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Sink Limitation Induces the Expression of Multiple Soybean Vegetative Lipoxygenase mRNAs while the Endogenous Jasmonic Acid Level Remains Low

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The response of individual members of the lipoxygenase multigene family in soybeans to sink deprivation was analyzed. RNase protection assays indicated that a novel vegetative lipoxygenase gene, *vlxC*, and three other vegetative lipoxygenase mRNAs accumulated in mature leaves in response to a variety of sink limitations. These data suggest that several members of the lipoxygenase multigene family are involved in assimilate partitioning. The possible involvement of jasmonic acid as a signaling molecule regulating assimilate partitioning into the vegetative storage proteins and lipoxygenases was directly assessed by determining the endogenous level of jasmonic acid in leaves from plants with their pods removed. There was no rise in the level of endogenous jasmonic acid coincident with the strong increase in both *vlxC* and vegetative storage protein *VspB* transcripts in response to sink limitation. Thus, expression of the vegetative lipoxygenases and vegetative storage proteins is not regulated by jasmonic acid in sink-limited leaves.

INTRODUCTION

Lipoxygenases are a class of enzymes found in plants and animals that catalyze the addition of molecular oxygen to (*Z*),(*Z*)-pentadiene motifs of unsaturated fatty acids to form hydroperoxide products and contain a non-heme iron, which is necessary for enzymatic activity. Animal lipoxygenases (and cyclooxygenases) use arachidonic acid (20:4) as a substrate and produce a plethora of eicosanoid products, including leukotrienes, lipoxins, and prostaglandins, which are important cellular mediators in inflammatory processes (Samuelsson et al., 1987). In addition, lipoxygenases associated with mammalian reticulocytes may be important in the degradation of intracellular membranes during red blood cell formation (Kuhn et al., 1990).

Plant lipoxygenases use linolenic acid (18:3) or linoleic acid (18:2) as substrates and are involved in the biosynthesis of regulatory compounds, such as traumatic acid and jasmonic acid, the latter derived from linolenic acid via an allene oxide intermediate (Vick and Zimmerman, 1987; Song and Brash, 1991). Lipoxygenase activities are induced during plant defense responses (Yamamoto and Tani, 1986; Keppler and Novacky, 1987; Croft et al., 1990; Bostock et al., 1992; Kato et al., 1992a; Koch et al., 1992; Fournier et al., 1993; Peng et al., 1994), and some lipoxygenase pathway products may directly mediate host resistance to insect (Kasu et al., 1994), fungal (Kauss et al., 1992; Vaughn and Gardner, 1993), and bacterial (Croft et al., 1993) pathogens. Lipoxygenases also may have a direct role in signaling plant defense and wounding responses via the production of jasmonic acid (Farmer and Ryan, 1992; Gundlach et al., 1992), and many jasmonate-inducible proteins appear to be involved in plant defense responses (Reinbothe et al., 1994). Creelman et al. (1992) have shown that wounding causes an increase in the level of endogenous jasmonic acid in soybean seedlings.

Soybeans contain at least seven different lipoxygenase activities that are distinguishable by chromatographic properties, pH optima, calcium activation, substrate specificity, and specific reaction products. Chromatofocusing has been the primary analytical method used to identify and characterize lipoxygenase isozymes and has delineated at least three peaks of activity in young soybean seedlings (Park and Polacco, 1989; Kato et al., 1992b) and mature leaves (Grayburn et al., 1991). Three additional isozymes (L-1, L-2, and L-3) are associated with the seed; L-1 is biochemically and structurally the best characterized lipoxygenase. Site-directed mutagenesis of L-1 has established the importance of several conserved histidine residues for catalysis (Steczko et al., 1992). The crystal

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structure of L-1 shows that soybean lipoxygenases consist of two major domains, an N-terminal β barrel and a larger α-helical domain containing the catalytic site and a single iron atom (Boyington et al., 1993; Minor et al., 1993). Although the β barrel domain is unique to plant lipoxygenases, its function is not known.

Despite the well-characterized enzymatic function of lipoxygenases and, in some cases, detailed knowledge of their primary and tertiary structures, the assignment of physiological roles for plant lipoxygenases has proceeded tentatively. Much of the ambiguity is due to the presence of multiple lipoxygenase isozymes and their association with a wide range of physiological processes, including normal growth and development, senescence, wounding, and pathogen defense responses (for review, see Siedow, 1991).

Identification of the ~94-kD vegetative storage protein (VSP94), which accumulates in leaves of pod-removed soybean plants as a lipoxygenase, and immunolocalization of VSP94 to the vacuoles of paraveinal mesophyll cells indicate that one or more vegetative lipoxygenases may also function as storage proteins (Tranbarger et al., 1991). Vegetative lipoxygenases respond to many of the same stimuli that induce *VSP* gene expression (Tranbarger et al., 1991; Grimes et al., 1992, 1993). For example, although low levels of nitrogen do not completely abrogate vegetative lipoxygenase protein accumulation, abundant nitrogen stimulates the accumulation of higher levels of lipoxygenases (Grimes et al., 1993); this is analogous to the VSP response to excess nitrogen (Staswick et al., 1991). Vegetative lipoxygenases and VSPs are induced by wounding, water stress, and jasmonic acid (Anderson, 1988; Mason and Mullet, 1990; Bell and Mullet, 1991), and low levels of airborne methyl jasmonate also result in their accumulation (Franceschi and Grimes, 1991; Grimes et al., 1992). Both vegetative lipoxygenases and VSPs are induced synergistically by methyl jasmonate and sugars (Mason et al., 1992), and intracellular phosphate may serve as an important regulatory control point for modulating both *VSP* and *vlx* gene expression (Sadka et al., 1994).

The primary objective of this research was to delineate the responses of individual members of the lipoxygenase multigene family to sink limitation. We report the cloning of a new vegetative lipoxygenase cDNA, *vlxC*. Together with other cloned *vlx* cDNAs, they provide the necessary nucleotide sequence data for the design of specific antisense RNA probes. RNase protection assays were used to distinguish between the *vlx* transcripts and to assay their individual responses to manipulation of the source-to-sink ratio. The data indicate that four *vlx* mRNAs accumulated in response to sink deprivation but that the magnitude of response varied for individual members of this multigene family. Finally, the level of endogenous jasmonic acid in sink-limited leaves was assayed to test directly the hypothesis that jasmonic acid is involved in the signaling cascade regulating assimilate partitioning into the VSPs and vegetative lipoxygenases.

