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Biochemical Characterization of Soybean Mutants Lacking Constitutive NADH:Nitrate Reductase[1]

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ABSTRACT

Two nitrate reductase (NR) mutants were selected for low nitrate reductase (LNR) activity by *in vivo* NR microassays of M_2 seedlings derived from nitrosomethylurea-mutagenized soybean (*Glycine max* [L.] Merr. cv Williams) seeds. The mutants (LNR-5 and LNR-6) appeared to have normal nitrate-inducible NR activity. Both mutants, however, showed decreased NR activity *in vivo* and *in vitro* compared with the wild-type. *In vitro* $FMNH_2$-dependent nitrate reduction and Cyt *c* reductase activity of nitrate-grown plants, and nitrogenous gas evolution during *in vivo* NR assays of urea-grown plants, were also decreased in the mutants. The latter observation was due to insufficient generation of nitrite substrate, rather than some inherent difference in enzyme between mutant and wild-type plants. When grown on urea, crude extracts of LNR-5 and LNR-6 lines had similar NADPH:NR activities to that of the wild type, but both mutants had very little NADH:NR activity, relative to the wild type. Blue Sepharose columns loaded with NR extract of urea-grown mutants and sequentially eluted with NADPH and NADH yielded a NADPH:NR peak only, while the wild-type yielded both NADPH: and NADH:NR peaks. Activity profiles confirmed the lack of constitutive NADH:NR in the mutants throughout development. The results provide additional support to our claim that wild-type soybean contains three NR isozymes, namely, constitutive NADPH:NR (c_1NR), constitutive NADH:NR (c_2NR), and nitrate-inducible NR (iNR).

Nitrate reduction represents a major pathway for assimilation of inorganic N to a biologically useful form for incorporation into plant protein. The NR[3] enzyme is regarded as catalyzing the rate-limiting step of nitrate assimilation (1), and as such has been the subject of numerous studies relating this enzyme to overall nitrogen nutrition of plants. Mutants with decreased or nil NR activity have been isolated in eucaryotic organisms such as fungi, algae, and higher plants (3). Within higher plants, NR mutants have been reported both in cell culture and in whole plants (3).

Most NR mutants have been selected by screening for chlorate resistance, while a few have been selected using a more direct approach involving an *in vivo* NR assay (3). In soybean, a mutant lacking constitutive NR activity has been previously described (6, 12). This mutant (gene designation nr_1) has proven to be a useful tool for the identification of NR isozymes (14). Using this mutant and the wild-type, three distinctive NR isozymes have been tentatively characterized: (a) a 'constitutive' NADPH:NR (c_1NR) with a pH optimum around 6.5; (b) a constitutive NADH:NR (c_2NR) with a pH optimum around 6.5; and (c) a nitrate-inducible NADH:NR (iNR) with a pH optimum around 7.5 (14). (The constitutive NR is that enzyme form which exists in soybean plants in the absence of any nitrate in the culture medium).

Prior to isolation of the nr_1 mutant in our laboratory, NR isozymes in soybean had been previously investigated by Jolly *et al.* (5), Campbell (2), and Orihuel-Iranzo and Campbell (9). These workers had successfully differentiated a NADPH:NR and a NADH:NR in both soybean cotyledons and leaves. A differential activity profile of the NR forms had been noted during development of cotyledons (9). However, the reason for this developmental profile difference was not known. The purpose of this work was to characterize two NR mutants (LNR-5 and LNR-6) having diminished NR activity compared with the wild-type when all were grown on urea. This observation indicated a mutation affecting the genes encoding constitutive NRs or proteins which process these enzymes. Furthermore, the mutants were investigated to determine the developmental isozyme patterns.

MATERIALS AND METHODS

Plant Material and Mutant Selection. Soybean (*Glycine max* [L.] Merr. cv Williams) seeds were mutagenized with NMU and then grown in the field as described (11). Two mutant lines were selected by rapid *in vivo* NR assay of field-grown M_2 plants. Two leaf discs (5 mm diameter) from leaflets of one plant were placed in a well of a microtitration plate containing 100 μl assay buffer (50 mM KNO_3, 100 mM K-phosphate (pH 7.5), 0.025% (v/v) Triton X-100, and 1% (v/v) propanol) in each well. After vacuum infiltration, plates were incubated at 30°C in the dark for 30 min. The presence of NR activity was then visualized by addition of 100 μl color developer (0.4% sulfanilic acid, 1.5 M HCl, and 0.35 mM *N*-1-naphthylethylene-diamine dihydrochloride). Plants exhibiting decreased NR activity were grown to maturity, and seed number was increased in subsequent selfed generations.

