The Plant Cell, Vol. 3, 973-987, September 1991 © 1991 American Society of Plant Physiologists

The Soybean 94-Kilodalton Vegetative Storage Protein Is a Lipoxygenase That Is Localized in Paraveinal Mesophyll Cell Vacuoles

Timothy J. Tranbarger,[a] Vincent R. Franceschi,[a] David F. Hildebrand,[b] and Howard D. Grimes[a,1]

[a] Department of Botany, Washington State University, Pullman, Washington 99164-4238
[b] Department of Agronomy, University of Kentucky, Lexington, Kentucky 40506

Soybean leaves contain three proteins (the vegetative storage proteins or VSPs) that respond to nitrogen status and are believed to be involved in the temporary storage of nitrogen. One of these proteins, with a molecular mass of 94 kD and termed vsp94, was microsequenced. Partial amino acid sequence indicated that vsp94 was highly homologous to the lipoxygenase protein family. Further evidence that vsp94 is a lipoxygenase was obtained by demonstrating that vsp94 cross-reacted with a lipoxygenase antibody. Also, a lipoxygenase cDNA coding region was able to detect changes in an mRNA that closely parallel changes in vsp94 protein levels resulting from alteration of nitrogen sinks. Extensive immunocytochemical data indicate that this vsp94/lipoxygenase is primarily expressed in the paraveinal mesophyll cells and is subcellularly localized in the vacuole. These observations are significant in that they suggest that plant lipoxygenases may be bifunctional proteins able to function enzymatically in the hydroperoxidation of lipids and also to serve a role in the temporary storage of nitrogen during vegetative growth.

INTRODUCTION

During vegetative growth, soybean leaves and immature organs accumulate three polypeptides of approximately 27, 29, and 94 kD. These three polypeptides constitute a class of proteins termed the vegetative storage proteins (VSPs), which were first described by Wittenbach (1982; 1983a; 1983b). Several factors increase the level of expression and accumulation of the VSPs, including removal of sink tissue (Wittenbach, 1983a; Staswick, 1989), water deficit (Mason and Mullet, 1990; Surowy and Boyer, 1991), wounding (Mason and Mullet, 1990; Staswick, 1990), light (Mason et al., 1988), jasmonic acid (Anderson, 1988; Anderson et al., 1989), and blockage of leaf phloem export (Wittenbach, 1983b; Staswick, 1989). All three VSPs accumulate during normal vegetative growth, but accumulation can be greatly increased by decreasing the sink demand for nitrogen by removing the pods (Wittenbach, 1983a; 1983b). During normal pod development, the VSPs are rapidly degraded, presumably to enhance the amino acid pool available for the critical early stages of pod and seed growth (Wittenbach, 1983a; 1983b; Franceschi and Giaquinta, 1983a; 1983b). A role in the early nutrition of developing seeds may be the major function of the VSPs because they accumulate in the leaves even under conditions of low nitrogen availability (deVeau et al., 1990) and in developing leaves, where the demand for nitrogen for cell division and growth must be very high (Klauer et al., 1991). In this way, the VSPs can be envisioned as an important determinant of seed protein quantity and quality.

Of the three soybean VSPs, vsp27 and vsp29 are the best characterized. These two VSPs are glycosylated (Wittenbach, 1983b) and consist of two related polypeptides which form heterodimers and homodimers (Spilatro and Anderson, 1989). The cDNAs for both vsp27 and vsp29 have been isolated and characterized (Staswick, 1988; Mason et al., 1988). The third VSP, vsp94, has been observed on SDS-PAGE gels to accumulate upon removal of sink tissue (Wittenbach, 1982) and to share other regulatory features with vsp27 and vsp29. Recently, Franceschi and Grimes (1991) demonstrated that all of the VSPs, including vsp94, accumulated in response to low levels of atmospheric methyl jasmonate. Other than these observations, however, nothing is known about the 94-kD vegetative storage protein in soybeans.

Both vsp27 and vsp29 accumulate in the vacuoles of a specialized tissue in the soybean leaf termed the paraveinal mesophyll or PVM (Fisher, 1967; Franceschi and Giaquinta, 1983a; 1983b; 1983c). Klauer et al. (1991) have demonstrated that vsp94 is also a major protein in protoplasts isolated from PVM cells, although its subcellular

[1] To whom correspondence should be addressed.

localization was not determined. The PVM is also present in other legumes (Lackey, 1978; Franceschi et al., 1983; Kevekordes et al., 1988) and is a distinct layer of many-branched cells forming a reticulum at the level of the phloem that spans the region between veins and includes the bundle sheath cells. The anatomical and morphological features of the PVM, relative to other leaf tissues, make it the most direct path for transfer of assimilates from the photosynthetically active tissue to the vasculature for subsequent translocation (Fisher, 1967; Franceschi and Giaquinta, 1983a; Franceschi et al., 1983; Franceschi, 1986) and also for distribution of xylem-borne nutrients (such as ureides, amides, NO_3, etc.) entering the leaf (Costigan et al., 1987; Everard et al., 1990). The PVM represents a significant storage compartment within the leaf, accounting for about 20% of the total mesophyll volume (Costigan et al., 1987; Everard et al., 1990) and about 40% of the total mesophyll vacuolar volume (Liljebjelke and Franceschi, 1991). Considering the structure of the PVM and its position in the leaf, its association with the vascular tissue, its biochemical capacities, and the fact that vsp27 and vsp29 are localized in PVM vacuoles, it becomes apparent that the PVM can have a major impact on the regulation of partitioning and transport of nitrogenous assimilates.

Even in plants that do not have a PVM layer, the concept of vegetative storage proteins is valid. All plants, during their life cycles, are confronted with surpluses and deficits of nitrogen assimilates. Flowering plants have a heavy demand for nitrogen during flower development and seed formation, when the well-characterized seed storage proteins are synthesized. There is also evidence that the amount of nitrogen available during pod filling can also affect translocation and partitioning of carbohydrate (Crafts-Brandner et al., 1984). During this stage in soybeans, there is a decline in the ability of the plant to reduce nitrogen from either the soil or atmosphere (Lawn and Braun, 1974; Thibodeau and Jaworski, 1975). Hence, the plant must rely on nitrogen reserves in the vegetative organs as a source of nitrogen for the developing seeds. It should not be surprising that vegetative tissues store these nitrogen reserves in the form of protein because plants assimilate, transport, and reutilize nitrogen throughout their life cycle (Pate, 1980; Simpson, 1986). As a storage product, proteins can provide a stable, concentrated, osmotically inactive form of reduced nitrogen with increased packing efficiency relative to small molecules such as amino acids (Mosse, 1985; Franceschi, 1986). The function of a protein in vegetative nitrogen storage would be substantiated by the identification of a protein with two distinct features. First, it must be clearly implicated in the temporary storage of nitrogen, i.e., a VSP or a VSP-like protein that responds to sink demand for nitrogen. Second, the protein, or protein class, would have to be of wide distribution in the plant kingdom. Such a protein would be implicated in serving a general role in plants as a nitrogen reserve.

In this paper, we focus our attention on the least-characterized VSP: vsp94. We demonstrate that vsp94 is potentially a bifunctional protein in that it has many characteristics that are identical to the other VSPs, but that it is also a member of the lipoxygenase protein family. This finding forces a reevaluation of the physiological roles of lipoxygenases and provides the first direct evidence that the lipoxygenases may play a role in the temporary storage of nitrogen during vegetative growth of plants.

RESULTS

vsp94 Accumulates during Vegetative Growth and Responds to Removal of Sink Tissue

To establish that vsp94 is present in vegetative tissue and responds to nitrogen status, soluble leaf proteins were extracted from plants at 2, 4, and 6 weeks after the plants had begun to flower (podded). For the depodded treatment, flowers were removed every day throughout the 6-week period, and leaves were harvested at 2, 4, and 6 weeks after the onset of flowering. The proteins were resolved on SDS-PAGE gels and the results are shown in Figure 1A. It is evident from these data that vsp94 is a major component of soybean leaves and accumulates to very high levels after removal of the nitrogen sinks that normally form during flowering. The rate of response to nitrogen status is quite rapid; within 2 weeks after pod removal, a dramatic increase in the level of vsp94 is apparent in the leaves (Figure 1A).

Partial Amino Acid Sequence Analysis of vsp94 Peptides Demonstrates Homology to Lipoxygenases

Our initial interest in vsp94 included the molecular regulation of this protein and, hence, it was decided to microsequence this protein. After several attempts, a successful approach to generating sequenceable peptides was found. This method included protection of the amino acids during initial electrophoresis, in situ cyanogen bromide (CNBr) cleavage (Jahnen et al., 1990), and resolving the peptides on a Tricine-SDS-PAGE gel system (Schägger and von Jagow, 1987). Peptides were blotted to polyvinylidene difluoride (PVDF) membrane and were then subjected to automated Edman degradation. The results are shown in Figure 2. Amino acid sequence analysis indicated that vsp94 was highly homologous to several lipoxygenases (Shibata et al., 1987, 1988; Ealing and Casey, 1988; Hildebrand et al., 1988; Matsumota et al., 1988; Yenofsky et al., 1988). Although the level of homology between vsp94 and the soybean seed lipoxygenases (termed LOX 1, 2, and 3) is quite high (65% for LOX 1, 76% for LOX 2, and 78% for LOX 3; Figure 2), there are several unique

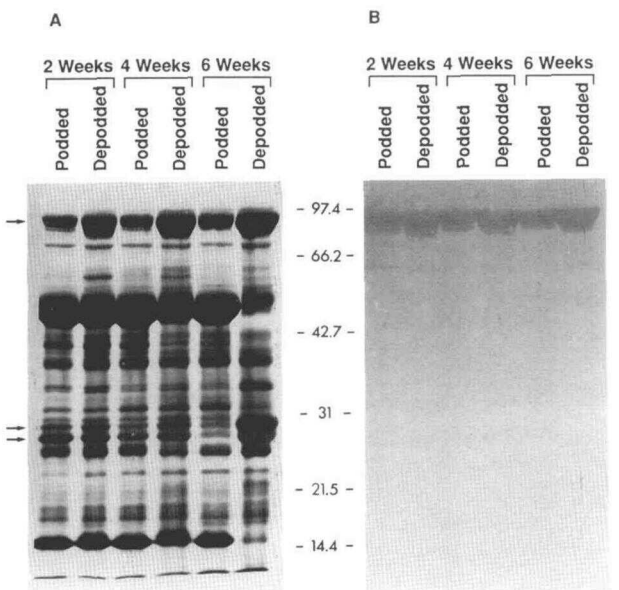

Figure 1. Accumulation of vsp94 Is Affected by Nitrogen Status and the Polypeptide Cross-Reacts with a Lipoxygenase Antibody.