RESULTS

Molecular Characterization of a Novel Vegetative Lipoxygenase

To clone the soybean lipoxygenase isozymes that accumulate when pods are removed, we first constructed a λZAP cDNA library using poly(A)$^+$ RNA isolated from leaves taken from depodded plants. This library was screened by nucleic acid hybridization using a random primed probe generated from a conserved region of a previously identified soybean seedling lipoxygenase cDNA clone (Park et al., 1994). Partial sequencing of 22 inserts from a total of 30 selected cDNAs revealed the presence of two different lipoxygenase cDNAs, with most of the cDNAs representing a single previously uncharacterized lipoxygenase gene. Figure 1 shows the 2819 nucleotide sequence of the longest novel cDNA, *vlxC*. In comparison with other plant lipoxygenase cDNAs, *vlxC* contains the initiating ATG codon and represents a full-length clone. The 3′ end of the *vlxC* cDNA matches the putative 3′ untranslated region of a partial lipoxygenase genomic clone found 1 kb upstream of the SC514 lipoxygenase gene (Shibata et al., 1991). The other lipoxygenase inserts isolated from the pod-removed leaf library corresponded to the pTK11 *vlx* cDNA for which a genomic subclone, SC514, also exists (Shibata et al., 1991).

In Table 1, we present a *vlx* gene nomenclature for members of this multigene family of soybean. In addition to the four *vlx* genes shown in Table 1, the soybean lipoxygenase multigene family contains three seed-specific lipoxygenase genes, which are designated *Lox1:Gm:1*, *Lox1:Gm:2*, and *Lox1:Gm:3*. Using the recommendations of the Commission on Plant Gene Nomenclature (Shibata et al., 1994), the four *vlx* genes are grouped in a single class designated *Lox1* and are, thus, given the additional designations of *Lox1:Gm:4*, *Lox1:Gm:5*, *Lox1:Gm:6*, and *Lox1:Gm:7* (as shown in Table 1). We have grouped *vlxA* with *vlxB* because of the sequence similarity implied by partial protection of *vlxB* RNAs with a *vlxA* antisense RNA probe. *vlxC* is grouped with *vlxD* because of sequence similarity and because *vlxC* is known to be the 5′ member of a direct tandem repeat with *vlxD* (see later discussion). Because *vlxA* and *vlxD* have been previously published but not yet assigned a gene member number, we have maintained the *vlx* nomenclature in this manuscript.

The translated *vlxC* sequence codes for an 859-residue lipoxygenase with a predicted molecular weight of 96,344 and a predicted isoelectric point of 6.71. Alignment of the deduced amino acid sequence of *vlxC* with that of the other soybean seed and vegetative lipoxygenases indicates that the soybean lipoxygenase family is highly conserved (data not shown). *vlxC* is most closely related to *vlxD*, showing 87% identity and 94% similarity. The first 154 nucleotides of the 3′ untranslated regions of these cDNAs are 71.4% identical. The homology of the

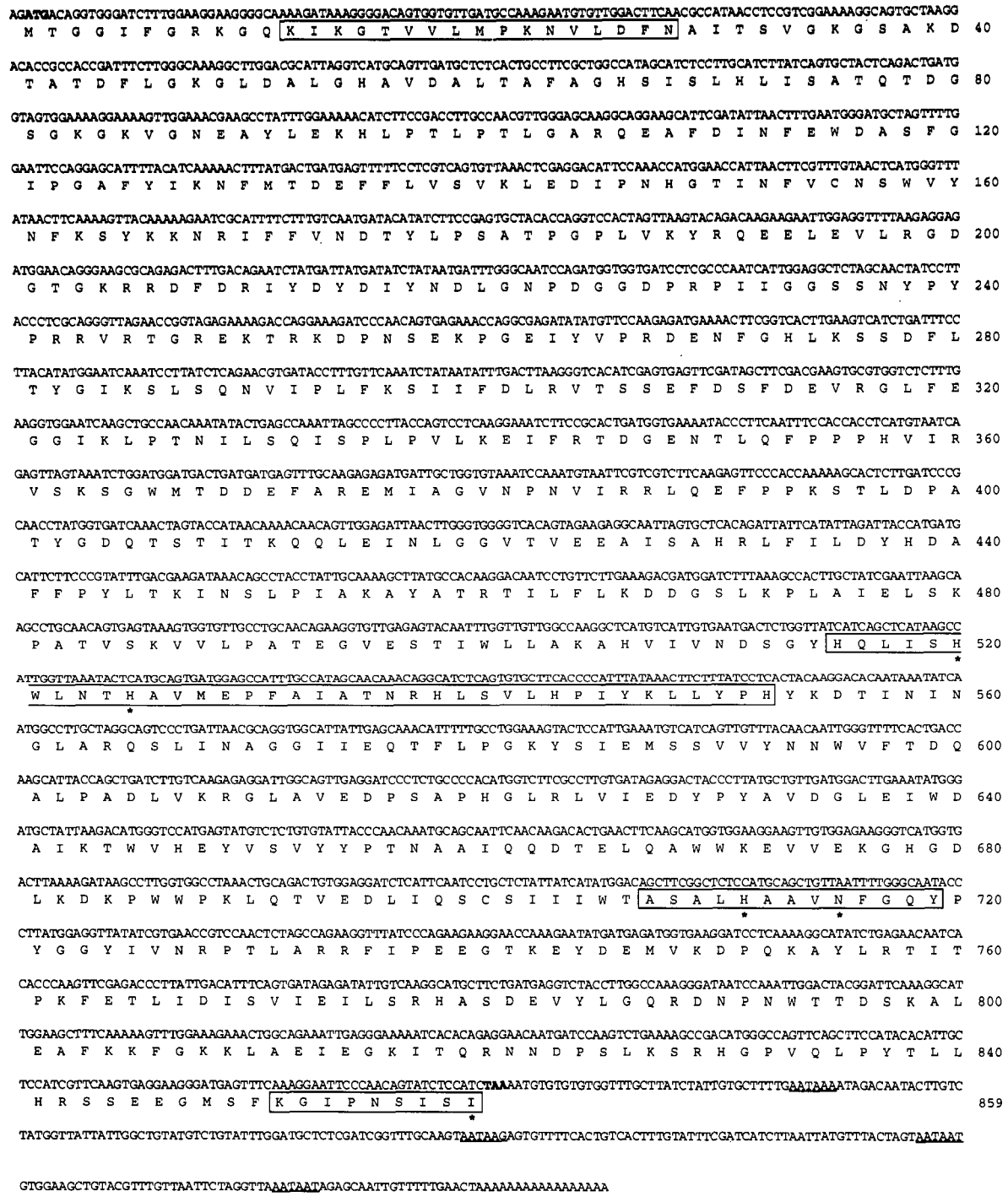

Figure 1. Nucleotide and Deduced Amino Acid Sequences of Soybean *vlxC*.

