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Expression and Accumulation Patterns of Nitrogen-Responsive Lipoxygenase in Soybeans[1]

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Gene expression and protein accumulation patterns of nitrogen-responsive lipoxygenase (LOX-NR), as a representative vegetative storage protein, were investigated in nonnodulated soybeans (*Glycine max* [L.] Merr. cv Wye). The form of available nitrogen (supplied as NH_4NO_3, NH_4^+, NO_3^-, or urea) influenced the mRNA level and the amount of LOX protein, indicating that preferential accumulation of LOX may occur. Soybeans were grown with 0, 2, 5, and 16 mM total nitrogen to determine the extent to which LOX accumulation responded to soil nitrogen levels. Analysis of both mRNA and protein levels was conducted in shoot tips, stems, pod walls, and leaves over the entire life cycle of the plant. A general correlation between increasing available nitrogen level and LOX level was seen in the shoot tip and other organs throughout the soybean life cycle. However, appreciable amounts of LOX-NR mRNA and protein accumulated even when plants were grown under conditions of nitrogen deficiency. The results indicate that LOX may play an important role as a temporary storage site for amino acids in the developing shoot tip. The expression patterns of LOX-NR in plants grown under nitrogen deficiency suggest that these proteins, although responsive to nitrogen status, may not function solely as temporary storage pools for amino acids.

Nitrogen is essential for normal plant growth, development, and reproduction. During germination, hydrolysis products of storage proteins provide the nitrogen for initial growth. After the seedling becomes photosynthetic, the roots take up nitrogen from the soil, which is assimilated into amino acids and proteins in the vegetative tissues. The need for nitrogen increases steadily with the multiplication and expansion of the developing vegetative organs and culminates during the reproductive stage with the synthesis of new seed storage proteins. However, mechanisms controlling the redistribution and partitioning of nitrogen in plant vegetative and reproductive tissues are poorly understood (Pate, 1980; Simpson, 1986). The regulation of specific protein accumulation and turnover in the vegetative tissues is only now becoming appreciated as having a significant impact on nitrogen partitioning in plants.

Soybean leaves have a set of polypeptides with approximate molecular mass values of 27, 29, and 94 kD that have some of the characteristics of storage proteins. These proteins accumulate in leaves during vegetative growth and decrease in abundance during seed set (Staswick, 1990). Their expression is dramatically increased in response to the removal of reproductive sink tissue (i.e. flowers and pods containing the developing seeds) or the inhibition of phloem transport (Wittenbach, 1982, 1983a, 1983b), and they are localized in the vacuoles of paraveinal mesophyll cells (Franceschi et al., 1983; Klauer et al., 1991; Tranbarger et al., 1991). The 27-kD (VSP-α) and 29-kD (VSP-β) proteins are glycosylated and represent between 6 and 15% of the total soluble leaf protein in leaves just prior to pod and seed development, after which their levels decrease (Wittenbach, 1983b; Staswick, 1989a; Mason and Mullet, 1990). The 94-kD VSP is a member of the LOX gene family and its regulation appears to be similar to that of the other VSPs (Tranbarger et al., 1991). Immunoblotting indicates that VSP-α and VSP-β proteins are present in flowers, germinating cotyledons, and pod walls (Staswick, 1989a). Collectively, these observations suggest a role for these proteins as a temporary storage pool for amino acids (Franceschi and Giaquinta, 1983a, 1983b; Wittenbach, 1983a, 1983b; Wittenbach et al., 1984; Staswick, 1989a, 1989b, 1990). VSP-α and VSP-β are referred to as vegetative storage proteins based on the premise that they store nitrogen and carbon during vegetative growth and turn these sources over after anthesis for import into the developing seed and embryo.

VSP-α and VSP-β cDNAs have been isolated and sequenced (Mason et al., 1988; Staswick, 1988, 1989b). A main attribute of the regulation of VSPs is that VSP mRNA levels increase in leaf tissues in response to removal of the reproductive sink tissue (Staswick, 1988, 1989a), phloem blockage (Staswick, 1989a; Staswick et al., 1991), and elevated nitrogen availability (Staswick et al., 1991). Thus, nitrogen availability seems to be a primary signal, although probably not a direct signal, regulating their expression. VSP mRNA and protein levels also increase in soybean stems in response to water deficit (Mason et al., 1988; Surowy and Boyer, 1991), wounding (Mason and Mullet, 1990; Staswick, 1990), and exposure to jasmonic acid or methyl jasmonate (Mason and Mullet, 1990; Franceschi and Grimes, 1991; Staswick et al., 1991; Grimes et al., 1992). During normal plant growth, however, VSP mRNA is highest in younger leaves and meristematic tissues (Mason and Mullet, 1990).

Although the nitrogen status of the plants seems to play a major role in regulating VSP mRNA levels and protein ac-

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Abbreviations: LOX, lipoxygenase; LOX-NR, nitrogen-responsive lipoxygenase; pvmLOX, paraveinal mesophyll lipoxygenase; VSP, vegetative storage protein.

cumulation, there is very little detailed information concerning the direct relationship between soil nitrogen availability and VSP expression and accumulation. Staswick et al. (1991) demonstrated that increasing the amount of NH_4NO_3 (up to 40 mM) supplied to soybeans resulted in an increased VSP transcript level. This increase of VSP mRNA in response to elevated nitrogen availability was most prominent in the younger leaf tissue, but also occurred in older leaves (Staswick et al., 1991). This study suggests that nitrogen availability influences VSP expression, which is consistent with the interpretation that these proteins serve as temporary storage sites for "excess" nitrogen during vegetative growth.

The present study examines the influence of nitrogen availability on the developmental expression of LOX-NRs, which probably include the previously termed paraveinal mesophyll lipoxygenase (pvmLOX) (Tranbarger et al., 1991). This change in nomenclature is predicated on the observations presented in this report but does not preclude the regulation of these LOXs by other agents. There are members of the LOX gene family that are responsive to nitrogen (hence, "NR" for "nitrogen-responsive"), but are not limited to expression in the leaf paraveinal mesophyll. At the outset it is important to realize that, in soybeans, the LOX gene family consists of 9 to 10 members (H.D. Grimes, unpublished data). The probes that we use in this study are generic, i.e. they recognize most, if not all, of the members of the LOX gene family. Although it may be preferable to examine specific gene responses, these probes are not yet available. The changes that we report, however, are valuable in that they chart global changes in LOX expression and accumulation throughout the entire life cycle of soybean under various nitrogen regimes.