(A) Leaf proteins were extracted from soybean leaves 2, 4, and 6 weeks after flowering had begun (podded treatment) or after continuous depodding (depodded treatment). Ninety micrograms of protein was loaded per lane. An increase in vsp94 is evident upon removal of the nitrogen sinks. Arrows point to vsp94 as well as two other VSPs, vsp27 and vsp29.
(B) The same treatments are shown as in **(A)** but the polypeptides were transferred to nitrocellulose, and an immunoblot was performed using antibody against a soybean leaf lipoxygenase. Ninety micrograms of protein was loaded per lane. The main band that is staining is identical to vsp94.

amino acid residues contained in the vsp94 sequence and only peptide 4 demonstrates 100% homology to the LOX 3 sequence. This sequence homology is very strong evidence that vsp94 is a lipoxygenase and that it may represent a unique lipoxygenase that has not yet been fully sequenced.

vsp94 Cross-Reacts with a Leaf Lipoxygenase Polyclonal Antibody

To gain additional evidence that vsp94 is indeed a lipoxygenase, we reacted this polypeptide with a polyclonal antiserum raised against chromatofocused soybean leaf lipoxygenase (peak 3 as described in Grayburn et al., 1991). Figure 1B shows the results of a protein gel blot of a similar time course SDS-PAGE gel as shown in Figure 1A. The lipoxygenase antiserum reacted primarily with a 94-kD polypeptide that corresponds to vsp94. This 94-kD polypeptide was seen to increase in response to pod removal within 2 weeks and continued to increase in week 4 and 6 depodded plants, whereas no comparable increase occurred in the 2-, 4-, and 6-week podded plants (Figure 1B). This pattern of expression in response to nitrogen status confirms the pattern observed on the Coomassie blue-stained gel (Figure 1A). Furthermore, the dominant staining of the vsp94 with lipoxygenase antiserum provides corroborative evidence that vsp94 is indeed a lipoxygenase. Its similar pattern of expression to vsp27 and vsp29 indicates that it is also involved in the temporary storage of nitrogen in vegetative tissue.

A Lipoxygenase Transcript Is Induced by Pod Removal

After having obtained the above data indicating that vsp94 is actually a member of the lipoxygenase protein family, it was of interest to determine whether the mRNA for this protein responded to nitrogen status or whether the protein accumulation was a post-translationally regulated event. Staswick (1988) demonstrated that the mRNAs for vsp27 and vsp29 respond directly to nitrogen status. It was further reasoned that if a lipoxygenase cDNA was able to detect an induced transcript in depodded plants, then additional evidence would be obtained indicating that vsp94 is a lipoxygenase. Total RNA was isolated from

PEPTIDE #1 (Approximate MW of 14 kD)

pvmLOX		X	Q	K	N	V	L	D	I	N	S	I	T	S	V
LOX 1	M^{15}	P	K	N	E	L	E	V	N	P	D	G	S	A	
LOX 2	M^{26}	R	K	N	V	L	D	F	N	S	V	A	D	L	
LOX 3	M^{19}	R	K	N	V	L	D	V	N	S	V	T	S	V	

PEPTIDE #2 (Approximate MW of 23 kD)

pvmLOX		Q	V	E	F	Y	L	K	S	L	I	L	E	D	I	P	N	H	G	T	I
LOX 1	M^{103}	Q	V	E	F	F	L	K	S	L	T	L	E	A	I	S	N	Q	G	T	I
LOX 2	M^{133}	Q	V	E	F	Y	L	K	S	L	T	L	E	D	V	P	N	Q	G	T	I
LOX 3	M^{121}	Q	T	E	F	F	L	V	S	L	T	L	E	D	I	P	N	H	G	S	I

PEPTIDE #3 (Approximate MW of 10 kD)

pvmLOX		X	X	X	D	E	F	A	R	E	R	I
LOX 1	M^{341}	T	D	E	E	F	A	R	E	M	I	
LOX 2	M^{369}	T	D	E	E	F	A	R	E	M	V	
LOX 3	M^{359}	T	D	E	E	F	A	R	E	M	L	

PEPTIDE #4 (Approximate MW of 9 kD)

pvmLOX		X	P	E	K	G	S	A	E	Y
LOX 1	L^{714}	P	E	K	G	T	P	E	Y	
LOX 2	L^{742}	P	E	K	G	S	P	E	Y	
LOX 3	M^{734}	P	E	K	G	S	A	E	Y	

Figure 2. Partial Amino Acid Sequence Data from vsp94 Indicates that It Is Highly Homologous to Soybean Seed Lipoxygenases.

Gel-purified vsp94 was used for in situ CNBr cleavage, and the peptides were isolated and microsequenced. Cleavage is at a methionine residue and the peptide fragments are aligned with the appropriate amino acid sequences from seed lipoxygenases (LOX 1, 2, and 3). The number on the M (or L) residue indicates its position in the intact protein.

soybean leaves at the following developmental stages: 7 weeks after cotyledon emergence from the soil, at the onset of flowering, 2 weeks after flowering (podded), 2 weeks after continuous depodding, 4 weeks after flowering (podded), 4 weeks after continuous depodding, and 6 weeks after flowering. This RNA was resolved on agarose gels containing formaldehyde, transferred to nitrocellulose, and probed with an EcoRI fragment from a plasmid (pTK11) containing a cDNA to a lipoxygenase expressed in hypocotyls and radicles of young soybean plants. Figure 3 indicates that there is a small amount of lipoxygenase transcript present at the 7-week-old stage (about 1 to 2 weeks before the plants start to flower). Transcript levels increase somewhat at the onset of flowering and then decrease during seed set and pod filling. In stark contrast to this, however, depodded plants demonstrate a dramatic increase in the lipoxygenase transcript level after 2 weeks (compare the 2-week podded to the 2-week depodded treatment). After this initial sharp increase, the mRNA level for the 94-kD protein tapers off (Figure 3). The transcript size, approximately 3 kb, is consistent with soybean seed lipoxygenase mRNAs (Altschuler et al., 1989) and is sufficiently large to encode for the intact 94-kD protein. It is not yet certain whether this hybridization represents a single transcript or a family of related transcripts. However, this pattern of mRNA expression is very similar to that observed for the other VSPs (Staswick, 1988). The fact that this lipoxygenase cDNA can hybridize with a transcript that is temporally expressed in a manner expected for vsp94 is also indicative of the fact that vsp94 is a lipoxygenase.

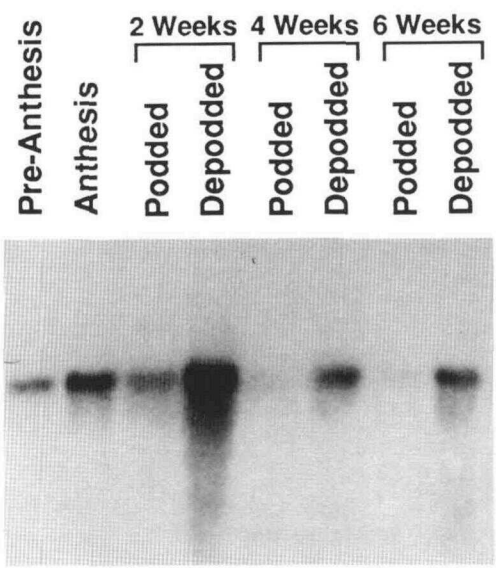

Figure 3. *A Lipoxygenase mRNA Responds to Alteration of* Nitrogen Sink Status.

Total RNA was isolated from soybean leaves 2 weeks before the onset of flowering (preanthesis), at anthesis, and at 2, 4, and 6 weeks after flowering had begun from plants that had nitrogen sinks left on (podded) or removed (depodded). Fourteen micrograms of total RNA was loaded into each lane, and the probe was a 1.4-kb EcoRI fragment from a germination-associated lipoxygenase. This fragment codes for a highly conserved region for plant lipoxygenases.