The predicted amino acid sequence derived from the continuous open reading frame of *vlxC* is shown in the standard one-letter code below the nucleotide sequence. Boxed regions of the translated sequence correspond to highly conserved regions between several members of the lipoxygenase gene family as described in the text. The asterisks indicate residues that may coordinate Fe near the catalytic site. These residues were determined by comparison with the soybean lipoxygenase L-1 amino acid sequence. The initiating methionine codon and the translation stop codon are shown in bold type. Potential polyadenylation sites are underlined in the 3′ untranslated region. The GenBank *vlxC* accession number is U26457.

Table 1. Proposed Nomenclature for Soybean *vlx* Multigene Family Members

Designation	Previous cDNA Epithet	Previous Genomic Clone Epithet (If Any)	Proposed Gene Member Designation
vlxA	TK18[a] (partial) SC501[c] *loxB1*[d] (partial)	SS12[b] λSCG501[c]	*Lox1:Gm:4*
vlxB			*Lox1:Gm:5*
vlxC	*loxN*[e]	λSCG514[b] (partial)	*Lox1:Gm:6*
vlxD	TK11[a] *loxA*[d] SC514[b]	λSCG514[b]	*Lox1:Gm:7*

[a] Park et al. (1994).
[b] Kato et al. (1993).
[c] Shibata et al. (1991).
[d] Bell and Mullet (1991).
[e] Koetje and Grimes (1992).

deduced *vlxC* gene product with the remaining soybean lipoxygenases varies from a low of 68% identity with the *vlxA* gene product to between 71 and 72% identity with the three seed isozymes. Because the C-terminal coding region of *vlxC* is <1 kb upstream of the *vlxD* coding region, the genes are in the same orientation (Shibata et al., 1991). This direct tandem repeated pair of lipoxygenase genes may have arisen by a gene duplication event.

Several regions of plant lipoxygenases are highly conserved, including nine amino acids at the extreme C terminus, a 38–amino acid domain containing five invariant histidine residues, a 13–amino acid domain conserved between plant lipoxygenases and mammalian 5-lipoxygenases, and a 17–amino acid region close to the N terminus (Siedow, 1991; Ferrie et al., 1994). These regions are also conserved in the *vlxC* gene product and are boxed in Figure 1. Crystallographic studies of L-1 indicate that four or five residues act as ligands in the coordination of the active site non-heme iron atom (Minor et al., 1993). Alignment of the amino acid sequence deduced from *vlxC* with the L-1 sequence demonstrates that all of the active site residues (indicated in Figure 1 by asterisks) are present

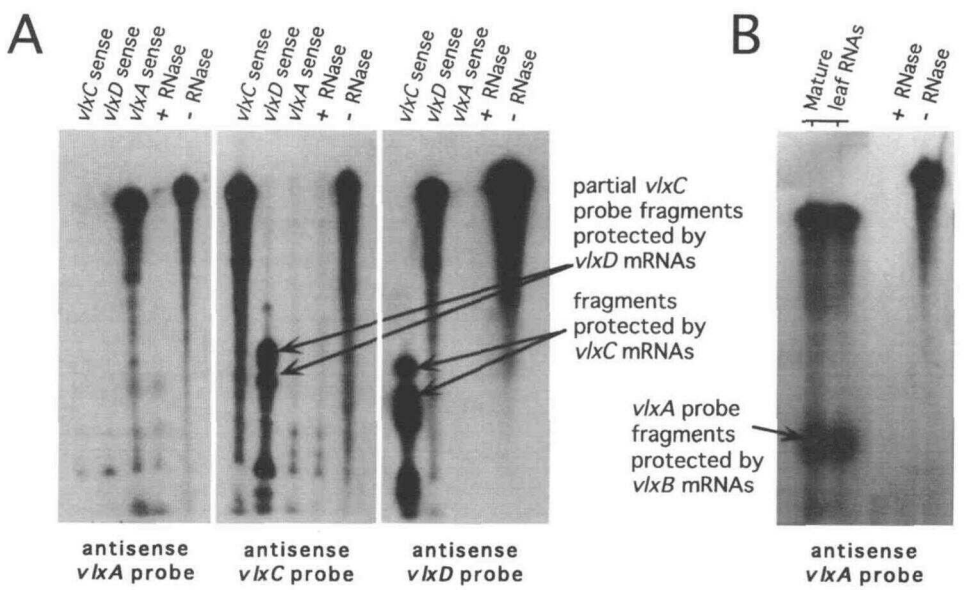

Figure 2. Specificity of the Lipoxygenase RNase Protection Assays and Detection of *vlxB* mRNA.

(A) Two femtomoles of the indicated *vlx* antisense RNA probes was hybridized with either *vlxA*, *vlxB*, or *vlxC* sense transcripts in RNase protection assays. **(B)** An RNase protection assay using 2 fmol of the *vlxA* antisense RNA probe and 5 μg of total RNA purified from mature soybean leaves indicates the presence of a fourth *vlx* gene, *vlxB*. After overnight hybridizations, digestions were performed with a mixture of RNase A/T1 for 30 min at room temperature. Digestion-resistant RNA:RNA duplex fragments were then denatured and resolved on a 3.5% SDS–polyacrylamide/8 M urea gel. The in vitro–transcribed RNA probes were gel purified but degraded rapidly due to radiolytic degradation that caused the observed tailing. One-twentieth of the amount of the RNA probe added to individual hybridizations was present in the (−) RNase lanes and was not subject to the RNase digestion. The (+) RNase label indicates the control for complete digestion of the *vlx* RNA probe in the absence of protecting RNA transcripts. For this latter control, 5 μg of yeast total RNA was hybridized with the indicated *vlx* RNA probe.

```
      1                                                   50
vlxC  ATGACAGGTG GGATGTTTGG AAGGAAGGGG CAAAAGATAA AGGGGACAGT
vlxD  ...-tgtt-- ----c--c-* **ac------ ---------- ----------
vlxA  ......atgt ttcct--c-- gcaa-----t ---------- -------ta-

      51                                                  100
vlxC  GGTGTTGATG CCAAAGAATG TGTTGGACTT CAACGCCATA ACCTCGGTCG
vlxD  ---------- ---------- ---------- ---------- ------a---
vlxA  ----g-t--- -ag------- -------ta- ----ag---c ---agt--t-

      101                                                 150
vlxC  GAAAAGGCAG TGCTAAGGAC ACCGCCACCG ATTTCTTGGG CAAAGGCTTG
vlxD  -t-----tg- --t--ttg-- --a------- gca----a-- -------g-c
vlxA  --******** ********** *********- gga--g-t-a -c-------a

      151                                                 200
vlxC  GACGCATTAG GTCATGCAGT TGATGCTCTC ACTGCCTTCG CTGGCCATAG
vlxD  ag-tt-g-t- --ggagt-a- ----a--gc- ---t-----t ta----g--a
vlxA  -g-ttca--- -cag------ cg----a--t ---***--t- -a-c-acc-a

      201                                                 250
vlxC  CATCTCCTTG CATCTTATCA GTGCTACTCA GACTGATGGT AGTGGAAAAG
vlxD  t------a-- --at-g---- ---------- ---------c --------t-
vlxA  a------a-c --gt-g--t- -------ca- -g------** *----g----

      251                                                 300
vlxC  GAAAAGTTGG AAACGAAGCC TATTTGGAAA AACATCTTCC GACCTTGCCA
vlxD  -g-------- --------t- ---------- ---------- ----------
vlxA  -----a---- ---gagta-a a----aag-g g-a-ga-*** a--a--a---

      301                                                 350
vlxC  ACGTTGGGAG CAAGGCAGGA AGCATTCGAT ATTAACTTTG AATGGGATGC
vlxD  ---------- ---------- t--g---ag- ---tt----- ----------
vlxA  --c------- -tg-cg-ac- -----a---- g----t---- -------cag

      351
vlxC  TAGTTTTGGA ATTC
vlxD  ---------- ----
vlxA  -cac--c--- ----
```