Plant Growth for Enzyme Characterization. Wild-type and mutant plants were grown hydroponically in growth chambers as described earlier (6). Nutrient solutions contained either 7.5 mM urea- or nitrate-N as the sole N source as previously described (7).

Assays and Enzyme Extraction. *In vivo* NR assays, and $NO_{(x)}$ evolution were done as described (4), with the exception that a second $NO_{(x)}$ assay was performed in which 25 mM KNO_2 was added to the *in vivo* NR assay medium so that $NO_{(x)}$ evolution was independent of the level of NR activity. Nitrite reductase was assayed as described (7) except that the final volume was 1

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[3] Abbreviations: NR, nitrate reductase; c_1NR, constitutive NADPH:NR; c_2NR, constitutive NADH:NR; iNR, inducible NADH:NR; NMU, nitrosomethylurea; $NO_{(x)}$, nitrogenous gases; DAP, days after planting.

ml. Nitrate reductase activity was extracted as described by Scholl *et al.* (13) and assayed for reduced pyridine nucleotide activity as described by Streit *et al.* (14). $FMNH_2$:NR activity was determined as described by Nelson *et al.* (8) and Cyt *c* reductase activity was measured according to Wray and Filner (15). For Cyt *c* reductase assays, 0.5 ml extract was dialyzed against two changes (0.5 L each) of extraction medium minus cysteine.

Blue Sepharose[4] Column Chromatography. NRs were purified from acetone powders as described previously (10, 14), except that the phosphate concentration in the washing- and elution-buffers was 50 mM. Lowering the pH of the extract to 7.0 immediately after addition of the Blue Sepharose increased binding of NRs considerably, thus increasing overall enzyme recovery.

RESULTS

Comparison of *in Vivo* NRA and $NO_{(x)}$ Evolution from Leaves. Both mutants showed decreased *in vivo* NR activity and $NO_{(x)}$ evolution compared with the wild-type when grown on urea (Table I). In the presence of nitrate in the nutrient solution, *in vivo* NRA was lower in the mutants than in the wild-type, while $NO_{(x)}$ evolution was similar between mutants and wild-type. That the increased $NO_{(x)}$ evolution from mutants grown on nitrate, compared with mutants grown on urea, was due to availability of more nitrite rather than any change in enzyme capability was verified by supplementing comparable *in vivo* NR assays with nitrite. In the presence of exogenous nitrite, all plant lines grown on either urea or nitrate evolved similar amounts of $NO_{(x)}$ (Table I). This observation indicated that the enzymic capability of evolving $NO_{(x)}$ was not saturated by levels of nitrite normally accumulating in the *in vivo* NR assay, and that both the mutant plant and the wild-type plant had similar $NO_{(x)}$ evolution capability, regardless of whether urea or nitrate was the nutrient N source. Thus, the low level of $NO_{(x)}$ evolution by the two mutant lines grown on urea, when compared with the wild type, was due to a limitation in nitrite availability rather than some inherent difference in enzyme expression. Nitrite reductase activity was also present and comparable in the mutants and the wild-type when grown on either urea or nitrate (Table I).

Developmental Profiles of Nitrate Reductases in Soybean Leaves. Constitutive NR activities were highest in very young leaves when calculated on a fresh weight basis (Fig. 1). The activity profiles for c_1NR and c_2NR were not affected by the nitrogen source (Fig. 1). However, less constitutive NR activity was measured in leaves of plants grown without N in the nutrient solution, compared with nitrate-grown plants (results not shown). The iNR appeared later than the cNRs and iNR was detectable only in plants grown on nitrate (Fig. 1). Thus, these results confirm a previous observation (6) that during development of soybean leaves, cNRs appear and peak early, while iNR peaks during full-leaf expansion at a time when cNRs have declined in activity.