Immunocytochemistry Demonstrates that vsp94 Is Localized in the Paraveinal Mesophyll

Three lines of evidence—amino acid sequence homology, lipoxygenase antibody cross-reactivity, and a lipoxygenase cDNA hybridizing with a transcript that shows the expected pattern of expression in relation to nitrogen status—demonstrate that the 94-kD VSP is a member of the lipoxygenase protein family. This result raises some interesting questions concerning the tissue and subcellular localization of the vsp94. A number of workers have examined the tissue and subcellular localization of lipoxygenases in different plant tissues (reviewed by Galliard and Chan, 1980; Mack et al., 1987; Vick and Zimmerman, 1987). The most reliable information to date on the cellular and subcellular distribution of soybean seed lipoxygenases indicates that they are initially localized in the storage tissues of etiolated soybean cotyledons during the first days after germination and later appear in the cytoplasm surrounding the protein bodies and within the protein bodies and various organelles in other tissues (Vernooy-Gerritsen et al., 1983; 1984). Although an extensive immunocytochemical study has not yet been performed, lipoxygenases are not thought to be tissue specific, and they are usually localized in the cytoplasm (Vernooy-Gerritsen et al., 1984). If, on the other hand, the 94-kD VSP is functioning as a storage protein in leaves, then we would expect it to be localized in a tissue-specific (i.e., the PVM) manner and subcellularly localized in the vacuoles in a manner similar to vsp27 and vsp29.

To address this issue, the lipoxygenase (peak 3) antibody was used for immunolocalization experiments. Figure 4A shows a cross-section of a leaf from a 2-week depodded plant that was immunostained with the lipoxygenase antibody. This result clearly demonstrates that the majority of the protein cross-reactive with the lipoxygenase antibody is associated with the paraveinal mesophyll cells. Although this micrograph demonstrates that there is some accumulation of vsp94 in the epidermal cells (Figure 4A), virtually no positive-staining material is present in the palisade parenchyma or the spongy mesophyll. In general, we observed a gradient of vsp94 accumulation in bundle sheath cells (where it is the highest) to the distal cells of the PVM between phloem veins (where it is the lowest). To quantify this distribution, and to obtain a firmer grasp on the quantitative relationship of vsp94 accumulation in

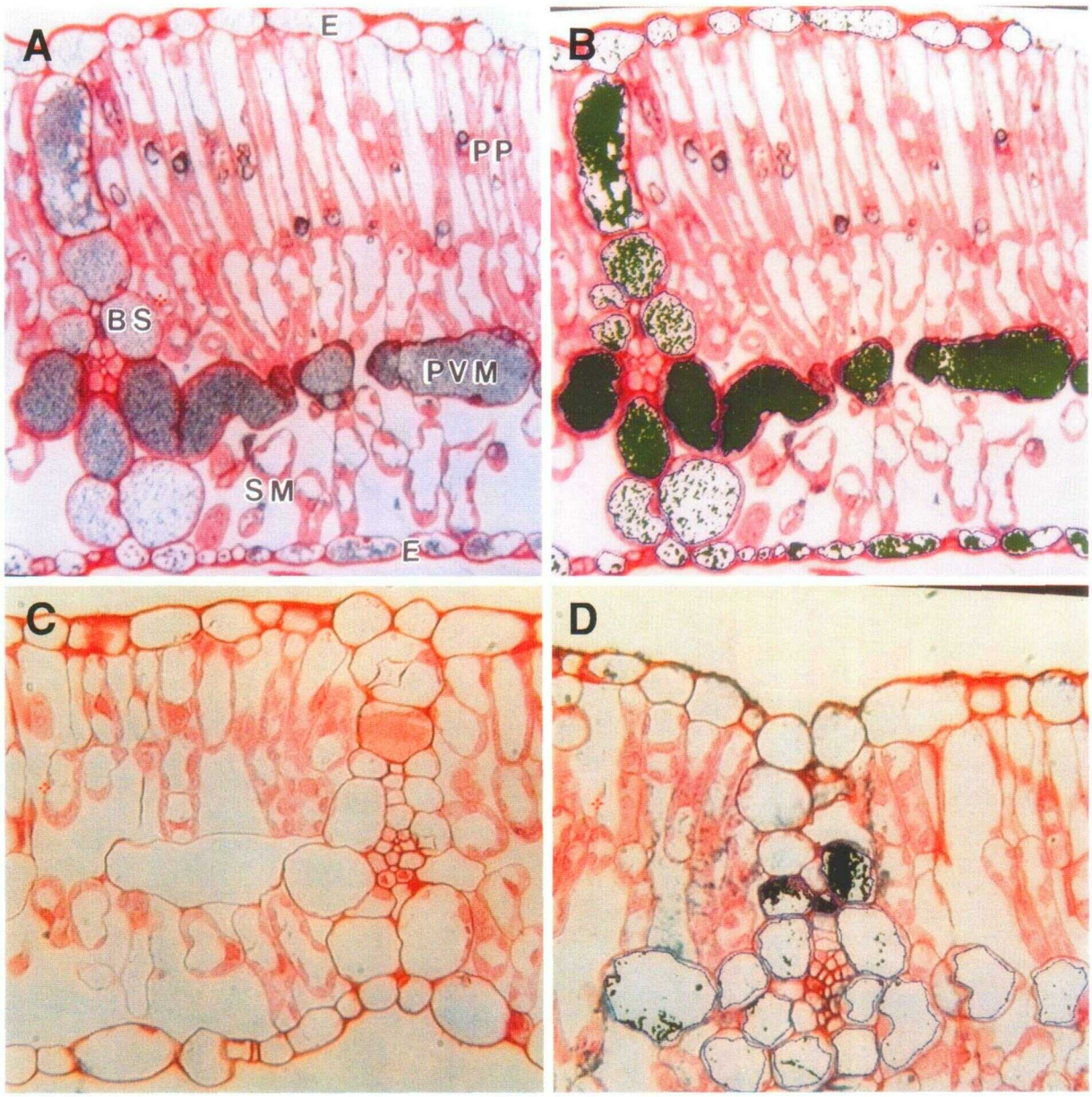

Figure 4. Lipoxygenase Is Associated with the PVM Cells in Depodded (2-Week) Soybean Leaves.

(A) A cross-section of a soybean leaf (2-week depodded) is shown. Silver grains are primarily associated with the PVM cells and, to a lesser degree, with epidermal cells. BS, bundle sheath; E, epidermis; SM, spongy mesophyll; PP, palisade parenchyma.
(B) The same cross-section as in **(A)** is shown but after the silver grains were converted to quantifiable pixels (green color).
(C) A cross-section of a soybean leaf (2-week depodded) that has been stained with nonimmune serum. No nonspecific staining is evident.
(D) A soybean leaf was taken from a plant 2 weeks before flowering and immunostained with lipoxygenase antibody, and the silver grains converted to pixels (dark green). Accumulation of vsp94/lipoxygenase is evident in the bundle sheath and PVM cells.

these different cell types, image analysis techniques were used. Figure 4B shows the same section as Figure 4A after the silver grains have been thresholded. Thresholding is accomplished by converting individual pixels that lie above a specific grey level to "marked" pixels, in this case, a green color. Because silver grains are a dark color, they are relatively easy to threshold. Although the threshold level is adjustable, a comparison of Figures 4A and 4B indicates that it is possible to obtain a very accurate conversion of silver grains to quantifiable pixels. Figure 5 presents the data from quantifying the relative amount of silver grains present in 4-week depodded leaves from various cell types (these quantitative data are expressed as the mean number of pixels per cell and represent the relative difference in number of silver grains in the different cell types). These data clearly indicate that the bulk of the antigen that cross-reacts with the lipoxygenase antibody lies in the bundle sheath and the PVM cells immediately surrounding the bundle sheath. In the region of the PVM that is distal to the bundle sheath cells, the amount of lipoxygenase is reduced (Figure 5). The epidermal cells, while containing some lipoxygenase, are not a major site of accumulation of lipoxygenase nor is the palisade parenchyma or the spongy mesophyll cells (Figure 5). When nonimmune serum was used, no staining was detected (Figure 4C).

Image analysis techniques were also used to examine the temporal pattern of vsp94/lipoxygenase accumulation in podded and depodded plants. First, however, we examined the amount of vsp94/lipoxygenase present in vegetative tissues prior to flowering. Because the VSPs are believed to play an important role in the storage of nitrogen before seed set, we would expect to find appreciable amounts of the vsp94/lipoxygenase in the PVM cells before the plants start to flower. Figure 4D shows that in leaves from plants 7 weeks after germination (about 2 weeks before flowering), demonstrable levels of the vsp94/lipoxygenase are present in the PVM and bundle sheath cells.

To examine the effects of altering nitrogen sinks on the localization and abundance of vsp94/lipoxygenase in PVM cells, an immunocytochemical analysis was performed with podded and depodded plants, as shown in Figure 6. In the podded plants, where the nitrogen sinks are present, the level of vsp94/lipoxygenase tapers off with increasing age (Figures 6A, 6C, and 6E). This is expected because the sink demand becomes quite strong during pod filling. When these sinks are removed by continuous depodding, however, a dramatic increase in the level of vsp94/lipoxygenase is seen after just 2 weeks (Figure 6B). The level of vsp94/lipoxygenase drops somewhat in 4- and 6-week depodded plants (Figures 6D and 6F), but remains quite high relative to the podded plants. This pattern reflects what we observed with the lipoxygenase mRNA level. It is evident that depodding results in a massive accumulation

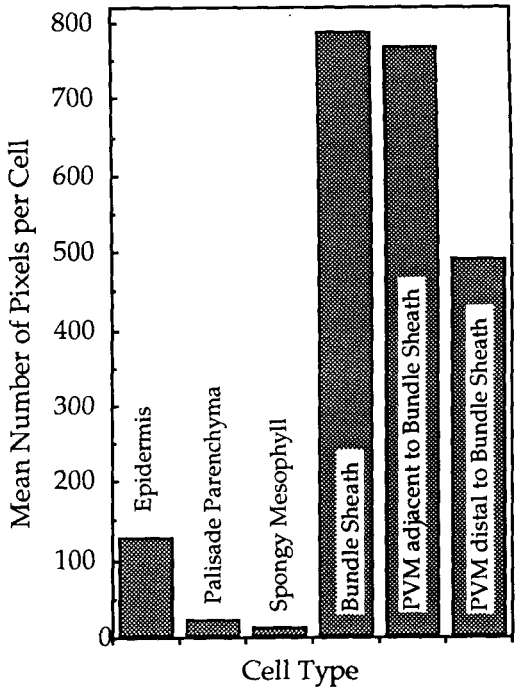

Figure 5. Quantification of Lipoxygenase in Different Cell Types of 4-Week Depodded Soybean Leaves.