Figure 3. Nucleotide Sequence Alignment of the 5′ Regions of *vlxA*, *vlxC*, and *vlxD* cDNAs.

The *vlx* antisense RNA probes and sense transcripts were generated from *vlx* cDNA EcoRI fragments subcloned into the pBluescript SK– multiple cloning site, which is flanked by opposing phage RNA polymerase promoters (see Methods). Alignment of the 5′ EcoRI *vlx* cDNA regions indicates that *vlxC* and *vlxD* are very similar and have 70- and 51-nucleotide regions of perfect identity (black boxes). The homologous *vlxA* region has multiple nucleotide substitutions, and the introduction of four gaps (indicated by asterisks) is necessary for alignment with *vlxC* and *vlxD*. Dashes indicate identity between *vlxA* or *vlxD* and the *vlxC* nucleotide sequence.

in the putative *vlxC* gene product; this suggests that *vlxC* may encode a functional lipoxygenase.

Specificity of the Lipoxygenase RNase Protection Assay

To establish the specificity of the RNase protection assay for members of the lipoxygenase multigene family, antisense *vlxA*, *vlxC*, and *vlxD* RNA probes were generated by in vitro transcription reactions using templates derived from N-terminal EcoRI fragments of the *vlx* cDNAs. Figure 2A shows the specificity of the individual lipoxygenase antisense RNA probes as determined by hybridization with their corresponding sense transcripts. The *vlxA* RNA probe was protected only by *vlxA* sense transcripts, the *vlxC* RNA probe was protected only by *vlxC* sense transcripts, and the *vlxD* RNA probe was protected only by *vlxD* sense transcripts. As expected, *vlxD* sense transcripts cross-hybridized with the antisense *vlxC* RNA probe, as evidenced by the presence of several short partially protected *vlxC* probe fragments. Similarly, several short *vlxD* RNA probe fragments were protected from RNase digestion by *vlxC* sense transcripts. Partial protection of these two RNA probe fragments occurred because the *vlxC* and *vlxD* nucleotide sequences share identical regions of 72 and 51 bp in the 5′ terminus of their cDNAs, as shown in Figure 3. Neither *vlxC* nor *vlxD* transcripts partially protected the less closely related *vlxA* RNA probe (70 to 73% identity with four gaps). The RNase A and RNase T1 mixture used to digest and degrade nonhybridized single-stranded RNA probe resolves many single-base mismatches in an RNA:RNA hybrid and cuts efficiently at dinucleotide and trinucleotide mismatches (Myers et al., 1985).

Figure 3 shows an alignment of the *vlxA* nucleotide sequence with the *vlxC* and *vlxD* sequences. After inspection of this alignment, we predicted that *vlxA* antisense RNA probe would not be protected by hybridization with either *vlxC* or *vlxD* mRNAs, and, as shown in Figure 2A, no partially protected *vlxA* fragments were observed. However, when the *vlxA* RNA probe was hybridized with soybean leaf RNA, as shown in Figure 2B, a protected probe fragment of ~50 bp was observed in addition to the expected full-length protected *vlxA* RNA probe (307 bases). This partially protected *vlxA* RNA probe fragment

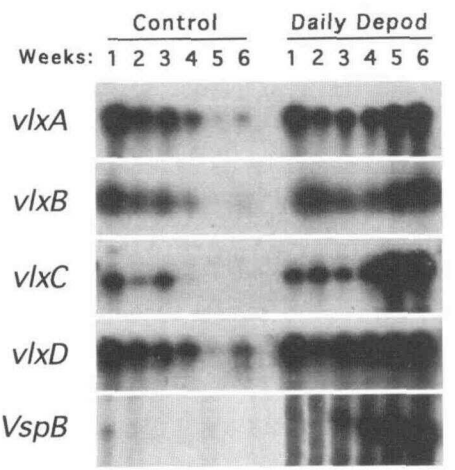

Figure 4. RNase Protection Assays of *vlx* and *VspB* mRNA Levels throughout 5 Weeks of Daily Pod Removal.

RNA isolated from the mature leaves from the main stem of either podded plants (Control) or daily depodded plants (Daily Depod) was used in *vlx* and *VspB* RNase protection assays. Leaves were first harvested 1 week after anthesis when the daily pod removal treatment was begun and thereafter at 1-week intervals for the next 5 weeks. RNase protection assays were performed as described in the legend to Figure 2B.

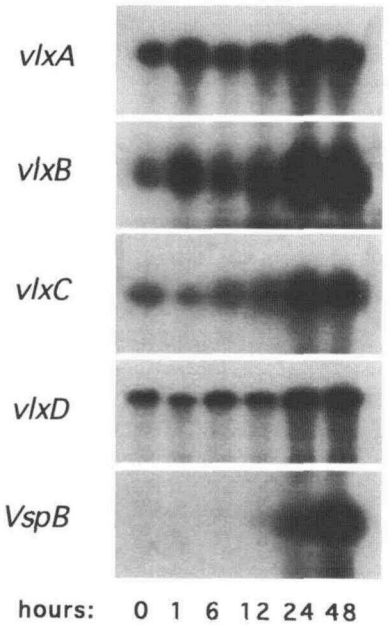

Figure 5. RNase Protection Assays of *vlx* and *VspB* mRNA Levels after Late Pod Removal.