Comparison of Developmental Profiles between Plant Types. Both LNR-5 and LNR-6 had considerably less NADH:NR activity at pH 6.5 compared with the wild-type (Fig. 1). This finding strongly indicates that the c_2NR (the isozyme with highest activity in the wild-type under these assay conditions) was less active or possibly absent in these mutants. (It is important to note that all three NR isozymes from soybean will have varying degrees of activity under the three assay conditions used, and that although the three assays used by our laboratory are optimized for each individual isozyme, this does not eliminate partial expression of activity of other NRs present in the extract). The diminished NADH:NR activity in the mutants was observed throughout leaf development, and was more pronounced in urea-grown plants (Fig. 1). A mutation causing NADH-degradation in the assays is an unlikely explanation of this decreased activity, since both mutants showed normal NO_3^--inducible NADH:NR activities (Fig. 1).

Elution Profile from Blue Sepharose. Affinity chromatography on Blue Sepharose confirmed the lack of c_2NR activity in urea-grown LNR-5 and LNR-6 (Fig. 2). NADH failed to elute NR activity from Blue Sepharose columns loaded with extracts from either LNR-5 or LNR-6 grown on urea. In contrast, both c_1NR and c_2NR were sequentially eluted with NADPH and NADH from Blue Sepharose loaded with wild-type extract (Fig. 2), and as previously shown (10, 14).

$FMNH_2$:NR and Cyt *c* Reductase Activity. Both LNR-5 and LNR-6 had diminished $FMNH_2$:NR and Cyt *c* reductase activities compared with the wild-type (Table II). These diminished activities were mainly observed in urea-grown plants, where nitrate-inducible activity did not occur. Thus, the lack of constitutive NADH:NR activity in both mutants was accompanied by a loss of $FMNH_2$:NR and Cyt *c* reductase partial activities as well as loss of NADH:NR activity.

DISCUSSION

Decreased NR activity and $NO_{(x)}$-evolution in urea-grown mutants, compared with the wild type, initially indicated that the gene(s) affecting expression of constitutive NR were affected, since simultaneous loss of constitutive NR activity and $NO_{(x)}$ evolution has been linked to a single nuclear locus in the genetic analysis of the nr_1 mutant (12). However, the similar evolution of $NO_{(x)}$ from LNR-5, LNR-6, and wild-type plants, when supplemented with exogenous NO_2^-, indicated that the loss of c_2NR

[4] Mention of a trademark, vendor, or proprietry 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.

Table I. *In Vivo NR Activity, $NO_{(x)}$ Evolution, and Nitrite Reductase Activity in Fully Expanded (12 DAP) Unifoliolate Leaves of Wild-Type (cv Williams) and Mutant (LNR-5, LNR-6) Soybean Plants*

Values represent means ± SD of four replicates.

Plant	N-Source	*In Vivo* NR	$NO_{(x)}$ Evolution		Nitrite Reductase
			$-NO_2^-$	$+NO_2^-$	
		μmol NO_2^- h^{-1} g^{-1} fresh wt[a]			
Williams	Urea	22.7 ± 0.5	10.8 ± 0.5	20.4 ± 1.4	23 ± 10
LNR-5	Urea	5.5 ± 0.5	5.2 ± 0.2	18.3 ± 1.0	33 ± 9
LNR-6	Urea	5.9 ± 0.2	7.3 ± 0.6	24.7 ± 1.6	27 ± 11
Williams	Nitrate	19.7 ± 0.9	7.9 ± 0.8	16.8 ± 0.3	154 ± 12
LNR-5	Nitrate	10.8 ± 0.8	7.0 ± 0.5	20.2 ± 1.0	155 ± 10
LNR-6	Nitrate	10.6 ± 0.4	7.6 ± 1.0	22.6 ± 2.5	134 ± 8

[a] Data values are for NO_2^- formed in the case of *in vivo* NR and $NO_{(x)}$ evolution, and for NO_2^- utilized in the case of nitrite reductase.