MATERIALS AND METHODS

Plant Material

All experiments were performed with nonnodulated soybean (*Glycine max* [L.] Merr. cv Wye). Seeds were planted in a mixture (1:1, v/v) of sterile vermiculite (fine grade) and sand (crushed basalt) in 1-gallon pots and grown in controlled environment chambers or growth rooms with a photon flux density of 360 to 400 μmol of photons m^{-2} s^{-1} PAR.

Nutrient Solutions

For the nitrogen source experiments, nitrogen was supplied using NH_4NO_3, NO_3^-, NH_4^+, and urea as the major forms of nitrogen at the following concentrations: (a) 2.4 mM NH_4NO_3; (b) 4.75 mM NH_4NO_3; (c) 4.3 mM NO_3^- [as 1.5 mM KNO_3, 1.05 mM $Ca(NO_3)_2 \cdot 6H_2O$, and 0.35 mM $Mg(NO_3)_2 \cdot 6H_2O$] plus 0.43 mM NH_4Cl; (d) 8.6 mM NO_3^- [as 3 mM KNO_3, 2.1 mM $Ca(NO_3)_2 \cdot 6H_2O$, and 0.7 mM $Mg(NO_3)_2 \cdot 6H_2O$] plus 0.86 mM NH_4Cl; (e) 4.3 mM NH_4Cl plus 0.43 mM KNO_3; (f) 8.6 mM NH_4Cl plus 0.86 mM KNO_3; (g) 2.4 mM urea; and (h) 4.75 mM urea. These mixtures supplied the plants with total nitrogen concentrations of 4.75 mM and 9.5 mM (1× and 2×). All other nutrient concentrations were maintained at levels found to be optimal for growth of soybean plants (Leggett and Frere, 1971) only varying Cl^- concentrations to allow for addition of different nitrogen forms. For the nitrogen concentration experiments, NH_4NO_3 was added to the solutions at 0, 1, 2.5, and 8 mM to give final concentrations of 0, 2, 5, and 16 mM total nitrogen. The pH of all nutrient solutions was adjusted to 6.3 with 1 M NaOH. Plants were fertilized in the morning with 100 mL of nutrient solution throughout the experiment and watered in the afternoon with 500 mL of tap water to eliminate salt accumulation.

Protein Extraction and Electrophoresis

Tissue was ground to a powder in liquid nitrogen with a chilled mortar and pestle, and total soluble protein was extracted with 2 volumes of homogenization buffer per gram of tissue for the trifoliolates and primary leaves, and 1 volume for all other tissues. The homogenization buffer consisted of 25 mM Tricine (pH 7.5), 1 mM EDTA, 10 mM β-mercaptoethanol, and 1% insoluble polyvinylpolypyrrolidone. Protease inhibitors were added to the homogenate to final concentrations of 1 μM leupeptin, 1 μM pepstatin, and 100 μg mL^{-1} PMSF. The homogenate was centrifuged at 10,000*g* for 15 min at 4°C and the supernatant was assayed for protein with Bio-Rad Protein Assay reagent according to the manufacturer's directions. Extracts were mixed (1:1, v/v) with 2× Laemmli sample buffer (Laemmli, 1970), heated at 90°C for 5 min, and centrifuged at 13,000*g* for 5 min. The supernatants were loaded onto SDS-PAGE gels on an equal protein basis and the proteins were resolved according to Laemmli (1970) except that a 7.5% to 15% acrylamide gradient was used with an accompanying 7.5% to 15% glycerol gradient. Gels were stained with Coomassie brilliant blue G-250.

Immunoblotting and Immunostaining

Soluble protein extracts resolved by SDS-PAGE were electroblotted onto nitrocellulose according to Towbin et al. (1979). The blot was blocked overnight (12 h) at 4°C with 10% nonfat Carnation instant milk in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) with the pH readjusted to 7.5. After blocking, the blots were incubated with soybean leaf LOX antiserum (provided by Dr. D. Hildebrand, University of Kentucky) diluted 1:10,000 (v/v) in the blocking solution for 1 to 3 h. The secondary antibody was goat anti-rabbit immunoglobulin G peroxidase (Pierce) in the blocking solution at a concentration of 1:5,000. Color was developed by immersing the blot in 30 mg of 4-chloro-1-naphthol (first dissolved in 10 mL of cold methanol) and 30 μL of 30% H_2O_2 in 50 mL of Tris-buffered saline.

RNA Extraction, Electrophoresis, and Gel and Slot-Blot Northern Hybridization

Tissue (0.5 g) was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Powder was immediately added 1:10 to a warm mortar containing 10 volumes of extraction buffer (0.81 M Tris, pH 9.0, 1% SDS, 0.02 M EDTA, pH 8.0, and 0.71 M β-mercaptoethanol added just before use) and ground for an additional 30 to 40 s. The slurry was then centrifuged at 31,000*g* for 15 min at 4°C and the supernatant was extracted with an equal volume of

phenol:chloroform (pH 8.0), mixed vigorously for 10 s, and centrifuged at 31,000*g* for 20 min at 4°C. The aqueous phase was collected and nucleic acids were precipitated with an equal volume of isopropanol at −20°C for at least 1 h. Nucleic acids were pelleted at 10,000*g* for 10 min at 4°C, the pellet was resuspended in 600 μL of TE/SDS buffer (0.01 M Tris, pH 7.0, 1 mM EDTA, pH 8.0, and 0.1% SDS), and the suspension was extracted twice with an equal volume of phenol:chloroform (pH 8.0) followed by centrifugation at 13,000*g* in a microcentrifuge for 10 min at room temperature. Nucleic acids in the aqueous layer were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol and incubated for 1 h at −20°C. Nucleic acids were pelleted by centrifugation at 13,000*g* for 10 min at 4°C, rinsed with 70% ethanol, and vacuum centrifuged until dry (Speedvac, Savant). Pellets were resuspended in 1 mL of TE/SDS buffer and precipitated with 0.25 mL of 10 M LiCl for 4 to 12 h at 4°C. RNA was pelleted by centrifugation at 13,000*g* for 10 min at 4°C and resuspended in 100 μL of diethyl pyrocarbonate-treated H_2O.