RNA isolated from leaves of plants that had all of their pods removed 4 weeks after anthesis (late depodded) was used in the *vlx* and *VspB* RNase protection assays. Mature leaves from the main stem were harvested 0, 1, 6, 12, 24, and 48 hr after pods were removed early in the 16-hr photoperiod. RNase protection assays were performed as described in the legend to Figure 2B.

indicates the presence of a fourth vegetative lipoxygenase transcript with high homology to *vlxA*. We named this transcript *vlxB*. Because the previously isolated *loxB2* partial cDNA has 96% identity with the *loxB1* partial cDNA for *vlxA* (Bell and Mullet, 1991), the *loxB2* cDNA may represent the same gene as *vlxB*. Although it is possible that *vlxB* is an allele of *vlxA*, this is unlikely because multiple, large partially protected fragments were not observed when using the *vlxA* antisense RNA probe. Rather, the observation of a partially protected ~50-bp fragment suggests a lower degree of identity, which is consistent with the interpretation that two distinct genes are present.

vlx mRNAs Accumulate in Response to Sink Limitation

RNase protection assays were used to determine the response of individual members of the lipoxygenase multigene family to daily pod removal. The levels of *VspB* transcript were monitored for comparative purposes because its response to manipulation of source-to-sink ratio and to induction by sugars, jasmonates, water stress, and wounding, and its responses to other factors are well characterized (Staswick, 1989a, 1989b, 1994; Mason and Mullet, 1990; Staswick et al., 1991; Mason et al., 1992, 1993; Sadka et al., 1994). Figure 4 shows that transcripts for *vlxA*, *vlxB*, *vlxC*, and *vlxD* are present in the leaves of soybean plants collected 1 week after anthesis (control, week 1). Subsequently, during pod expansion and pod filling (control plants in Figure 4), the *vlx* mRNA transcripts gradually decreased. The *vlxC* transcript levels declined steadily and were undetectable 4 weeks after anthesis. The *vlxA*, *vlxB*, and *vlxD* mRNA levels also decreased during pod development but not as rapidly or as completely as *vlxC*. In response to daily pod removal, the levels of *vlx* mRNAs increased to a maximum at 5 and 6 weeks after anthesis (Figure 4). The *vlxC* mRNA level showed the greatest relative increase in response to daily pod removal, and the pattern of its mRNA accumulation was comparable to the *VspB* transcript.

To test further the hypothesis that the vegetative lipoxygenases of soybean are responsive to plant carbon and nitrogen status, *vlx* mRNA levels were examined in leaves undergoing a rapid transition from source to "storage" status. This was accomplished by removing all of the pods (1 to 3 inches in length) from plants 28 days after flowering had commenced. Figure 5 shows that *vlx* mRNAs as well as *VspB* mRNAs increased within 24 hr after this late pod removal. The rapid and strong induction of both *VspB* and *vlx* mRNAs is consistent with the sudden need of the sink-limited plant to store excess nitrogen (and carbon) in an osmotically inactive form.

As an alternative sink deprivation, *vlx* mRNA levels were examined in mature leaves following the inhibition of normal vegetative growth. Figure 6 shows that daily removal of new shoot tips and leaf buds from 1-month-old plants resulted in an increase in the *VspB* and *vlx* mRNA levels within 8 days after shoot tip removal was begun, and their levels continued to rise throughout the remainder of the 16-day time course. In contrast to the pattern of expression of *vlx* mRNAs after pod removal, *vlxD* mRNA was most responsive to tip removal, whereas *vlxC* was the least responsive. Daily tip removal was terminated after 16 days, and the response of the *vlx* mRNAs was assayed 7 and 14 days later (23 and 30 days after tip removal began). Figure 6 shows that there was a decrease in *vlx* mRNA levels 7 days after vegetative growth was allowed to resume; this may indicate increased partitioning of assimilates into developing organs. Within 14 days, the *vlxA*, *vlxB*, and *vlxD* mRNA levels rose again and were similar to the basal levels of expression observed at day 0. In this experiment, the increase in *vlxD* mRNA levels, except for a higher basal level of expression, closely paralleled the increase in *VspB* mRNA levels. To verify that the lipoxygenase and VSP protein levels correlate with their steady state mRNA levels, leaf proteins were extracted and resolved on SDS–polyacrylamide gels as shown in Figure 7. As expected, VSPα, VSPβ, and lipoxygenase(s) accumulated after tip removal, reflecting the higher levels of transcripts for these proteins. Ribulose-1,5-bisphosphate carboxylase/oxygenase large and small subunits, however, decreased between 9 and 16 days of tip removal. After 2 weeks of tip removal, the fully expanded leaves assumed a dark green, wrinkled appearance, which is similar to the leaf morphology

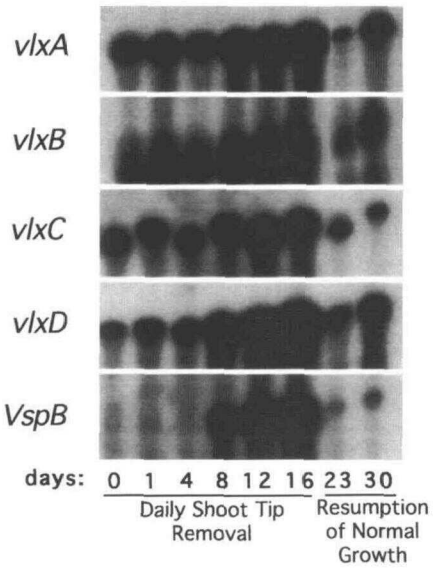

Figure 6. Leaf *vlx* and *VspB* mRNA Levels during Limitation of Vegetative Growth.

RNA isolated from main stem leaves of 1-month-old soybean plants subject to daily removal of shoot tips and leaf buds was used in *vlx* and *VspB* RNase protection assays. Leaves were collected on days 0, 1, 4, 8, 12, and 16 during the shoot tip removal time course. Leaves were also collected 1 (day 23) and 2 (day 30) weeks after growth was allowed to resume. RNase protection assays were performed as described in the legend to Figure 2B.

associated with pod-removed plants. Thus, the "storage" phenotype of leaves from tip-removed plants is comparable with the storage phenotype observed for leaves from pod-removed plants.

Jasmonate Levels Do Not Correlate with *vlx* or *VspB* mRNA Levels in Mature Leaves

Lipoxygenase(s), VSPα, and VSPβ increase in response to low levels of exposure to methyl jasmonate (Mason and Mullet, 1990; Bell and Mullet, 1991, 1993; Grimes et al., 1992; Kato et al., 1993; Melan et al., 1993). Because vegetative lipoxygenases and VSPs are strongly implicated in the storage of amino acids (this study; Franceschi et al., 1983; Staswick, 1989a; Mason and Mullet, 1990), it seemed obvious to ask whether jasmonates are involved in the signaling cascade regulating partitioning of carbon and nitrogen assimilates into these proteins, as suggested by Anderson et al. (1989) and Staswick (1990). This possibility was addressed by assaying the endogenous levels of jasmonic acid in mature leaves after daily pod removal and comparing the levels of this putative signaling molecule with the levels of *vlxC* and *VspB* mRNAs. Figure 8A demonstrates that the endogenous level of jasmonic acid in mature leaves throughout the 5-week time course of daily pod removal never rose above 10 ng per gram fresh weight of tissue. Because mechanical wounding of soybean seedling hypocotyl tissue caused jasmonic acid levels to increase to ~500 ng per gram fresh weight of tissue at 8 hr after wounding (Creelman et al., 1992), the jasmonic acid levels measured in leaves of flowering plants were low, and they remained low throughout the 6 weeks of daily pod removal. Importantly, after 4 and 5 weeks of daily pod removal, the levels of *vlxC* and *VspB* mRNAs increase sixfold, with no corresponding rise in the endogenous level of jasmonic acid.