Four-week depodded leaves were prepared for immunocytochemistry and immunostained with lipoxygenase antibody, and a number of different cross-sections were thresholded and quantitated.

of this lipoxygenase in the PVM that continues throughout the time course examined.

Transmission Electron Microscopy (TEM) Demonstrates that vsp94 Is Localized in the Vacuoles of Paraveinal Mesophyll Cells

To further confirm and extend the observations made by the light microscopy studies, TEM immunocytochemistry was performed. Preimmune controls were free of gold particles, as shown in Figure 7A, whereas lipoxygenase antibody demonstrated significant and specific labeling (Figure 7B). The enhanced resolution of the TEM made possible visualization of label associated with specific subcellular structures of PVM cells that were unresolved at the light microscope level. Electron-dense material within the vacuole was most heavily labeled, whereas electron-lucent areas of the vacuole were not labeled (Figure 7B). Some label could also be seen within the cytoplasm (Figures 7B and 7C) and did not appear to be associated with a particular organelle. Endoplasmic reticulum, Golgi apparatuses, mitochondria, chloroplasts, and cell walls were

not labeled. However, a significant amount of label was associated with the tonoplast itself (Figure 7C). The amount of label on the tonoplast was greater in the 2-week depodded samples and decreased in the 4-week depodded samples, but was still present.

Because several lines of evidence demonstrate that vsp94 is a lipoxygenase, and because this lipoxygenase is found primarily in the vacuoles of the PVM cells, we now refer to vsp94 as the "paraveinal mesophyll lipoxygenase" or the "pvmLOX."

Is the pvmLOX an Active Lipoxygenase?

The next question that was addressed is whether the pvmLOX is an active enzyme or whether it is merely homologous to other, active, lipoxygenases. This is a pertinent question, especially considering the fact that the pvmLOX is localized in acidic vacuoles. To address this question, protein extracts were made from preflowering leaves, leaves from 6-week podded plants, and leaves from 6-week depodded plants. These protein extracts were assayed for lipoxygenase activity, and the data are presented in Figures 8A and 8B. These results indicate that when an increase in the pvmLOX is observed (i.e., on SDS-PAGE gels or immunolocalized tissue sections), there is a corresponding increase in lipoxygenase activity when expressed on a per leaf volume basis (Figure 8A). When the specific activity is assayed, however, no change is observed (Figure 8B). This latter result is because the total protein level in the leaf is also rising during this time (Wittenbach, 1982; 1983a; Staswick, 1989), and this increase in protein compensates for the parallel increase in lipoxygenase activity. One observation here is confusing concerning the apparent lipoxygenase activity in the 6-week podded treatments. We have shown that the lipoxygenase mRNA level (Figure 3), the vsp94/lipoxygenase protein level (Figure 1), and the amount of this protein present in the PVM cells (Figure 6E) are all significantly reduced in the 6-week podded plants. Because of this, it is unclear why the lipoxygenase activity is higher in the 6-week podded plants relative to the 2-week preflowering plants. More extensive characterization of the leaf lipoxygenases may be necessary before this apparent conflict can be addressed.

DISCUSSION

Five major lines of evidence presented here demonstrate that the 94-kD vegetative storage protein in soybean leaves is a member of the lipoxygenase protein family. First, partial amino acid sequence data obtained from gel-purified vsp94 showed a high level of homology to other known lipoxygenases. Second, an antibody prepared against soybean leaf lipoxygenases cross-reacted with vsp94. Third, a lipoxygenase cDNA (from a hypocotyl/radicle-associated lipoxygenase) hybridized with a transcript that responded to nitrogen status, i.e., the transcript level increased dramatically upon removal of nitrogen sink tissue. Hence, the transcript detected with the lipoxygenase cDNA displayed a pattern of temporal expression that was identical to the other known VSPs (Staswick, 1988). Fourth, extensive immunocytochemical data obtained with a lipoxygenase antibody demonstrated that lipoxygenase accumulated in the vacuoles of PVM cells in the soybean leaf. Again, this pattern of localization, both tissue and subcellular, is coincident with the pattern observed for the other soybean VSPs (Franceschi et al., 1983). Finally, we were able to demonstrate that removing the sink tissue by depodding resulted in an increase in the level of vsp94 observed on SDS-PAGE gels, protein gel blots, and at the immunocytochemical level. This observed increase in the level of vsp94 was correlated with an increase in the level of lipoxygenase activity when assayed from leaves. Because of the fact that vsp94 is now proven to be a member of the lipoxygenase protein family, and because its expression in soybean leaves is almost exclusively associated with the PVM cells, we refer to this protein as the paraveinal mesophyll lipoxygenase or the pvmLOX.

This work has broad significance for plant biology in that it provides evidence that lipoxygenases are regulated as a function of nitrogen status and presumably play a role in the temporary storage of nitrogen during vegetative growth. Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a class of enzymes that catalyze the hydroperoxidation of polyunsaturated lipids containing *cis*, *cis*-pentadiene moieties (Hildebrand et al., 1988). Lipoxygenases are essentially ubiquitous among eukaryotic organisms and have been demonstrated to exist in many tissues of numerous higher plants and animals. The principal substrates for plant lipoxygenases are the di-unsaturated and tri-unsaturated fatty acids linoleic (18:2) and linolenic (18:3) acids (Vick and Zimmerman, 1986; Hatanaka et al., 1987). Lipoxygenase isozymes have been isolated or characterized from soybean seeds (Axelrod et al., 1981; Hildebrand and Hymowitz, 1983), young seedlings (Peterman and Siedow, 1985a, 1985b; Park and Polacco, 1989), and leaves (Grayburn et al., 1991).

There are three major areas of plant biology where lipoxygenases have been implicated: (1) growth and development, (2) senescence, and (3) wound response and pest resistance (reviewed in Hildebrand et al., 1988). Our work has added another major function for lipoxygenases in higher plants: the temporary storage of nitrogen in vegetative tissue. Although workers have speculated that seed lipoxygenases may play a storage role (Vernooy-Gerritsen et al., 1984; Peterman and Siedow, 1985a), the results presented here clearly demonstrate that lipoxygenases (or at least one lipoxygenase) are regulated by nitro-

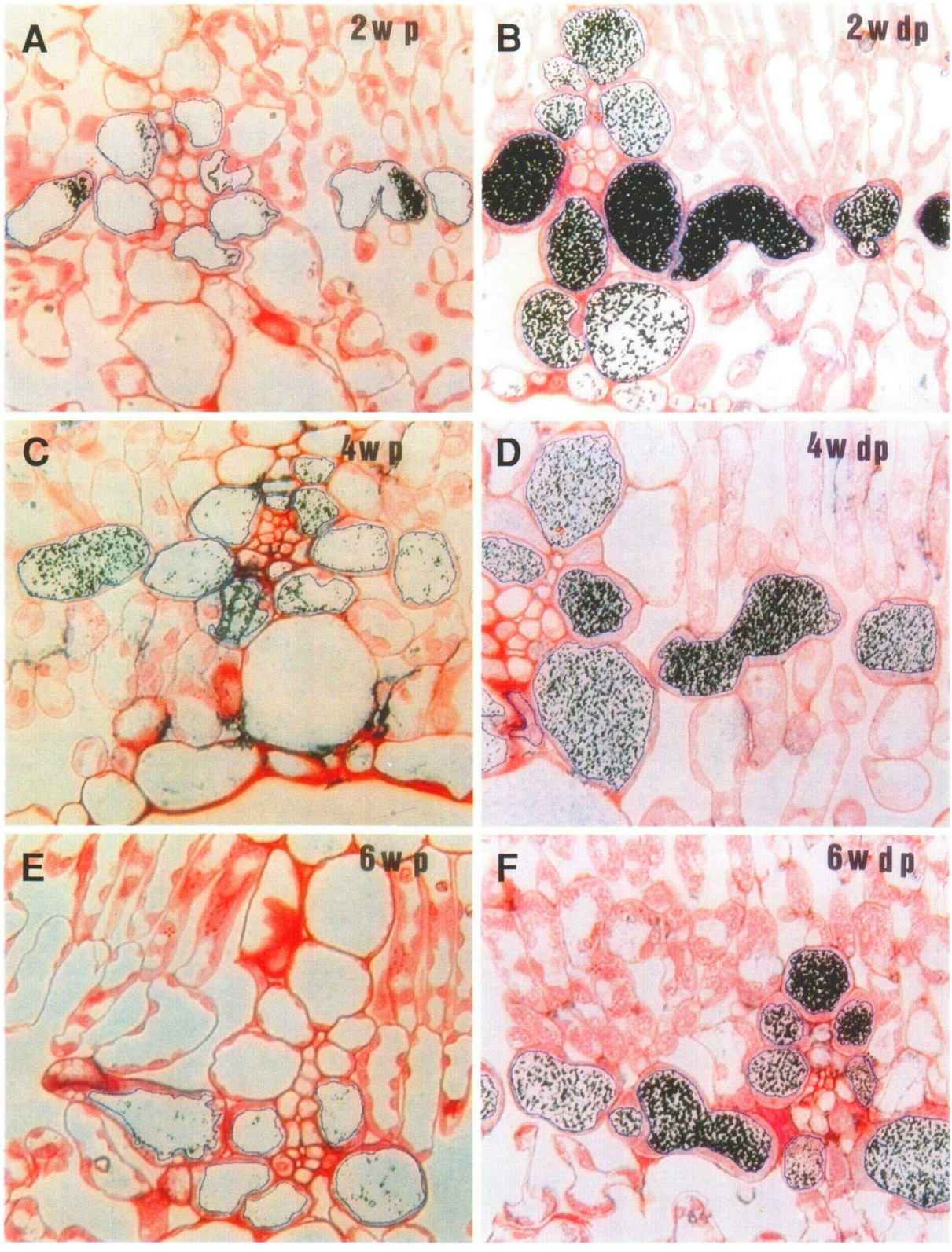

Figure 6. The vsp94/Lipoxygenase Is Strongly Regulated by Alteration of Nitrogen Sinks.

gen status. It seems quite probable at this point that plant lipoxygenases are bifunctional proteins in that they can function enzymatically in fatty acid metabolism and can also be expressed and targeted to the vacuole of PVM, cells where they apparently have been recruited to serve as a nitrogen reserve.