RNA was electrophoretically separated on agarose gels (10 μg of total RNA per lane) containing formaldehyde (Sambrook et al., 1989) and transferred to GeneScreen Plus membranes (Dupont). A 1.4-kb *Eco*R1 fragment isolated from pTK11 (generously supplied by Dr. J.C. Polacco, University of Missouri; Park and Polacco, 1990) was labeled by random priming according to the manufacturer's directions (Amersham). Prehybridization was done using 2 mL of prehybridization solution (1% SDS, 1 M NaCl, 10% dextran sulfate), and hybridizations were carried out in the same prehybridization solution overnight at 65°C in a hybridization incubator (Robbins Scientific). Blots were rinsed twice with 2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate) at room temperature for 5 min, twice with 2× SSC:1% SDS at 65°C for 30 min, and twice with 0.1× SSC at room temperature for 30 min.

Slot blotting was performed by mixing a 10-μg aliquot of RNA with an equal volume of 100% formamide and then mixing this 1:5 (v/v) with formaldehyde. This RNA solution was incubated for 1 h at 50°C and chilled quickly on ice before being loaded on the slot-blot apparatus. RNA was allowed to stand in wells for 30 min before a slight vacuum was applied for 30 s. Slot blots were probed using the same procedure described above for gel blots.

Chl Extraction and Quantitation

Leaf discs (4.5 cm^2) were taken from the trifoliolates collected at each time point (4, 7, 10, and 13 weeks after planting) for the nitrogen concentration and nitrogen source experiments. Chl was extracted from leaf discs with 10 mL of 95% ethanol for 72 h in the dark with gentle agitation. A_{665}, A_{649}, and A_{652} were determined spectrophotometrically and total Chl concentration was calculated according to Knudson et al. (1977).

RESULTS

The Effect of Source Nitrogen Form on Expression and Accumulation of LOX-NR

Plants were fertilized with nutrient solution containing nitrogen as NH_4NO_3, NO_3^-, NH_4^+, or urea at two different concentrations, 4.75 mM (=1×) or 9.5 mM (=2×). The effect of these different treatments was first assessed by determining leaf Chl content, plant height, and the number of expanded trifoliolates on the main axis at 4, 7, 10, and 13 weeks after planting. These data are shown in Table I and indicate that these nutrient solutions and nitrogen forms were adequate for normal plant growth and development. As expected, when the nitrogen level was doubled to 9.5 mM (2×), an increase in Chl content, plant height, and number of expanded trifoliolates was observed (Table I). The value that we refer to as "1×" closely approximates the nitrogen level in our usual fertilization regime, which is to fertilize three times per week with the manufacturer's recommended dilution of Peter's Professional Water Soluble Fertilizer (20–20–20). In general, the value referred to as "2×" results in greater plant height, number of trifoliolates, and Chl content, and indicates that although 1× adequately supports growth and development, 2× is able to enhance most of the growth parameters that we have measured.

Although the rate of uptake and assimilation of different nitrogen forms into soybeans is equitable if the rhizosphere pH is constant (Tolley-Henry and Raper, 1986, 1989), it is not known whether different nitrogen forms are assimilated into the VSPs or LOX-NR at equal efficiencies. To test this for LOX-NR, we fertilized soybean plants with a nutrient solution containing nitrogen supplied in four different forms (NH_4NO_3, NO_3^-, NH_4^+, or urea) and at two different concentrations. Soluble proteins were extracted from leaves, shoot tips, and stems at 4, 7, 10, and 13 weeks after planting and subjected to SDS-PAGE and immunoblotting to detect and semi-quantitatively estimate the level of LOX-NR. This cultivar flowers at approximately 8 weeks and, thus, the 10- and 13-week-old samples represent 2 and 5 weeks after anthesis and are further divided into podded (controls) and depodded.

Figure 1 shows the response of LOX-NR to these different nitrogen forms and levels at different developmental stages of the soybean. This figure is a composite of data from 12 separate immunoblots and is presented in this manner to facilitate comparisons of LOX-NR accumulation patterns under the different conditions. In all cases, equal protein loading among the lanes was confirmed by SDS-PAGE gels and Coomassie staining but is not presented because of space limitations. The data in Figure 1 suggest that there are subtle differences in the level of LOX-NR accumulation in response to different nitrogen forms. Although there are numerous differences, the data from 10-week-old podded plants best illustrate differences in the amount of LOX-NR accumulated in the third trifoliolates, with NO_3^- showing more accumulation than NH_4NO_3. In shoot tips, it appears as though NH_4^+ and urea (which is probably taken up as NH_4^+) are preferentially accumulated into LOX-NR. These differences, however, are not consistently found throughout the life cycle of the soybean plant and so it is difficult to make firm conclusions as to whether any of these nitrogen forms are consistently preferentially accumulated into LOX-NR. Also, some of the variability of these data may stem from problems associated with different rates of uptake of these nitrogen sources. Although we endeavored to provide nutrient solutions under defined conditions, we were not able to control rhizosphere

Table I. *Effect of different forms and concentrations of available nitrogen on plant growth parameters*

Plants were grown with NH_4NO_3, NO_3^-, NH_4^+, or urea as the sole source of available nitrogen. 1× and 2× refer to total nitrogen concentrations of 4.75 mM and 9.75 mM, respectively. Plants flowered at 8 weeks after planting, so the 10- and 13-week samples represent 2 and 5 weeks after anthesis. Ten- and 13-week plants were either allowed to pod normally (*P*) or were depodded daily (*DP*). –, No data were collected.