It is possible that daily pod removal results in transient spikes of jasmonic acid immediately after the pod removal due to the wounding that results from the removal of these sink organs. Over several weeks, these hypothetical spikes could result in the induction of *vlxC* and *VspB*. To determine whether transient jasmonic acid spikes exist after pod removal, the levels of jasmonic acid were assayed in mature leaves 0, 1, 6, 12, 24, and 48 hr after late "massive" pod removal and compared with *vlxC* and *VspB* mRNA levels. Figure 8B indicates that over a 48-hr period after this massive pod removal episode, the endogenous level of jasmonic acid never rose above 10 ng per gram fresh weight of tissue, which is well below the level known to mediate wounding and other responses. Thus, no transient spikes in the level of jasmonic acid were observed after pod removal.

DISCUSSION

During vegetative growth, distinct polypeptides accumulate in the vegetative organs of many plant species, including soybean,

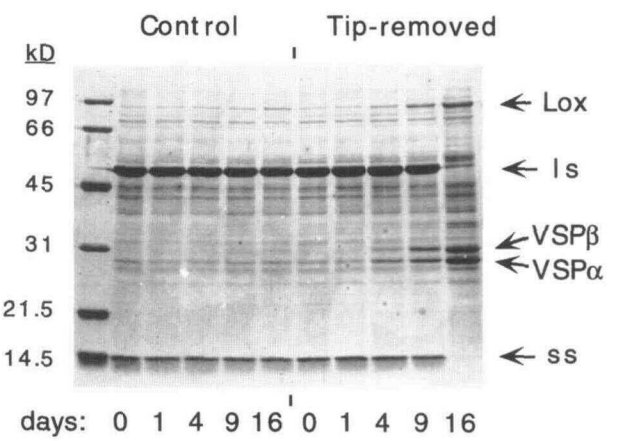

Figure 7. SDS–Polyacrylamide Gel Analysis of Protein Accumulation during the Limitation of New Vegetative Growth.

Soluble proteins were extracted from leaves of control and soybean plants with their shoot tips and leaf buds removed daily (Tip-removed) at time points corresponding to 0, 1, 4, 9, and 16 days after tip removal. Lipoxygenases (Lox) along with VSPα and VSPβ accumulated in the tip-removed plants but not in the control plants. The amount of ribulose-1,5-bisphosphate carboxylase/oxygenase large (ls) and small (ss) subunits declined between 9 and 16 days of growth limitation.

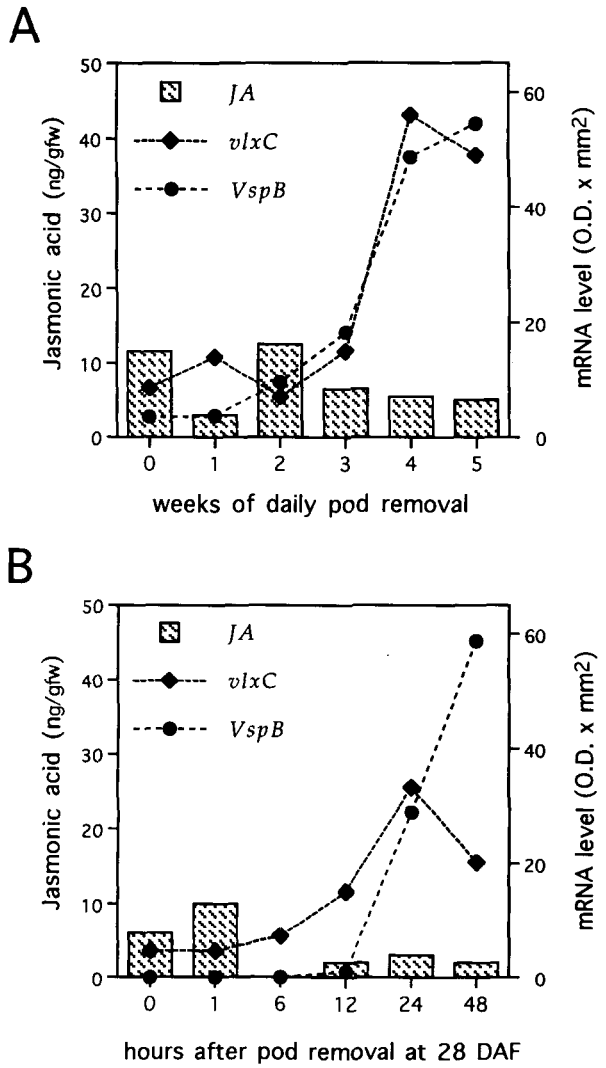

Figure 8. Endogenous Jasmonic Acid Levels in Mature Leaves Do Not Correlate with *vlxC* and *VspB* mRNA Levels after Pod Removal Treatments.

At the indicated times, two mature main stem leaves were collected from different plants for either jasmonic acid quantitation or total RNA purification. Relative mRNA levels are shown in units of optical density times square millimeter after densitometric quantitation of RNase protection assay signals from autoradiographs. Jasmonic acid (JA) was extracted, HPLC fractionated, and detected by gas chromatography–mass spectrometry as described in Methods. gfw, grams fresh weight of tissue.

(A) Daily pod removal time course. Pod removal was begun 1 week after anthesis. The *vlxC* and *VspB* daily pod removal RNase protection assay is also shown in Figure 4.

(B) Late pod removal at 28 days postanthesis (DAF). Trifoliolate leaves were collected from identically treated plants at 0, 1, 6, 12, 24, or 48 hr after massive pod removal. The *vlxC* and *VspB* late pod removal RNase protection assay is also shown in Figure 5.

pea, poplar, and Arabidopsis that are postulated to function as storage proteins (Coleman et al., 1992; Davis et al., 1993; Staswick, 1994). First described by Wittenbach (1982) in soybean leaves, these proteins and their mRNAs accumulate in response to sink deprivation, nitrogen, water deficit, wounding, light, sugars, jasmonic acid, and blockage of leaf phloem export (Wittenbach, 1983a, 1983b; Mason et al., 1988; Anderson et al., 1989; Staswick, 1989a, 1990; Mason and Mullet, 1990; Franceschi and Grimes, 1991; Grimes et al., 1992, 1993). The amino acid sequences of two of these three polypeptides (VSPα and VSPβ) are similar to acid phosphatases, and purified VSP displays acid phosphatase activity (DeWald et al., 1992).