Franceschi and Grimes (1991) have demonstrated that low levels (approximately 10^{-7} M) of atmospheric methyl jasmonate induce the accumulation of all three VSPs in soybean seedlings. Staswick et al. (1991) demonstrated that methyl jasmonate induced the expression of the vsp27/vsp29 genes, but the effect was obtained by immersing the petioles in relatively high (50 μM) concentrations of methyl jasmonate. The fact that one of these induced proteins is now known to be a lipoxygenase is interesting in that jasmonic acid and methyl jasmonate are derived from the lipoxygenase-dependent oxidation of 18:3 fatty acids in plants (Vick and Zimmerman, 1984). At this time, it is not known whether the methyl jasmonate-induced increase in the pvmLOX can result in a coincident further increase in the biosynthesis of jasmonate-related messengers in plant cells. The fact that the pvmLOX is localized in the vacuole, whereas most of the 18:3 fatty acids are associated with plastids and plasma membranes in leaves, may indicate that the proper substrate for the biosynthesis of jasmonate-related messengers is spatially segregated from the enzyme. These questions, however, deserve further attention in terms of signal transduction involvement in nitrogen partitioning.

The tissue and subcellular localization of the pvmLOX demonstrate that, at the cellular level, it behaves as a vegetative storage protein. Although we have observed some expression of a cross-reacting lipoxygenase in the epidermal cells, our quantitative analysis indicates that the PVM contains roughly an order of magnitude (adding together the bundle sheath and all of the PVM cells) more pvmLOX than the epidermal cells. This tissue-specific localization is even more dramatic when one considers that the palisade parenchyma cells and spongy mesophyll cells contain almost no cross-reacting lipoxygenases and yet comprise a large volume of the leaf organ. Overall, the PVM layer (which includes the bundle sheath cells) contains approximately 96% of the lipoxygenase cross-reactive material. Thus, the major lipoxygenase activity in soybean leaves is associated primarily with the PVM layer. This report demonstrates tissue-specific expression of lipoxygenases in a plant tissue.

In general, published immunocytochemical data indicate that lipoxygenases are cytoplasmic-associated or plastid-associated enzymes. Only two reports have indicated that a lipoxygenase may be associated with "lysosomal vesicles" (Wardale and Galiard, 1975, 1977). Our immunocytochemical experiments, at both the light and electron microscope level, indicate that the pvmLOX is targeted directly to the vacuole. It was observed with the TEM that the pvmLOX is distributed in the vacuole in electron-dense regions, a pattern that was observed also for vsp27 and vsp29 (Klauer et al., 1991). The vacuolar localization of the pvmLOX is interesting because the seed lipoxygenases have been sequenced (Shibata et al., 1987, 1988; Yenofsky et al., 1988), and no signal sequences characteristic of the secretory pathway have been reported. The intracellular pathway for pvmLOX arrival in the vacuole is dissimilar to the pathway for vsp27 and vsp29. Both vsp27 and vsp29 are associated with the Golgi apparatus in PVM cells and can be observed in Golgi-derived vesicles adjacent to the tonoplast (Franceschi and Giaquinta, 1983a). This has been interpreted as evidence that vsp27 and vsp29 are synthesized in membrane-bound ribosomes, moved through the Golgi apparatus, and packaged into vesicles that fuse with the tonoplast to deliver the proteins to the vacuolar compartment (Staswick, 1990). The pvmLOX, however, does not demonstrate this pattern. We were unable to observe any gold label associated with the Golgi apparatus, and we never observed any staining associated with any intracellular vesicles. Hence, the pvmLOX would appear not to follow the same intracellular pathway for delivery into the vacuole. This conclusion is corroborated by the observation that gold label was consistently seen to be in the cytoplasmic matrix and also to be in direct association with the tonoplast itself. From these data, it would appear that the pvmLOX is synthesized on free ribosomes, moved directly to the tonoplast without the involvement of membrane vesicles, and then,

Figure 6. (continued).

In all panels, the number refers to the number of weeks after flowering began. w, week; p, podded; dp, depodded.
(A) Thresholded cross-section through a 2-week podded soybean leaf.
(B) Thresholded cross-section through a 2-week depodded soybean leaf.
(C) Thresholded cross-section through a 4-week podded soybean leaf.
(D) Thresholded cross-section through a 4-week depodded soybean leaf.
(E) Thresholded cross-section through a 6-week podded soybean leaf.
(F) Thresholded cross-section through a 6-week depodded soybean leaf.

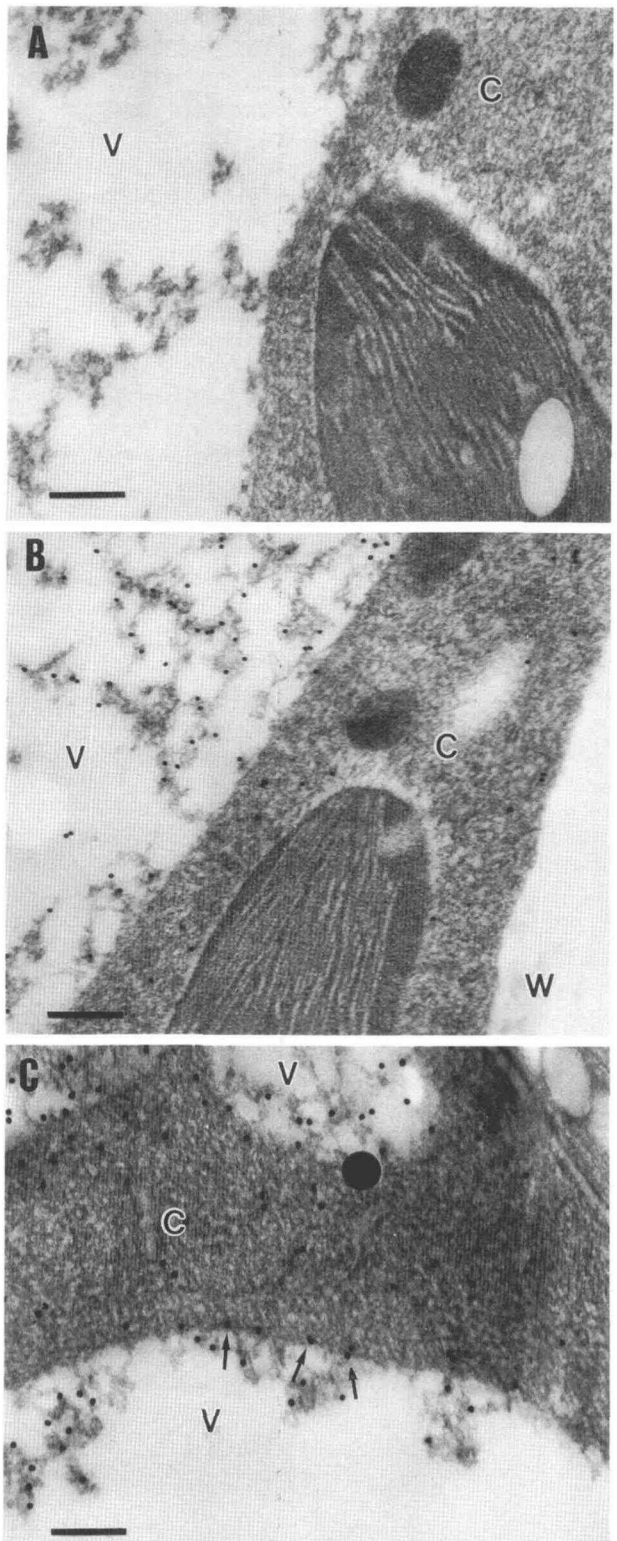

Figure 7. The vsp94/Lipoxygenase Is Localized in the Vacuole of Paraveinal Mesophyll Cells.

by an as yet undetermined mechanism, deposited into the vacuole.

Another question that was addressed was whether the pvmLOX is an active lipoxygenase. The data on this point are equivocal. We observed an increase in the lipoxygenase activity in 6-week depodded plants that coincided with previously observed increases in the pvmLOX protein (as documented on SDS-PAGE gels and immunocytochemical analysis). Even with these increases in lipoxygenase activity, it is not entirely clear that they matched the level of protein increase observed by SDS-PAGE and protein gel blot analyses. Another complicating factor in answering this question concerns the localization of the pvmLOX in the vacuole. Although it is clear that after extraction from the leaf a measurable increase in lipoxygenase activity is present in depodded plants, it is unclear whether the pvmLOX can function under the conditions that would be present in the vacuole. Because of the fact that there was a significant, measurable increase in extracted protein, however, it seems as though at least some of the pvmLOX is potentially functional.