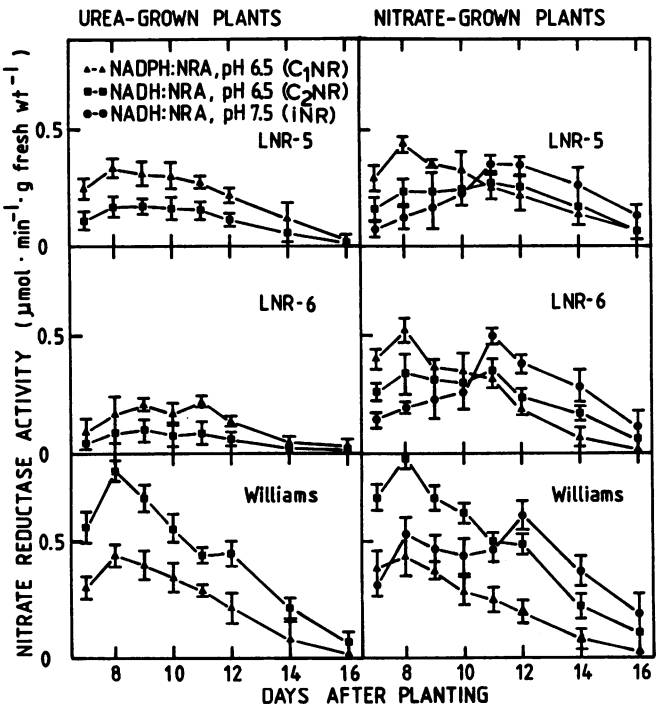

FIG. 1. Developmental profile of NR isozymes in unifoliolate leaves of urea-grown and nitrate-grown mutant and wild-type soybean plants. Symbols represent activities ± SD from six parallel extractions.

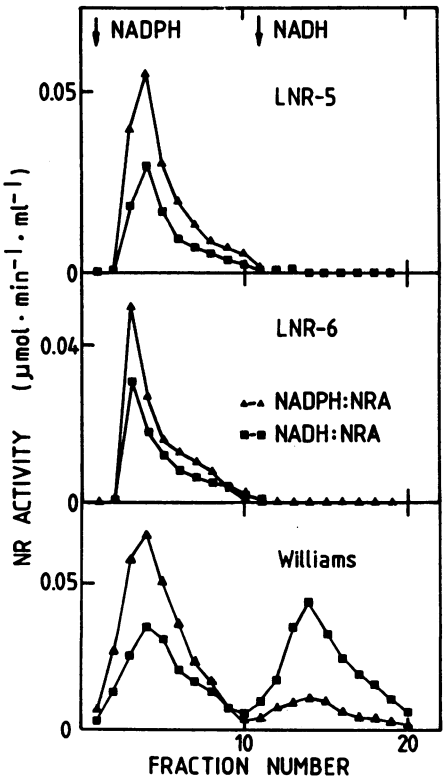

FIG. 2. Elution profile of NR activities from urea-grown soybean plants (12 DAP) recovered from Blue Sepharose columns after application of 50 μM NADPH followed by 50 μM NADH, both in washing buffer.

Table II. *Comparison of in Vitro Enzyme Activities Associated with NR in Wild-type (cv Williams) and Mutant (LNR-5, LNR-6) Soybean Leaves*

Fully expanded unifoliolate leaves from nitrate-grown plants (12 DAP) were harvested, extracted, and assayed as described in "Materials and Methods." Values represent means ± SD of six replicates.

Plant	NADH:NR	$FMNH_2$:NR	Cyt *c* Reductase
Williams	0.39 ± 0.04	0.42 ± 0.05	2.8 ± 0.4
LNR-5	0.28 ± 0.01	0.27 ± 0.03	1.6 ± 0.2
LNR-6	0.21 ± 0.06	0.24 ± 0.05	1.5 ± 0.2

from the two mutants was not linked to $NO_{(x)}$ evolution. The possibility that the c_1NR enzyme component is responsible for $NO_{(x)}$ evolution seems consistent with the available data, but remains to be verified.

The developmental profiles show that both constitutive NR isozymes appear earlier in leaf development than does inducible NR (Fig. 1). This is in accordance with the findings of Orihuel-Iranzo and Campbell (9), who found a shift from NADPH:NR to NADH:NR as soybean cotyledons matured. Similarly, Nelson *et al.* (6) had shown with the comparison between Williams grown on urea and nr_1 grown on nitrate that cNR activity profiles preceded iNR activity expression. Thus, constitutive NR isozymes may reduce nitrate *in situ* during leaf expansion, while in mature leaves inducible NR is the major nitrate-reducing enzyme form. However, endogenous leaf nitrate levels are low during leaf expansion which minimizes the role of cNR isozymes in overall nitrate metabolism.