Our laboratory purified and sequenced peptide fragments of the ~94-kD VSP and demonstrated that these peptide sequences are homologous with known lipoxygenase amino acid sequences (Tranbarger et al., 1991). Lipoxygenase mRNA(s) and protein(s) respond to plant nitrogen status in a developmentally regulated manner (Grimes et al., 1993), and airborne methyl jasmonate induces the expression of lipoxygenase mRNAs and lipoxygenase enzymatic activity (Grimes et al., 1992). Because the soybean lipoxygenase multigene family consists of several members, it has proven difficult to ascertain whether a single gene or multiple lipoxygenase genes are involved in these responses. Anion exchange chromatography previously indicated that the vegetative lipoxygenase isozyme L-4 (equivalent to the *vlxA* gene product) increases in the leaves of pod-removed plants (Kato et al., 1993) along with three other minor peaks of lipoxygenase activity. Saravitz and Siedow (1995) demonstrated that pod removal results in the appearance of a neutral lipoxygenase isozyme with a pI of 6.9, which is similar to the predicted pI of 6.7 for *vlxC*, and that multiple acidic lipoxygenase isoforms also increase.

To determine directly which members of the lipoxygenase multigene family respond to manipulation of source-to-sink ratio, RNase protection assays were used to assess individually the expression of four distinct members of this multigene family in response to sink limitation. The results of this study clearly indicate that multiple lipoxygenase mRNAs increase in response to daily pod removal. This generalized increase in lipoxygenase mRNA levels after pod removal is consistent with earlier data from our laboratory (Grimes et al., 1993) as well as the work of others (Kato et al., 1992b, 1993; Saravitz and Siedow, 1995). A primary advantage of the RNase protection assay is its ability to delineate the magnitude of individual *vlx* mRNA levels in response to pod removal and to relate these changes to specific genes, which has not been possible in other studies.

Examination of the *vlx* expression patterns of several different plants (one representative experiment is shown in Figure 4) suggests that transcription of the *vlx* genes is inhibited or terminated after anthesis, as shown by the decrease in *vlx* mRNA levels in the leaves of control plants. In comparison with the expression pattern of *VspB*, one noticeable difference is the higher constitutive level of all four *vlx* mRNAs, perhaps reflecting a constitutive need for the enzymatic function of the

lipoxygenase gene products. Although all of the *vlx* mRNAs increased in response to pod removal, it is clear that specific members of this multigene family respond more strongly than others (compare the response of *vlxC* with that of *vlxD* in Figure 4). One consistent trend in the pattern of *vlx* mRNA expression after pod removal is the tendency of the *vlx* mRNAs to decrease slightly 2 or 3 weeks after pod removal had begun and then to increase after 4 to 6 weeks of daily pod removal. The *VspB* transcript accumulated in a more linear fashion after pod removal, and the reasons for this slight variation are not known. Collectively, however, these experiments indicate that specific members of the lipoxygenase multigene family are regulated transcriptionally in a manner that closely parallels that of *VspB* and provides important support for the hypothesis that multiple lipoxygenases function in the temporary storage of assimilates during vegetative growth.

The function of the lipoxygenase multigene family in temporary assimilate storage prior to anthesis is further supported by the accumulation of *vlx* mRNAs within 24 hr after removing all of the pods 28 days after anthesis. After this "massive" late pod removal episode, a rapid buildup of both carbon and nitrogen assimilates occurs and the leaf becomes sink regulated (Wittenbach, 1982; Goldschmidt and Huber, 1992; Krapp et al., 1993). As a result, we would expect the transcripts of proteins involved in storage of these assimilates to increase coordinately with the rising level of assimilates. This predicted pattern was verified in the patterns of *VspB* transcript accumulation as well as in the observed increases of the four *vlx* mRNAs. As another test of the response of the lipoxygenase multigene family to manipulation of the source-to-sink ratio, young developing leaves (that is, one-eighth expanded or younger) and shoot tips were removed from the apex of 1-month-old soybean plants. Subsequently, mature leaves were harvested, and RNase protection assays were used to assay the response of the *vlx* mRNAs in comparison with the *VspB* transcript. This experiment again demonstrated that after sink removal, the *vlx* mRNAs accumulated in leaves in much the same manner as the *VspB* mRNA. Interestingly, the *vlxD* transcript is generally among the least responsive to pod removal treatments, although this particular mRNA is responsive to shoot tip removal. The dissimilar response of individual *vlx* multigene family members at different developmental stages to source-to-sink ratio manipulations may reflect organ-specific differences in regulatory components and their interaction with *cis*-acting elements associated with individual lipoxygenase genes.

The induction of all four of the *vlx* mRNAs in sink-limited leaves suggests that differential gene regulation in response to the need of the leaf to store excess sugars and amino acids is not the primary reason for genetic redundancy of this multigene family. Therefore, the reason for multiple lipoxygenase isozymes in soybean leaves must be ascribed to some other factor, of which at least three possibilities exist. First, the vegetative lipoxygenases may vary with respect to their pH optima, calcium activation, substrate specificity, and product formation (Vick and Zimmerman, 1987; Maccarrone et al., 1994). The fact that all of the *vlx* members are constitutively expressed suggests that the observed genetic redundancy is necessary to encode discrete enzymatic activities. Second, the *vlx* genes may have distinct developmental expression patterns. There may be overlapping developmental expression patterns as noted for the bean lipoxygenase genes (Eiben and Slusarenko, 1994) and the two lipoxygenases expressed in tomato fruit (Ferrie et al., 1994), or there may be distinct expression patterns as observed in Arabidopsis for *LOX1* and *LOX2* (Bell and Mullet, 1993; Melan et al., 1994). This would allow fine-tuning of the biochemical activities by providing temporal and spatial refinement to the expression of individual members of this multigene family. Third, individual *vlx* genes may be associated with different cellular or subcellular locations. Plant lipoxygenases have been localized to cytoplasmic, plasma membrane, microsomal, and vacuolar sites (Todd et al., 1990; Tranbarger et al., 1991; Bowsher et al., 1992; Rouet-Mayer et al., 1992; Droillard et al., 1993; Feussner and Kindl, 1994; Macri et al., 1994), and recently several plant lipoxygenase genes with putative N-terminal chloroplast transit peptides have been cloned (Bell and Mullet, 1993; Peng et al., 1994). We have developed isozyme-specific antisera to specific *vlx* members and are using these antisera for both cellular and subcellular localization studies. Fourth, the genetic redundancy may be an outcome of the intense artificial selection that humans have placed on crop species, including soybean, to increase productivity. If the VSPs and vegetative lipoxygenases play an important role in nitrogen partitioning, then human selection pressure might have resulted in a duplication of one or more *vlx* genes. Indeed, the high degree of sequence conservation and the inverted linkage of two *VspB* genes in the soybean genome indicate a recent gene duplication event (Rapp et al., 1990). The observation that genotypes of soybean lacking one or more of the seed lipoxygenase isozymes have normal phenotypes also supports the idea that some plant lipoxygenases do not have an essential biochemical activity (Hildebrand and Hymowitz, 1981; Kitamura, 1984).