In summary, we have shown that one of the vegetative storage proteins present in soybean leaves is a member of the lipoxygenase protein family that we have termed the pvmLOX. This lipoxygenase is expressed primarily in the PVM layer and is localized in the vacuoles. Its method of import into the vacuole, however, appears not to involve the normal Golgi-derived vesicles seen with the other VSPs (Wittenbach et al., 1984). These results raise the interesting question of whether lipoxygenases in other species may be similarly regulated in response to nitrogen supply and demand.

METHODS

Plant Material

Soybean (*Glycine max* cv Wye) seeds were planted in potting compost in 1-gallon pots and grown in a controlled environment

---

TEM of PVM cells in soybean leaf sections immunolabeled for localization of the vsp94/lipoxygenase. c, cytoplasm; v, vacuole; w, cell wall.

(A) Preimmune control on a 4-week depodded leaf. Magnification ×34,420; bar = 0.25 μm.

(B) A 4-week depodded leaf immunostained with lipoxygenase antibody. Gold label is primarily associated with material within the vacuole, but some cytoplasmic labeling is also evident. Magnification ×34,420; bar = 0.25 μm.

(C) A 2-week depodded leaf immunostained with lipoxygenase antibody. Label is closely associated with the tonoplast (arrows) as well as the vacuolar material and cytoplasmic matrix. Magnification ×51,820; bar = 0.2 μm.

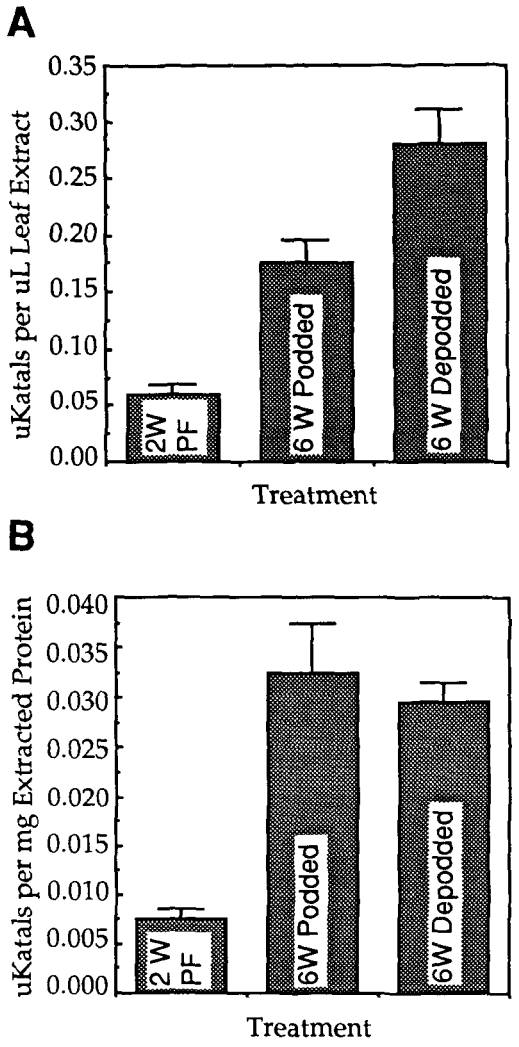

Figure 8. Lipoxygenase Activity Increases in Parallel with Increases in the vsp94/Lipoxygenase.

(A) Protein was extracted from soybean leaves 2 weeks before flowering (2W PF), 6 weeks after flowering and podding (6W Podded), and 6 weeks after continuous depodding (6W Depodded) and assayed for lipoxygenase activity using linoleic acid as a substrate. Data are expressed on a volume of leaf extract basis that is equivalent, in this case, to a leaf area basis.
(B) As in panel **(A)** except that the data are expressed as specific activity.

chamber with a photon flux density of 360 to 400 $\mu E\ m^{-2}\ sec^{-1}$ PAR at canopy height, with a 16-hr photoperiod and 24°C/18°C day/night temperatures. Plants were allowed to flower for 1 week, then flowers and developing pods were removed continuously (referred to as depodded plants) for the remainder of the time course. Control plants were allowed to flower (referred to as podded plants) and develop pods and seeds. Leaf samples were collected at the same weekly intervals as the depodded leaf samples.

Protein Extraction and Electrophoresis

Fully expanded leaf samples were collected from the main stem only once from each plant. Samples were weighed and quick frozen in liquid nitrogen and stored at −80°C. 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. The 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 in the following concentrations: 1 μM leupeptin, 1 μM pepstatin, and 100 $\mu g\ mL^{-1}$ (4-amidinophenyl)-methanesulfonyl fluoride. The homogenate was centrifuged at 10,000*g* for 15 min at 4°C and the supernatant was filtered through Miracloth and assayed for protein with the Bio-Rad Protein Assay reagent according to the manufacturer's directions (Bio-Rad Laboratories) or by the modified Lowry method of Bensadoun and Weinstein (1976). 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, and the supernatants were loaded onto SDS-PAGE gels on an equal protein basis. 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 to nitrocellulose according to Towbin et al. (1979) at 100 mA for 12 hr, followed by 200 mA for 1 hr. The blot was blocked for 2 to 3 hr with 10% Nonfat Carnation Instant Milk in Tris-buffered saline (TBS buffer: 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 lipoxygenase antiserum (1:5,000 dilution) in the blocking solution. Secondary antibody was goat anti-rabbit IgG peroxidase (Pierce Chemical Co.) in the blocking solution at a concentration of 1:5,000 for 1 hr. Color was developed by immersing the blot in 60 mg of 4-chloro-1-naphthol (first dissolved in 20 mL of cold methanol) and 60 μL of H_2O_2 in 100 mL of Tris-buffered saline.

In Situ CNBr Treatment and Amino Acid Sequence Analysis

Protein was extracted from the leaves of a plant that had been depodded for 6 weeks. The proteins were resolved on preparatory SDS-PAGE gels, and the prominent 94-kD band was excised. During electrophoresis, it was necessary to protect the amino acid residues from chemical modifications that can occur during electrophoresis by modifying the SDS-PAGE conditions as follows. The preparatory separating gels were polymerized overnight at room temperature, and the stacking gels were polymerized the next morning. The gels were prerun at 20mA per gel with Laemmli cathode buffer containing 5 μM reduced glutathione. After the ion front just entered the separating gel, the current was stopped,

the protein sample (prepared as described above except it was heated at 60°C for 5 min) was loaded into the preparatory well, and a fresh cathode buffer containing 0.1 mM sodium thioglycolate was used during electrophoresis. The thio compounds act as scavengers to oxidants and free radicals in the acrylamide gel and keep them from reacting with the N-terminal amino acid and other amino acid side chains, which could affect amino acid sequence analysis (Hunkapiller et al., 1983). The gels were stained with 0.1% Coomassie Brilliant Blue G-250 for no longer than 15 min, followed by rapid destaining until the protein bands were just visible when placed over a light box. Gel pieces containing vsp94 were cut to 3 mm^2, lyophilized, and stored at −20°C.

In situ CNBr cleavage of vsp94 contained in gel pieces was done according to Jahnen et al. (1990). The protein:CNBr mass ratio was approximately 1:50 with the reaction carried out in 70% formic acid (v/v) in 0.5-mL volumes. The reaction mixtures were flushed with nitrogen gas before being capped and wrapped in foil and incubated for 16 hr on a rotary shaker at room temperature. At the end of the reaction, the gel pieces were transferred to new microcentrifuge tubes, which were filled with water (to dilute the formic acid and CNBr within the gel pieces) and then vacuum centrifuged to dryness (Speedvac, Savant).

Peptides generated by in situ CNBr treatment of protein-containing gel slices were resolved by Tricine-SDS-PAGE as described by Schägger and von Jagow (1987) using a 1.5-mm gel composed of a separating gel (16.5% T, 3% C, 10 cm long), a spacer gel (10% T, 3% C, 2 cm long) and a stacking gel (4% T, 3% C, 4 cm long) (where T = total percent of acrylamide:Bis and C = percent of Bis [crosslinker]). After CNBr treatment, gel slices containing peptides were equilibrated with three changes of 1 M Tris-HCl (pH 8) for 5 min before being loaded directly into the sample wells of the stacking gel. Gel slices were overlayed with 2 × Laemmli sample buffer (Laemmli, 1970) and electrophoresed at 20 mA for 3 hr , followed by 50 mA for 13 hr. The resolved peptides were then electroblotted to PVDF membrane with a current of 200 mA for 3 hr, followed by 250 mA for 15 min. Peptides were visualized by staining the blot with 0.1% Coomassie Brilliant Blue G-250 for 5 min, rinsing with water, and destaining with two quick changes of 50% MeOH and 10% acetic acid. Peptides bound to the PVDF membrane were cut out, transferred to microcentrifuge tubes, and rinsed in several changes of water before being subjected to automated Edman degradation directly on the PVDF membrane. PVDF membrane pieces binding peptides were stored with a drop of water at −20°C if not analyzed immediately. The microsequencing was performed using an Applied Biosystems 470 protein sequencer by the laboratory for Biotechnology and Bioanalysis, Biochemistry and Biophysics Department, Washington State University.