Dramatically decreased NADH:NR activity at pH 6.5 in both LNR-5 and LNR-6 throughout leaf development indicates that c_2NR, the isozyme having highest activity under these conditions, is absent. The absence of c_2NR in urea-grown mutants was further confirmed by failure of elution of NR activity from Blue Sepharose columns by NADH (Fig. 2). Measuring *in vitro* NR activities under three different conditions, each optimized for one isoform, can therefore be very helpful in assessing the NR isozyme distribution in soybean.

A mutation in LNR-5 and LNR-6 affecting the molybdenum cofactor per se can be ruled out, since the presence of both c_1NR and iNR necessitates the presence of an active molybdenum cofactor (8). The mutation in both the LNR-5 and LNR-6 mutants appears to affect the whole c_2NR enzyme, since the Cyt *c* reductase and $FMNH_2$:NR activities are affected as well as NADH:NR activity. Our data and the work of Robin *et al.* (10) indicate that the NR mutants isolated in soybean appear to be deficient of the whole enzyme, a feature rather uncommon among NR mutants of other species (3).

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LITERATURE CITED

1. BEEVERS L, RH HAGEMAN 1980 Nitrate and nitrite reduction. *In* PK Stumpf, EE Conns, eds, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 115–168
2. CAMPBELL WH 1976 Separation of soybean leaf nitrate reductases by affinity chromatography. Plant Sci Lett 7: 239–254
3. DUNN-COLEMAN NS, J SMARELLI JR, RH GARRETT 1984 Nitrate assimilation in eucaryotic cells. Int Rev Cytol 92: 1–50
4. HARPER JE 1981 Evolution of nitrogen oxide(s) during *in vivo* nitrate reductase assay of soybean leaves. Plant Physiol 68: 1488–1493
5. JOLLY SO, WH CAMPBELL, NE TOLBERT 1976 NADPH- and NADH-nitrate reductase from soybean leaves. Arch Biochem Biophys 174: 431–439
6. NELSON RS, SA RYAN, JE HARPER 1983 Soybean mutants lacking constitutive nitrate reductase activity. I. Selection and initial plant characterization. Plant Physiol 72: 503–509
7. NELSON RS, L STREIT, JE HARPER 1984 Biochemical characterization of nitrate

and nitrite reduction in the wild-type and a nitrate reductase mutant of soybean. Physiol Plant 61: 384–390
8. NELSON RS, L STREIT, JE HARPER 1986 Nitrate reductases from wild-type and nr_1-mutant soybean (*Glycine max* [L.] Merr.) leaves. II. Partial activity, inhibitor, and complementation analyses. Plant Physiol 80: 72–76
9. ORIHUEL-IRANZO B, WH CAMPBELL 1980 Development of NAD(P)H: and NADH:nitrate reductase activities in soybean cotyledons. Plant Physiol 65: 595–599
10. ROBIN P, L STREIT, WH CAMPBELL, JE HARPER 1985 Immunochemical characterization of nitrate reductase forms from wild-type (cv Williams) and nr_1 mutant soybean. Plant Physiol 77: 232–236
11. RYAN SA, JE HARPER 1983 Selection and inheritance of nitrate reductase mutants in soybeans. Soybean Gen Newslett 10: 33-35
12. RYAN SA, RS NELSON, JE HARPER 1983 Soybean mutants lacking constitutive nitrate reductase activity. II. Nitrogen assimilation, chlorate resistance, and inheritance. Plant Physiol 72: 510–514
13. SCHOLL RL, JE HARPER, RH HAGEMAN 1974 Improvements of the nitrite color development in assays of nitrate reductase by phenazine methosulfate and zinc acetate. Plant Physiol 53: 825–828
14. STREIT L, RS NELSON, JE HARPER 1985 Nitrate reductases from wild-type and nr_1-mutant soybean (*Glycine max* [L.] Merr.) leaves. I. Purification, kinetics, and physical properties. Plant Physiol 78: 80–84
15. WRAY JL, P FILNER 1970 Structural and functional relationships of enzyme activities induced by nitrate in barley. Biochem J 119: 715–725