Treatment			No. of Expanded Trifoliolates				Plant Height (cm)				Chl Content (μg/g fresh weight)		
Nitrogen Form	[N]	P/DP	4	7	10	13	4	7	10	13	4	10	13
NH_4NO_3	1×	P	2	7	8	9	12.8	24	27	30	901	558	183
NH_4NO_3	2×	P	3	9	10	8	19.3	39	40	40	1008	987	231
NH_4NO_3	1×	DP	–	–	9	8	–	–	21	28.5	–	859	961
NH_4NO_3	2×	DP	–	–	9	11	–	–	38.5	40	–	1256	902
NO_3^-	1×	P	2	7	11	9	12.3	25	25.5	33	459	1151	156
NO_3^-	2×	P	3	9	9	9	15.3	31	41	33	830	1111	684
NO_3^-	1×	DP	–	–	9	9	–	–	27	32.5	–	708	599
NO_3^-	2×	DP	–	–	9	8	–	–	37.5	27	–	1460	1256
NH_4^+	1×	P	2	7	9	9	14.3	28	33.5	30.5	373	625	626
NH_4^+	2×	P	3	9	10	9	19.8	43	44.5	42	851	964	–
NH_4^+	1×	DP	–	–	10	8	–	–	30.5	25.5	–	822	1225
NH_4^+	2×	DP	–	–	10	9	–	–	42	38	–	1111	801
Urea	1×	P	2	7	9	7	27.5	25	35	25	407	685	275
Urea	2×	P	3	7	7	8	16.8	32	35.5	35	781	986	95
Urea	1×	DP	–	–	9	8	–	–	25	24.5	–	962	1295
Urea	2×	DP	–	–	8	8	–	–	35	33.5	–	1381	1045

pH, and changes in the rhizosphere pH may result in differential uptake rates of these different nitrogen sources.

Figure 1 also shows that LOX-NR accumulation is responsive to nitrogen level but that this response is dependent on the developmental stage. At 4 weeks after planting, doubling the nitrogen level results in slight increases in the levels of LOX-NR in the trifoliolates, shoot tips, and primary leaves (Fig. 1). These increases in nitrogen level also result in increases in the level of LOX-NR accumulation in organs of more mature plants (Fig. 1). For example, close examination of Figure 1 indicates that many different organs at various developmental stages in podded plants exhibit an increase in the accumulation of LOX-NR by increasing the nitrogen concentration from 1× to 2× (e.g. NH_4^+ treatment at 10 weeks, all tissues). LOX-NR levels also change among different organ systems during different stages of plant maturation. At 4 weeks, the level of LOX-NR is fairly similar in the trifoliolates, shoot tips, and primary leaves. At 7 weeks, LOX-NR preferentially accumulates in the shoot tips and stems, and the leaves show less accumulation (Fig. 1). Some of these changes may be due to alterations in competing sinks and changes in photosynthetic activity during plant growth.

If LOX-NR functions as a site for temporary storage of nitrogen, then we might predict that providing more nitrogen would result in more accumulation of LOX-NR after depodding (i.e. the plants supplied with 9.5 mM nitrogen [2×] would accumulate more LOX-NR after depodding than the plants supplied with 4.75 mM nitrogen [1×]). This prediction would hold unless the depodding treatment alone induces LOX gene expression to its maximum level. The data presented at 10 and 13 weeks in Figure 1 demonstrates that removal of sink tissues (i.e. 2 and 5 weeks of depodding) results in a very strong accumulation of LOX-NR in all organs examined. There is essentially no difference between LOX-NR levels after depodding between plants supplied with either 4.75 mM or 9.5 mM nitrogen (Fig. 1) at 13 weeks. These experiments were repeated and representative blots are shown in Figure 1; however, less exposed immunoblots were also obtained and these blots also indicated that there were no appreciable differences after depodding between 1× and 2× nitrogen levels. LOX-NR levels in 10 week tissue also showed no differences except, inexplicably, in the NH_4^+ and urea treatments (Fig. 1). Collectively, these results suggest that either removal of sink tissue in the presence of excess nitrogen does not result in significantly more nitrogen being partitioned into LOX-NR or that the depodding treatment alone induces maximal levels of accumulation.

Figure 2 indicates that the pod walls of 10-week-old plants contain a significant amount of LOX-NR and that this accumulation is not strongly dependent on nitrogen form. The accumulation of LOX-NR in this tissue does appear to be responsive to nitrogen concentration, which is more evident from the Coomassie-stained gel. Besides LOX-NR, several other pod wall proteins became more abundant in response to increasing nitrogen level. These are evident on the Coomassie-stained SDS-PAGE gel in Figure 2 and have molecular mass values of approximately 90 and 85 kD. The 90-kD polypeptide cross-reacts somewhat with the LOX antiserum, but the 85-kD polypeptide does not. At this time, it is uncertain whether these polypeptides are LOX degradation products or novel nitrogen-responsive polypeptides.

RNA gel-blot experiments were performed to determine if LOX-NR mRNA levels responded to different nitrogen regimes. Total RNA was isolated from trifoliolates at 4, 7, and 10 weeks after planting and were subjected to either formaldehyde gel electrophoresis (4 weeks) or slot-blot analysis