RNA Isolation and RNA Gel Blot Analysis

Total leaf RNA was isolated according to Nelson and Ryan (1980) with only minor modifications. The tissue (2 g) was ground to a fine powder with a prechilled mortar and pestle and transferred to a clean mortar at room temperature containing 20 mL of extraction buffer (5 M sodium perchlorate, 0.64 M Tris, 5% [w/v] SDS, 100 mM EDTA, pH 8, 50 mM NaCl, and 8.5% [w/v] PVP) and ground for an additional 5 min. The slurry was centrifuged at 30,000*g* for 15 min at 4°C in an SW28 rotor. The aqueous layer under the insoluble plug that formed was extracted using a sterile syringe, filtered through cheesecloth, and precipitated in ethanol. The precipitate was dissolved in 2 mL of TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and extracted sequentially with equal volumes of phenol, twice with phenol:chloroform (1:1), and twice with chloroform. The organic phase was reextracted with an additional equal volume of TE and the aqueous layers were pooled and RNA was precipitated with 0.5 volume of 7.5 M ammonium acetate, pH 7.8, and 2 volumes of ice-cold 95% ethanol. RNA was precipitated overnight before being pelleted by centrifugation at 13,000*g* at 4°C for 10 min in a JA17 rotor. The supernatant was removed and the pellet was dissolved in 250 μL of TE, and the RNA concentration was determined spectrophotometrically. Total RNA samples were reprecipitated with 0.5 volume of 7.5 M ammonium acetate, pH 7.8, and 2 volumes of ethanol and stored at −20°C.

For RNA gel blot analysis, total RNA (14 μg per lane) was separated on agarose gels containing formaldehyde and transferred to nitrocellulose membranes (Sambrook et al., 1989). The probe was a 1.4-kb EcoRI fragment that was isolated from pTK11 (this plasmid contains a cDNA to a hypocotyl/radicle-associated lipoxygenase that was isolated by Park and Polacco and generously provided to us) and labeled by random priming according to the manufacturer's directions (United States Biochemicals). Prehybridization was done for 2 hr in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS, and 100 μg/mL sheared salmon sperm DNA. Hybridizations were carried out in the same buffer overnight at 65°C. The blots were washed once in 2 × SSC and 0.5% SDS at room temperature for 5 min, then in 2 × SSC and 0.1% SDS at room temperature for 15 min, then in 0.1 × SSC and 0.5% SDS at 37°C for 45 min, and finally in 0.1 × SSC and 0.5% SDS at 65°C for 45 min.

Tissue Preparation and Immunolocalization

Leaf tissue was sliced into pieces approximately 2 mm^2 and placed immediately into a vessel containing a freshly prepared fixative with the following components: 2% (v/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde, 50 mM Pipes, pH 7.2, and 2 mM $CaCl_2$. Tissue was allowed to fix for 6 hr at room temperature with gentle agitation before being rinsed with 50 mM Pipes, pH 7.2, three times for 15 min and dehydrated with an ethanol series. Tissue was infiltrated with L. R. White resin and polymerized in gelatin capsules at 60°C for 24 hr. One-micrometer sections were cut and dried to a gelatin-coated slide. Sections were blocked with TTBS+BSA (10 mM Tris, 500 mM NaCl, 0.3% Tween 20, 1% [w/v] BSA) for 60 min, then incubated in soybean leaf lipoxygenase antiserum (1:150 dilution) at room temperature for 3 hr. Nonimmune serum was used at the same dilution. Sections were then washed four times for 5 min with the diluted blocking solution before being incubated with protein A-gold conjugate (15 nm; Janssen, Olen, Belgium; U.S. distributor Ted Pella, Inc., Tustin, CA; 1:50 in TBS) for 2 hr. The sections were rinsed in the blocking solution three times for 5 min, then rinsed with TTBS+BSA (10 mM Tris-Cl, 500 mM NaCl, 1% BSA [v/v], 0.1% Tween 20 [v/v]) three times for 5 min. Sections were then rinsed in water for 30 to 60 sec and allowed to air dry and then enhanced with silver (IntenseM; Amersham) twice for 5 min. Sections were then stained with 1% Safranin at room temperature.

Light microscopy was performed using a Leitz (Wetzlar, Germany) Aristoplan microscope. The Bioquont (R and M Biometrics, Inc., Nashville, TN) Meg IV image analysis programs were used for background correction and quantification of silver grains within cells. For the quantification of relative protein level in individual cell types, a 4-week depodded section was used. At least six different sections were thresholded and between 15 and 60 individual cells were counted for each cell type.

TEM Immunocytochemistry

Leaf samples were those used for light microscopy immunolocalization. Thin sections were picked up onto nickel grids and incubated in TTBS + BSA for 1 hr at room temperature to block nonspecific protein binding sites. The grids were then incubated for 4 hr in soybean leaf lipoxygenase antiserum at a dilution of 1:100 with TTBS+BSA. They were rinsed four times by immersion (5 min) in TTBS+BSA and then incubated for 1 hr in protein A-gold (15 nm; Janssen) diluted 1:50 with TTBS+BSA. After extensive washes by immersion in TTBS+BSA, TTBS, and then water, the sections were poststained with uranyl acetate, potassium permanganate, and lead citrate. Sections were examined and photographed with a Hitachi H-300 transmission electron microscope.

Activity Measurements for Lipoxygenase

Leaves from preflowering, 6-week podded, and 6-week depodded plants were harvested, weighed, and ground in liquid nitrogen, and an equal volume of water (per gram fresh weight) was added. Samples were centrifuged at 13,000*g* for 15 min and supernatant was collected and filtered through Miracloth. Lipoxygenase activity was determined with a substrate containing 1.4 mM linoleic acid and 20 μM hydroperoxylinoleic acid using the standard spectrophotometric assay (Hildebrand et al., 1991), which involves measurement of conjugated diene formation at 235 nm.

ACKNOWLEDGMENTS

The authors are especially indebted to Tae Kyung Park and Dr. Joseph Polacco (Biochemistry Department, University of Missouri-Columbia) for their generous gift of the lipoxygenase cDNA (pTK11). A grant from the National Science Foundation (DIR9016138) allowed us to establish the Plant Biology Light Microscopy and Image Analysis Facility. We thank Paul Overvoorde for his excellent assistance with the image analysis. The amino acid sequencing was performed with the aid of Dr. Gerhard Munske of the Laboratory for Biotechnology and Bioanalysis at Washington State University (supported by grants from the U.S. Department of Health and Human Services and the National Institutes of Health). This work was supported in part by the U.S. Department of Agriculture (Grant No. 84-CRSR-2-2496).

Received June 11, 1991; accepted July 25, 1991.

REFERENCES

Altschuler, M., Grayburn, W.S., Collins, G.B., and Hildebrand, D.F. (1989). Developmental expression of lipoxygenases in soybeans. Plant Sci. **63,** 151–158.

Anderson, J.M. (1988). Jasmonic acid-dependent increases in the level of specific polypeptides in soybean suspension cultures and seedlings. J. Plant Growth Regul. **7,** 203–211.

Anderson, J.M., Spilatro, S.R., Klauer, S.F., and Franceschi, V.R. (1989). Jasmonic acid-dependent increases in the level of vegetative storage protein in soybean. Plant Sci. **62,** 45–52.

Axelrod, B., Cheesbrough, T.M., and Laasko, S. (1981). Lipoxygenase from soybeans. Methods Enzymol. **71,** 441–451.

Bensadoun, A., and Weinstein, D. (1976). Assay of proteins in the presence of interfering materials. Anal. Biochem. **70,** 241–250.

Costigan, S.A., Franceschi, V.R., and Ku, M.S.B. (1987). Allantoinase activity and ureide content of mesophyll and paraveinal mesophyll protoplasts of soybean leaves. Plant Sci. **50,** 179–187.

Crafts-Brandner, S.J., Below, F.E., Harper, J.E., and Hageman, R.H. (1984). Effects of pod removal on metabolism and senescence of nodulating and nonnodulating soybean isolines. J. Exp. Bot. **75,**318–322.

deVeau, E.J., Robinson, J.M., Warmbrodt, R.D., and Van Berkum, P. (1990). Photosynthesis and photosynthate partitioning in N_2-fixing soybeans. Plant Physiol. **94,** 259–267.

Ealing, P.M., and Casey, R. (1988). The complete amino acid sequence of a pea (*Pisum sativum*) seed lipoxygenase predicted from a near full-length cDNA. Biochem. J. **253,** 915–918.

Everard, J.D., Ku, M.S.B., and Franceschi, V.R. (1990). Distribution of metabolites and enzymes of nitrogen metabolism between the mesophyll and paraveinal mesophyll of non-nodulated *Glycine max*. J. Exp. Bot. **41,** 855–861.

Fisher, D.B. (1967). An unusual layer of cells in the mesophyll of the soybean leaf. Bot. Gaz. **128,** 215–221.

Franceschi, V.R. (1986). Temporary storage and its role in partitioning among sinks. In Phloem Transport, J. Cronshaw, W.J. Lucas, and R.T. Giaquinta, eds. (New York: Alan R. Liss), pp. 399–409.

Franceschi, V.R., and Giaquinta, R.T. (1983a). The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. I. Ultrastructure and histochemistry during vegetative development. Planta **157,** 411–421.

Franceschi, V.R., and Giaquinta, R.T. (1983b). The paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. II. Structural, metabolic, and compartmental changes during reproductive growth. Planta **157,** 422–431.

Franceschi, V.R., and Giaquinta, R.T. (1983c). Specialized cellular arrangements in legume leaves in relation to assimilate transport and compartmentation. Comparison of the paraveinal mesophyll. Planta **159,** 415–422.

