and NR mutants (LNR-2, LNR-3, LNR-4). Plants were grown on NO_3^- and harvested at 16 DAP (18 DAP for LNR-4). Each point is the mean ± SE for three replicates.

Table I. *Total-N, NO_3^--N, and Dry Weight of Wild-Type and LNR-2 Plant Parts in the Field*

Tissue	Plant Age	Total-N		NO_3^--N		Dry Wt	
		Wild type	LNR-2	Wild type	LNR-2	Wild type	LNR-2
	DAP	*mg N·g⁻¹ dry wt*				*g·plant⁻¹*	
Leaf	34	51.4	50.8	1.1	1.0	2.0	1.9
Stem + petiole		31.9	31.8	5.9	6.1	0.9	0.9
Leaf	61	60.1	59.2	0.2	0.2	14.2	12.7
Stem + petiole		19.4	21.5*[a]	2.2	2.6	17.5	14.8
Stem + pod	122	11.2	10.0*	0.3	0.3	23.3	25.6
Seed		69.8	70.8	0.1	0.1	25.0	25.7

[a] (*), Significant difference between wild type and LNR-2; $P < 0.05$.

exogenously and, therefore, $NO_{(x)}$ evolution should not have been dependent on NR activity to supply substrate. However, no recombinants were found even when exogenous NO_2^- was supplied. The ability of exogenous NO_2^- to act as a substrate for $NO_{(x)}$ 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 $NO_{(x)}$ evolution as a single dominant nuclear gene was confirmed in 16 F_3 lines of a wild-type × 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 ($\chi^2_2 = 0.38$, $0.75 < P <$ 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 × LNR-3, and 10 F_2 seedlings of LNR-2 × LNR-4 showed that all lacked both constitutive NR activity and $NO_{(x)}$ 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 pleiotropic 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

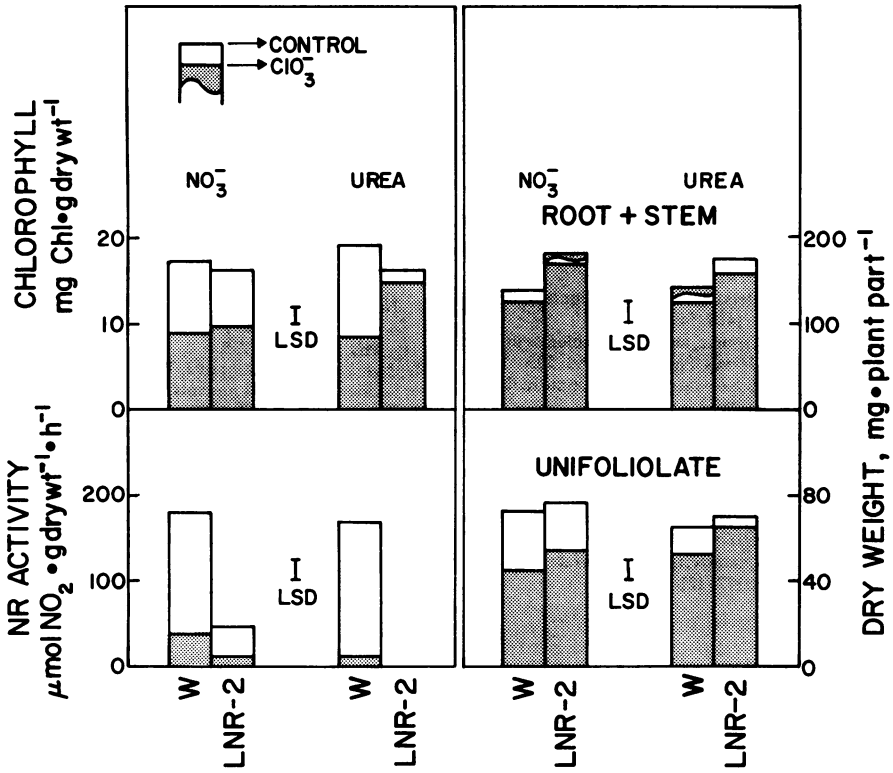

FIG. 2. Effect of ClO_3^- on NR activity and Chl concentration of the unifoliolate leaf, and dry weight of plant parts for wild type (W) and an NR mutant (LNR-2). Seedlings were transplanted at 5 DAP into NO_3^- or urea nutrient solutions, with or without 0.5 mM ClO_3^-, and were harvested at 9 DAP. Cotyledons were included in the root plus stem fraction.

Table II. *Presence of Constitutive NR Activity and $NO_{(x)}$ Evolution in F_2 Seedlings of Reciprocal Crosses between Williams and LNR-2, LNR-3, and LNR-4*

Cross	No. of F_2 Seedlings		χ^2 (3:1)	P
	With NR and $NO_{(x)}$	Without NR and $NO_{(x)}$		
W[a] × LNR-2	54	21	0.36	0.50–0.70
LNR-2 × W	16	12	4.75	0.02–0.05
W × LNR-2	100	32	0.04	0.80–0.90
LNR-2 × W	31	9	0.13	0.70–0.80
W × LNR-3	42	6	4.00	0.02–0.05
LNR-3 × W	41	7	2.78	0.05–0.10
W × LNR-4	36	18	2.00	0.10–0.20
LNR-4 × W	19	5	0.22	0.50–0.70
Total	339	110	0.06	0.80–0.90

[a] This reciprocal set tested for presence of NR activity only.

ClO_3^- *in situ*. Presumably, it is also capable of reducing the analog NO_3^-.

The absence of ClO_3^- toxicity symptoms in the urea-grown mutant suggested that the major effect of ClO_3^- on soybeans was due to its reduction by NR to ClO_2^-, at least with the concentration and treatment duration used. These results contrast with barley where the wild type was more sensitive to ClO_3^- when NR was not induced, and where mutants having 10% of normal NR activity were still sensitive to ClO_3^- (23). Although the response to ClO_3^- appears to be somewhat species dependent, ClO_3^- 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 ClO_3^- 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 $NO_{(x)}$ evolution in soybeans cannot be determined at this stage. The biochemical nature of $NO_{(x)}$ 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 $NO_{(x)}$ 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 mutagens 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 E_1 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 $NO_{(x)}$ evolution in our mutants are controlled by one or two genes.

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