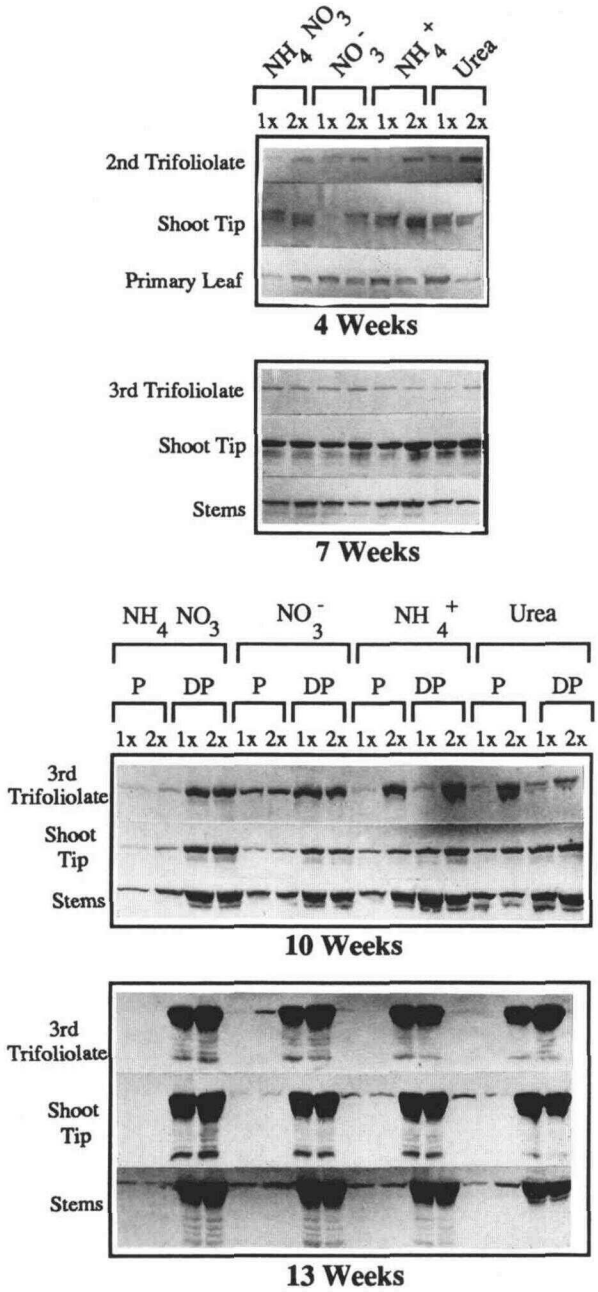

Figure 1. Immunoblot analysis of LOX-NR levels during vegetative and reproductive growth of soybeans fertilized with NH_4NO_3, NO_3^-, NH_4^+, or urea at 4.75 mM total nitrogen (1×) or 9.5 mM total nitrogen (2×). Plants were fertilized with the indicated nitrogen sources and samples were harvested at the indicated times. Soluble proteins were extracted, separated by SDS-PAGE, transferred to nitrocellulose, and probed with a LOX antibody. This figure is a composite of 12 different immunoblots with only the LOX-reactive region of the blot shown. Plants flowered at 8 weeks after planting and, for the depodded (DP) samples, flowers were removed daily after this time. Plants with the flowers and pods not removed are referred to as podded (P) samples. At 10 and 13 weeks after planting, no new vegetative growth occurred and, in these time points, shoot tip refers to the terminal trifoliolate on the main axis. Each lane contains 70 μg of protein.

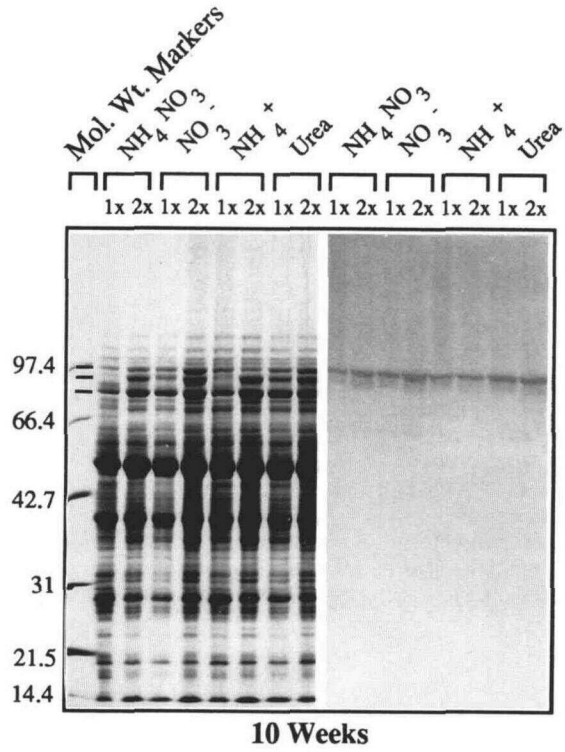

Figure 2. Immunoblot analysis of LOX-NR levels in the pod walls at 10 weeks after planting. Plants were fertilized with the indicated nitrogen sources and samples were harvested at the indicated times. Soluble proteins were extracted, separated by SDS-PAGE, transferred to nitrocellulose, and probed with a LOX antibody. 1× refers to 4.75 mM total nitrogen and 2× is 9.5 mM total nitrogen. Left side of figure is a Coomassie-stained SDS-PAGE gel and the right side is the immunoblot. Each lane contains 70 μg of protein. The bars to the left of the figure point out LOX-NR and the 90- and 85-kD nitrogen-responsive polypeptides.

(7 and 10 weeks). Figure 3 demonstrates that the level of LOX-NR mRNA increases significantly in response to increasing NH_4NO_3, NO_3^+, and NH_4^+ levels in 4-week-old plants. The magnitude of LOX-NR mRNA response to increasing nitrogen concentration is much more marked than increases in the protein level (compare Fig. 3 with Fig. 1). Although Figure 4 indicates that LOX-NR transcript levels do not vary greatly between the 1× and 2× (4.75 and 9.5 mM) nitrogen levels in 7-week-old plants (preanthesis) and 10-week-old depodded plants, there are some examples of small changes (i.e. NO_3^- and urea at 7 weeks and NH_4^+ and urea at 10 weeks).

The Effect of Nitrogen Concentration on the Expression and Accumulation of LOX-NR

Because our data indicate that LOX-NR is somewhat responsive to varying nitrogen concentrations, we used only one nitrogen form but expanded the concentration range to 0, 1, 2.5, and 8 mM NH_4NO_3 (equivalent to 0, 2, 5, and 16 mM total nitrogen) to further define these responses. Figure 5 shows various physiological and growth parameters from

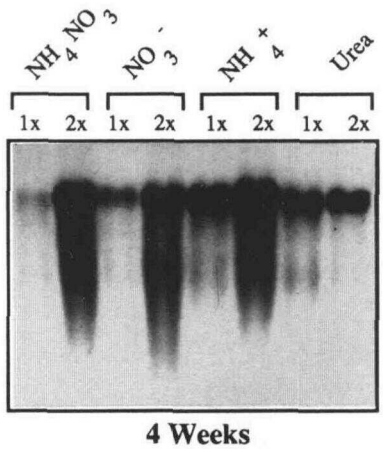

Figure 3. RNA gel-blot analysis of LOX-NR mRNA level in second trifoliolate leaves 4 weeks after planting. Plants were fertilized with the indicated nitrogen sources and samples were harvested at the indicated times. 1× refers to 4.75 mM total nitrogen and 2× is 9.5 mM total nitrogen. Each lane contains 10 μg of total RNA.