Franceschi, V.R., and Grimes, H.D. (1991). Low levels of atmospheric methyl jasmonate induce the accumulation of soybean vegetative storage proteins and anthocyanins. Proc. Natl. Acad. Sci. USA **88,** 6745–6749.

Franceschi, V.R., Wittenbach, V.A., and Giaquinta, R.T. (1983). Paraveinal mesophyll of soybean leaves in relation to assimilate transfer and compartmentation. III. Immunohistochemical localization of specific glycopeptides in the vacuole after depodding. Plant Physiol. **72,** 586–589.

Galliard, T., and Chan, H.W.-S. (1980). Lipoxygenases. In The Biochemistry of Plants, Vol. 4, P.K. Stumpf and E.E. Conn, eds (Orlando, FL: Academic Press), pp. 131–161.

Grayburn, W.S., Schneider, G.R., Hamilton-Kemp, T.R., Bookjans, G., Ali, K., and Hildebrand, D.F. (1991). Soybean leaves contain multiple lipoxygenases. Plant Physiol. **95,** 1214–1218.

Hatanaka, A., Kajimara, T., and Sekiya, J. (1987). Biosynthetic pathway for C6-aldehyde formation from linolenic acid in green leaves. Chem. Phys. Lipids **44,** 341–361.

Hildebrand, D., and Hymowitz, T. (1983). Lipoxygenase activities in developing and germinating soybean seeds with and without lipoxygenase-1. Bot. Gaz. **144,** 212–216.

Hildebrand, D.F., Hamilton-Kemp, T.R., Legg , C.S., and Bookjans, G. (1988). Plant Lipoxygenases: Occurrence, properties, and possible functions. Curr. Top. Plant Biochem. Physiol. **7,** 201–219.

Hildebrand, D.F., Versluys, R.T., and Collins, G.B. (1991). Changes in lipoxygenase isozyme levels during soybean embryo development. Plant Science, in press.

Hunkapiller, M.W., Lujan, E., Ostrander, F., and Hood, L.E. (1983). Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. **91,** 227–235.

Jahnen, W., Ward, L.D., Reid, G.E., Moritz, R.L., and Simpson, R.J. (1990). Internal amino acid sequencing of proteins by in situ cyanogen bromide cleavage in polyacrylamide gels. Biochem. Biophys. Res. Commun. **166,** 139–145.

Kevekordes, K.G., McCully, M.E., and Canny, M.J. (1988). The occurrence of an extended bundle sheath system (paraveinal mesophyll) in the legumes. Can. J. Bot. **66,** 94–100.

Klauer, S.F., Franceschi, V.R., and Ku, M.S.B. (1991). Protein compositions of mesophyll and paraveinal mesophyll of soybean leaves at various developmental stages. Plant Physiol., in press.

Lackey, J.A. (1978). Leaflet anatomy of Phaseolae (Leguminosae: Papilionoideae) and its relation to taxonomy. Bot. Gaz. **139,** 436–446.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lawn, R.J., and Braun, W.A. (1974). Symbiotic nitrogen fixation in soybeans I. Effect of photosynthetic source-sink manipulation. Crop Sci. **14,** 11–16.

Liljebjelke, K.A., and Franceschi, V.R. (1991). Differentiation of mesophyll and paraveinal mesophyll in soybean leaf. Bot. Gaz. **152,** 34–41.

Mack, A.J., Peterman, T.K., and Siedow, J.N. (1987). Lipoxygenase isozymes in higher plants: Biochemical properties and physiological role. Isozymes Curr. Top. Biol. Med. Res. **13,** 127–154.

Mason, H.S., and Mullet, J.E. (1990). Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. Plant Cell **2,** 569–579.

Mason, H.S., Guerrero, F.D., Boyer, J.S., and Mullet, J.E. (1988). Proteins homologous to leaf glycoproteins are abundant in stems of dark grown soybean seedlings. Analysis of proteins and cDNAs. Plant Mol. Biol. **11,** 845–856.

Matsumota, T., Funk, C.D., Radmark, O., Hoog, J.O., Jornvall, H., and Samuelsson, B. (1988). Molecular cloning and amino acid sequence of human 5-lipoxygenase. Proc. Natl. Acad. Sci. USA **85,** 26–30.

Mosse, J. (1985). Considerations on storage product physiology and biochemistry. Physiol. Veg. **23,** 37–43.

Nelson, C.E., and Ryan, C.A. (1980). In vitro synthesis of preproteins of vacuolar compartmented proteinase inhibitors that accumulate in leaves of wounded tomato plants. Proc. Natl. Acad. Sci. USA **77,** 1975–1979.

Park, T.K., and Polacco, J.C. (1989). Distinct lipoxygenase species appear in the hypocotyl/radicle of germinating soybean. Plant Physiol. **90,** 285–290.

Pate, J.S. (1980). Transport and partitioning of nitrogenous solutes. Annu. Rev. Plant Physiol. **31,** 313–340.

Peterman, T.K., and Siedow, J.N. (1985a). Behavior of lipoxygenase during establishment, senescence, and rejuvenation of soybean cotyledons. Plant Physiol. **78,** 690–695.

Peterman, T.K., and Siedow, J.N. (1985b). Immunological comparison of lipoxygenase isozymes-1 and 2 with soybean seedling lipoxygenase. Arch. Biochem. Biophys. **238,** 476–483.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Schägger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kD. Anal. Biochem. **166,** 368–379.

Shibata, D., Steczko, J., Dixon, J.E., Hermodson, M., Yagdanparast, R., and Axelrod , B. (1987). Primary structure of lipoxygenase 1. J. Biol. Chem. **262,** 10080–10085.

Shibata, D., Steczko, J., Dixon, J.E., Andrews, P.C., Hermodson, M., and Axelrod, B. (1988). Primary structure of lipoxygenase L-2. J. Biol. Chem. **263,** 6816–6821.

Simpson, R.J. (1986). Translocation and metabolism of nitrogen: Whole plant aspects. In Fundamental, Ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants, H. Lambers, J.J. Neetson, and I. Stulin, eds (Dordrecht, The Netherlands: Martinus Nijhoff), pp. 71–96.

Spilatro, S.R., and Anderson, J.M. (1989). Characterization of a soybean leaf protein that is related to the seed lectin and is increased with pod removal. Plant Physiol. **90,** 1387–1393.

Staswick, P.E. (1988). Soybean vegetative protein structure and gene expression. Plant Physiol. **87,** 250–254.

Staswick, P.E. (1989). Developmental regulation and the influence of plant sinks on vegetative storage protein gene expression in soybean leaves. Plant Physiol. **89,** 309–315.

Staswick, P.E. (1990). Novel regulation of vegetative storage protein genes. Plant Cell **2,** 1–6.

Staswick, P.E., Huang, J.F., and Rhee, Y. (1991). Nitrogen and methyl jasmonate induction of soybean vegetative storage protein genes. Plant Physiol. **96,** 130–136.

Surowy, T.K., and Boyer, J.S. (1991). Low water potentials affect expression of genes encoding vegetative storage proteins and

plasma membrane proton ATPase in soybean. Plant Mol. Biol. **16,** 251–262.

Thibodeau, P.S., and Jaworski, E.G. (1975). Patterns of nitrogen utilization in the soybeans. Planta **127,** 133–147.

Towbin, H., Staehlin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76,** 4350–4354.

Vernooy-Gerritsen, M., Bos, A.L.M., Veldink, G.A., and Vliegenthart, J.F.G. (1983). Localization of lipoxygenases-1 and -2 in germinating soybean seeds by an indirect immunofluorescence technique. Plant Physiol. **72,** 262–267.

Vernooy-Gerritsen, M., Leunissen, J.L.M., and Veldink, G.A. (1984). Intracellular localization of lipoxygenases-1 and -2 in germinating soybean seeds by indirect labelling with protein A-colloidal gold complexes. Plant Physiol. **76,** 1070–1079.

Vick, B.A., and Zimmerman, D.C. (1984). Biosynthesis of jasmonic acid by several plant species. Plant Physiol. **75,** 458–461.

Vick, B.A., and Zimmerman, D.C. (1986). Characterization of 12-oxo-phytodienoic acid reductase in corn. Plant Physiol. **80,** 202–205.

Vick, B.A., and Zimmerman, D.C. (1987). Oxidative systems for modifications of fatty acids: The lipoxygenase pathway. In The Biochemistry of Plants: A Comprehensive Treatise, Vol. 9, P.K. Stumpf and E.E. Conn, eds (Orlando, FL: Academic Press), pp. 53–90.

Wardale, D.A., and Galiard, T. (1975). Subcellular localization of lipoxygenase and lipolytic acyl hydrolase enzymes in plants. Phytochemistry **14,** 2323–2329.

Wardale, D.A., and Galiard, T. (1977). Further studies on the subcellular localization of lipid-degrading enzymes. Phytochemistry **16,** 333–338.

Wittenbach, V.A. (1982). Effect of pod removal on leaf senescence in soybeans. Plant Physiol. **70,** 1544–1548.

Wittenbach, V.A. (1983a). Effect of pod removal on leaf photosynthesis and soluble protein composition of field-grown soybeans. Plant Physiol. **73,** 121–124.

Wittenbach, V.A. (1983b). Purification and characterization of a soybean leaf storage glycoprotein. Plant Physiol. **73,** 125–129.

Wittenbach, V.A., Franceschi, V.R., and Giaquinta, R.T. (1984). Soybean leaf storage proteins. Curr. Top. Plant Biochem. Physiol. **3,** 19–30.

Yenofsky, R.L., Fine, M., and Liu, C. (1988). Isolation and characterization of a soybean (*Glycine max*) lipoxygenase-3 gene. Mol. Gen. Genet. **211,** 215–222.