these different nitrogen levels, and these data indicate that 0 and 1 mM NH_4NO_3 were not able to sustain adequate growth. The 2.5 mM NH_4NO_3 concentration sustained normal growth and the 8 mM NH_4NO_3 was clearly enough for vigorous growth. Plants grown at 0 and 1 mM NH_4NO_3 demonstrated signs of nitrogen deficiency such as stunted growth and chlorotic leaves (the plants grown with no added nitrogen died after 5 weeks). Using the soybean Wye cultivar and these growth conditions, these data indicate that 0 and 1 mM NH_4NO_3 do not support adequate growth and plants grown at these nitrogen levels are nitrogen deficient, 2.5 mM NH_4NO_3 is adequate for normal growth and is used as a control, and 8 mM NH_4NO_3 supplies the plants with a surplus of nitrogen.

At 4, 7, 10, and 13 weeks after planting, samples were collected from leaves (primary leaf at 4 weeks), shoot tips, and stems grown at various nitrogen levels. Soluble proteins

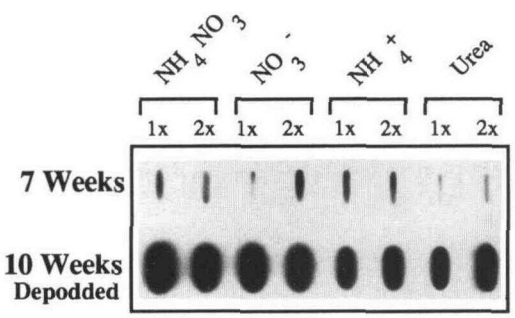

Figure 4. RNA slot-blot analysis of LOX-NR mRNA level in third trifoliolate leaves 7 and 10 weeks after planting. The 10-week samples were from plants that had the pods removed daily for 2 weeks prior to sampling. Plants were fertilized with the indicated nitrogen sources and samples were harvested at the indicated times. 1× refers to 4.75 mM total nitrogen and 2× is 9.5 mM total nitrogen. Each slot contains 10 μg of total RNA.

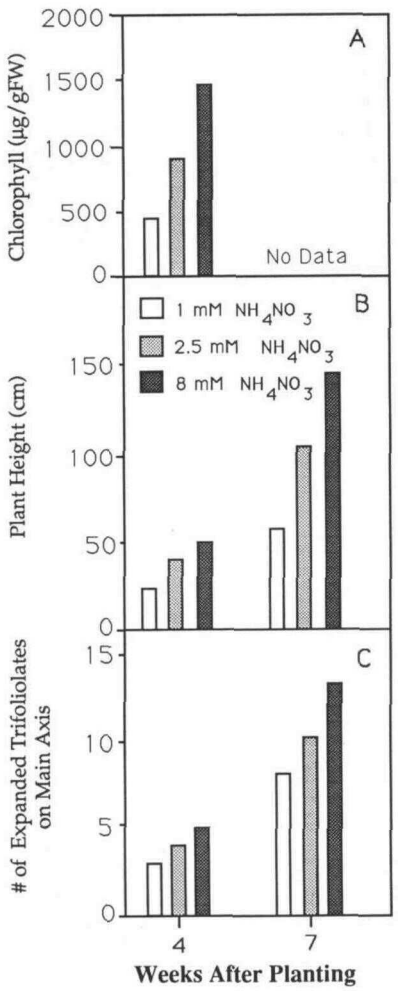

Figure 5. Effect of increasing amounts of available nitrogen on various plant growth parameters. Plants were grown with 1, 2.5, and 8 mM NH_4NO_3 and measurements were taken at 4 and 7 weeks after planting.

were extracted and subjected to immunoblot analysis. Figure 6 demonstrates that at 4 weeks after planting, a significant amount of LOX-NR accumulated in all tissues even when the plants were supplied with no nitrogen. The plants supplied with no nitrogen died shortly after this time point was collected (at about 5 weeks of age), indicating the severity of the nitrogen stress. The data from this 4-week-old sample thus indicate that even under extreme (0 and 2 mM total nitrogen) nitrogen-deficient conditions, an appreciable amount of LOX-NR is accumulated. There are consistent increases in LOX-NR level as the concentration increases to 16 mM total nitrogen in virtually all tissues at all developmental stages. In the 10- and 13-week-old samples (2 and 5 weeks after anthesis, respectively), it is again apparent that depodding has a much larger impact on LOX-NR accumulation than does the nitrogen concentration. Furthermore, there is essentially no difference in the amount of LOX-NR after depodding between plants grown under nitrogen-deficient conditions and plants grown with a surplus of nitrogen (compare 1 mM to 8 mM NH_4NO_3 at 13 weeks in Fig. 6).

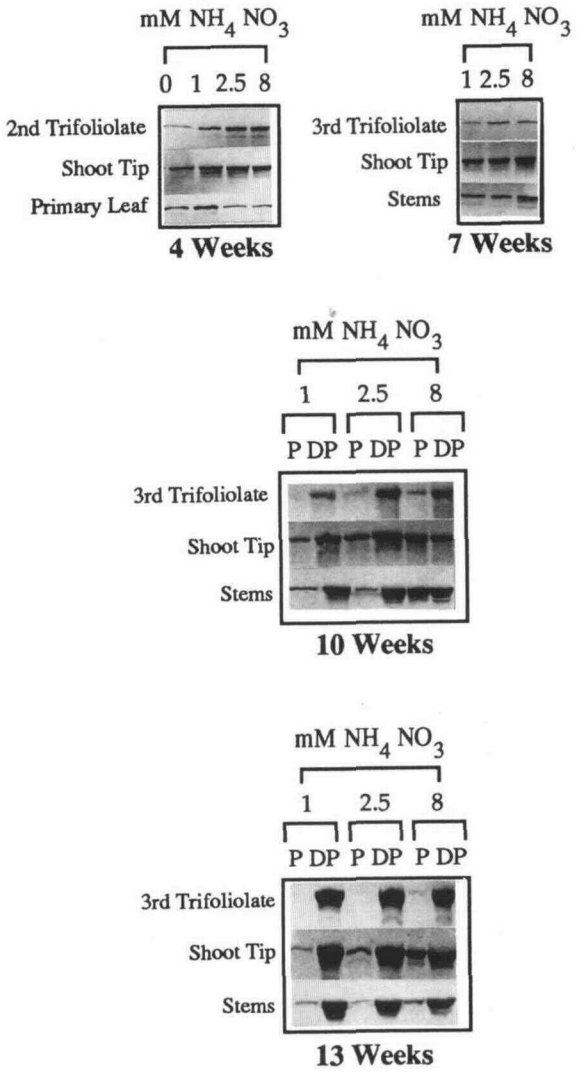

Figure 6. Immunoblot analysis of LOX-NR levels during vegetative and reproductive growth of soybeans fertilized with 0, 1, 2.5, or 8 mM NH_4NO_3 (total nitrogen of 0, 2, 5, and 16 mM). Samples were harvested at the indicated times and soluble proteins were extracted, separated by SDS-PAGE, transferred to nitrocellulose, and probed with a LOX antibody. This figure is a composite of 12 different immunoblots with only the LOX-reactive region of the blot shown. No data are supplied for 0 nitrogen after 4 weeks due to plant death. Plants flowered at 8 weeks after planting and, for the depodded (DP) samples, flowers were removed daily after this time. Plants with the flowers and pods not removed are referred to as podded (P) samples. At 10 and 13 weeks after planting, no new vegetative growth occurred and, in these time points, shoot tip refers to the terminal trifoliolate on the main axis. Each lane contains 70 μg of protein.

The Coomassie-stained lanes in Figure 7 indicate that the level of LOX-NR in the pod walls also increased with increasing nitrogen availability, demonstrating that the pod walls were somewhat responsive to nitrogen status. LOX-NR levels appeared to decrease between 10 and 13 weeks, indicating that this pod wall LOX-NR was also degraded during seed maturation. As described earlier, the pod walls contained two

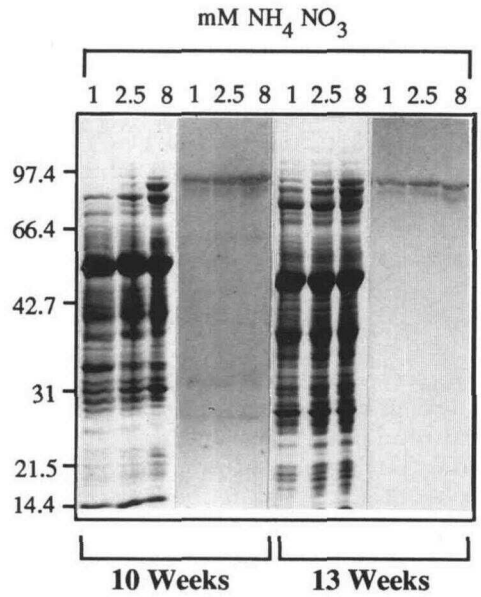

Figure 7. Immunoblot analysis of LOX-NR levels in the pod walls at 10 and 13 weeks after planting. Plants were fertilized with NH_4NO_3 at the indicated concentrations and samples were harvested at the indicated times. Soluble proteins were extracted, separated by SDS-PAGE, transferred to nitrocellulose, and probed with a LOX antibody. The indicated mM amounts are for NH_4NO_3 as the nitrogen source, and total nitrogen is 2, 5, and 16 mM (for 1, 2.5, and 8 mM NH_4NO_3, respectively). Both Coomassie-stained SDS-PAGE gels (lanes 1–3 and 7–9) and immunoblots (lanes 4–6 and 10–12) are shown. Each lane contains 70 μg of protein.

additional polypeptides (of 90 and 85 kD) that respond to nitrogen.

In the experiments comparing LOX-NR expression and accumulation in response to different forms of nitrogen, we observed that LOX-NR transcript appeared to be more sensitive to changes in nitrogen concentration than did the protein. Staswick et al. (1991) also reported that this was the case for VSP-α and VSP-β. Slot-blot analysis of total RNA extracted from trifoliolates from 10-week-old plants grown

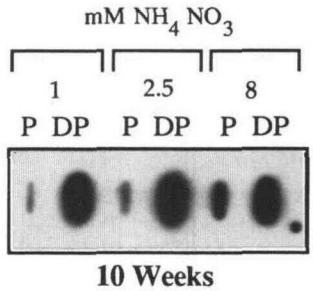

Figure 8. RNA slot-blot analysis of LOX-NR mRNA levels in third trifoliolate leaves 10 weeks after planting. Plants were fertilized with NH_4NO_3 at the indicated concentration (total nitrogen was 2, 5, and 16 mM nitrogen for 1, 2.5, and 8 mM NH_4NO_3, respectively). Pods either remained on the plants ("P" for podded) or were removed daily after 8 weeks ("DP" for depodded). Each slot contains 10 μg of total RNA.

with 1, 2.5, or 8 mM NH_4NO_3 shown in Figure 8 demonstrates that LOX-NR transcript increased in response to increasing nitrogen level (compare the "P" for podded or control lanes). Again, however, we observed that manipulation of the sink ("DP" for depodding) resulted in a much more dramatic increase in LOX-NR mRNA level than did the manipulation of source nitrogen concentration, and that even under severe nitrogen deficiency an appreciable amount of LOX-NR transcript was present.

DISCUSSION

The experiments presented in this paper constitute a thorough analysis of both mRNA expression and protein accumulation patterns of a representative soybean VSP, LOX-NR, in response to varying nitrogen regimes coupled with artificial manipulation of source-sink relationships (by depodding). Because of this, the degree of LOX-NR responsiveness, at both the transcript and protein levels, to source nitrogen is worth considering in some detail.

Shoot tips consistently contained relatively high levels of LOX-NR protein. This pattern is consistent with LOX-NR playing a storage protein role in this organ. Since the emerging shoot tip is primarily a sink, LOX-NR may be recruited as a temporary storage site for nitrogen. Because the expanding leaf makes the transition to becoming a source organ, LOX-NR may be hydrolyzed and the amino acids reassimilated into other proteins, such as Rubisco. In shoot tips, then, LOX-NR may function primarily as a storage protein for very short-term storage.

Work in other laboratories indicates that LOX activity and protein levels are elevated in the developing embryo axis (Altschuler et al., 1989; Hildebrand et al., 1991), expanding hypocotyl or radicle tissue (Kubacka-Aebalska and Kacperska-Palacz, 1980; Park and Polacco, 1989), and expanding leaves (Holden, 1970; Altschuler et al., 1989; Ievinsh, 1992). The association of high LOX activity and protein with young developing tissues suggests a role in plant growth and development (Siedow, 1991). The present results and those of Tranbarger et al. (1991) also suggest that LOXs play an important role in plant growth and development. It remains to be determined whether their role is enzymic or for short-term nitrogen storage or both.

Our results consistently demonstrate that LOX-NR mRNA is responsive to changes in source nitrogen concentration. This response is greatest in young tissues (4 weeks after planting) where 2- and 3-fold increases in nitrogen, from an adequate level of nitrogen to a slight surplus, instigate a response. If LOX-NR is functioning as a VSP, then we might expect situations of nitrogen deprivation to result in little or no LOX-NR expression or translation. Experiments with plants that were grown with either no added nitrogen (i.e. the only available nitrogen is from the cotyledons) or with only 2 mM total nitrogen indicated that an appreciable amount of LOX-NR transcript was made under these conditions. Analysis of LOX-NR protein level also indicated that there was an appreciable accumulation of LOX-NR even under nitrogen deficiency. This result is in contrast to the other VSPs, VSP-α and VSP-β, both of which exhibit no detectable expression or accumulation until approximately 5 mM nitrogen is supplied (Staswick et al., 1991).

The experiments where LOX-NR expression and accumulation was analyzed as a function of both available nitrogen concentration and artificial manipulation of the source-sink relationships offer important insight into the role of LOX-NR in storage of nitrogen. Previous work demonstrates that leaf LOX responds to depodding with a dramatic increase in both mRNA level and protein accumulation in the vacuoles of paraveinal mesophyll cells (Tranbarger et al., 1991). Indeed, these results were critical in our determination that the 94-kD VSP was, in fact, a member of the LOX gene family. If LOX-NR functions as a VSP, then one expectation might be that simultaneous removal of the sink tissues, or pods, while providing a nitrogen surplus, would result in more expression and accumulation of LOX-NR than depodding alone. Conversely, depodding coupled with conditions of nitrogen deprivation would be expected to result in less expression and accumulation of LOX-NR. Our results found that there was no difference between mRNA levels in depodded plants when the plants were nitrogen starved or grown in the presence of a nitrogen surplus. Analysis of LOX-NR protein level also indicated no difference between nitrogen-starved versus nitrogen-surplus plants. Depodding always produced a dramatic increase in transcript level and protein accumulation, irrespective of whether the nitrogen available in the root zone was extremely limiting or in surplus.

The fact that LOX-NR is expressed and accumulated even under conditions of severe nitrogen deprivation indicates that this protein is a necessary component of plant metabolism and may not function solely as a storage site for "excess" nitrogen. This conclusion is corroborated by the finding that depodding plants under severe nitrogen deficiency or nitrogen surplus results in the same amount of LOX-NR accumulation. Also, there is much more LOX-NR accumulated in nitrogen-deficient (2 mM nitrogen) plants after depodding than is accumulated in podded plants grown under surplus nitrogen (16 mM nitrogen) conditions.

At this time, we see three possible explanations for these results. First, the depodding treatment may be an extreme manipulation of the plant, which serves as a massive disruption of the normal nitrogen partitioning homeostasis. The severity of this source-sink manipulation may result in an "over-compensatory" remobilization of existing nitrogen into LOX-NR. In this case, the difference between nitrogen-deficient and nitrogen-surplus conditions may be overwhelmed. Analysis of transcripts and proteins in this situation, then, would result in the depodding showing a dramatic change with little impact of the available source nitrogen. Second, it is possible that the nitrogen status per se is not the sole determining factor for LOX-NR expression and accumulation. Perhaps the wounding that accompanies depodding also is serving as a signal and induces maximal expression of LOX-NR, and this response overwhelms the differences in nitrogen status. In this regard, note that several laboratories observed an increase in VSP-α and VSP-β expression after wounding soybean seedlings (Mason and Mullet, 1990; Staswick, 1990; Bell and Mullet, 1991) and other workers report an increase in LOX level after wounding (Hildebrand et al., 1989; Siedow, 1991). Third, amino acid availability from protein turnover,

due to senescence (Wittenbach, 1982), may be very high 2 weeks after depodding. These high levels of amino acids, because they are deprived of a sink, may be reassimilated into VSPs, such as LOX-NR. If this were the case, then changes in the amino acid pool may be more influential on LOX-NR transcription and translation than the available soil nitrogen during vegetative growth. The fact that appreciable amounts of LOX-NR accumulate even under conditions of nitrogen deficiency, however, suggests that there may be another role for these proteins besides solely storage.

It is important to stress that several LOXs exist as members of a gene family in soybeans. Although they probably play a wider role in nitrogen metabolism, or even plant growth and development, than previously envisioned (Tranbarger et al., 1991; results presented here), there are members of this family that appear to be regulated in part by the nitrogen status of the plant. The name "LOX-NR" was used consistently in this paper to reflect that fact. Tranbarger et al. (1991) refer to the 94-kD VSP as the pvmLOX. The results presented here, however, confirm that LOX expression and accumulation is not limited to one type of cell or organ. Grimes et al. (1992) also demonstrated that methyl jasmonate induced the expression and accumulation of at least two soybean seedling LOXs and that methyl jasmonate-responsive LOXs were present in shoot tips, stems, and primary leaves. It is possible, indeed probable, that the pvmLOX is not a single LOX but, rather, several different LOX isoforms. If this is the case, then it becomes important to determine which members of this gene family are nitrogen-responsive and involved in nitrogen partitioning or other aspects of growth and development in soybeans. It is feasible that several different LOX genes are involved in nitrogen partitioning due to tissue and organelle specificity of their expression. Apparently, the role of LOXs in plant growth and development is far from elucidated.

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