Jasmonic acid, or its methylated ester, induces the accumulation of vegetative lipoxygenases and VSPs and has been proposed to participate in a signaling pathway mediating the induction of plant defense–related proteins and wound responses in plants (Bell and Mullet, 1991; Franceschi and Grimes, 1991; Farmer and Ryan, 1992; Fournier et al., 1993; Kato et al., 1993; Park et al., 1994). Indeed, soybean vegetative lipoxygenase and *Vsp* mRNAs are wound inducible, and new lipoxygenase isoforms appear to accumulate after mechanical wounding (Saravitz and Siedow, 1995). Because jasmonates induce the expression of VSPs and lipoxygenases and because they function as molecular signals, it has been speculated that jasmonic acid may be involved in the signal transduction cascade regulating nitrogen partitioning into the VSPs and lipoxygenases (Anderson et al., 1989; Staswick, 1990). If this hypothesis has merit, the endogenous levels of jasmonic acid in leaves should increase after pod removal and accumulate at times preceding the increase in levels of *vlx* and *Vsp* mRNA levels. To test this hypothesis directly, we

concurrently assayed the endogenous level of jasmonic acid in leaves from depodded plants and compared these levels with the relative increase in *vlxC* and *VspB* transcript level in the same leaves. No correlation between jasmonic acid level and *vlxC/VspB* mRNA level was observed. Because wounding stimulates both jasmonic acid level and vlx/*VspB* mRNA levels, we decided to determine whether pod removal was a strong enough wound treatment to cause small, transient "spikes" in jasmonic acid levels. After removal of >200 pods from plants 28 days after anthesis, endogenous jasmonic acid levels were assayed and again compared with both *vlxC/VspB* mRNA levels in the same leaves. Despite the fact that both *vlxC* and *VspB* mRNA levels increased more than sixfold, no detectable increase occurred in the jasmonic acid level during the 48 hr of this experiment. The level of jasmonic acid, as opposed to methyl jasmonate, was assayed in these experiments because previous work has shown that jasmonic acid is the major jasmonate in wounded soybean hypocotyls (Creelman et al., 1992). The results of these experiments seem to preclude the involvement of lipoxygenase-derived jasmonic acid in the in vivo gene regulation of vegetative lipoxygenases and VSPs under sink-limited conditions.

In summary, this research establishes a clear link between the "storage" phenotype of sink-regulated leaves and the induction of members of the lipoxygenase multigene family. When individual members of the lipoxygenase multigene family were examined using RNase protection assays, it became evident that *vlx* transcript levels decreased after anthesis when the sinks were present but increased when developing sinks were removed. Although there are variations in the magnitude of response to manipulation of the source-to-sink ratio between individual *vlx* members, regulation of these genes is very similar to that of *VspB*. One consistent difference between regulation of the *vlx* genes as contrasted with the *VspB* transcript is that the *vlx* mRNAs seem to be constitutively produced before anthesis. This may be because the *vlx* gene product storage function is required during vegetative growth; alternatively, lipoxygenase enzymatic function may be required throughout vegetative development. Importantly, the endogenous levels of jasmonic acid do not rise prior to or in conjunction with the sharp increase observed in the amount of *vlxC* and *VspB* transcripts in the leaves of pod-removed plants. Thus, jasmonic acid does not appear to mediate expression of the vegetative lipoxygenase or VSP genes as a result of sink limitation. Other signals or combinations of signals involved in the coordination of carbon and nitrogen metabolism may be important in regulating the soybean *vlx* genes.

METHODS

Plant Material

All experiments were performed with unnodulated soybean (*Glycine max* 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 growth chambers with a photon flux density of 360 to 400 μmol m^{-2} sec^{-1}. Beginning at 1 month, all plants were fertilized once per week with 500 mL Peters Professional nutrient solution and once per week with 500 mL Peters Excell nutrient solution (USA/Grace-Sierra Horticulture Products Co., Allentown, PA) prepared by diluting 4 g of fertilizer per L of water.

cDNA Library Construction and Screening

Poly(A)$^+$ RNA was prepared from leaves of soybean plants that had young pods removed daily for 1 or 2 weeks and treated with methyl mercury prior to reverse transcription. The pod-removed leaf Uni-ZAP XR cDNA library was constructed using size-selected cDNAs following the manufacturer's protocols (Stratagene). A 1408-bp EcoRI fragment of pTK11 was used to generate a lipoxygenase probe by the random primer method using a Megaprime Kit (Amersham). Screening by plaque hybridization of the amplified phage library was performed by standard methods (Sambrook et al., 1989) using Colony/Plaque Screen filters (Du Pont–New England Nuclear Research Products). In vivo excision was performed on positive phage as described by Stratagene. The resulting pBluescript SK– plasmids (Stratagene) containing unidirectional cDNA inserts were screened for lipoxygenase sequences by double-stranded DNA sequencing using the Sequenase 2.0 kit (U.S. Biochemical Corp.).

Protein and RNA Extraction and Electrophoresis

Soluble protein extraction and SDS–PAGE were performed as described by Grimes et al. (1993). Total RNA was isolated from 0.5 g of soybean tissues as described previously (Grimes et al., 1993).

Jasmonic Acid Detection and Quantitation

Harvested leaves were frozen immediately in liquid nitrogen and stored at −80°C until analyzed. Four to 6 g of frozen leaf tissue was homogenized in 50:50 acetone–methanol, and a known amount of ^{13}C-jasmonic acid was added as an internal standard. After almost complete removal of the solvents by rotary evaporation, 50 mL of 0.1 M sodium phosphate, pH 7.8, 5% NaCl was added, and rotary evaporation continued for several minutes. The resulting aqueous solution was extracted two times with dichloromethane, acidified to approximately pH 2.0 by the addition of 6 N HCl, extracted twice with hexane, and then twice with dichloromethane (equal volumes each extraction). Under acidic conditions, the jasmonic acid partitioned into dichloromethane, which was collected, dried by rotary evaporation, and dissolved in a small amount of the initial solvent used for HPLC. Additional purification was performed by analytical gradient HPLC with a Phenomenex (Torrance, CA) amino column (300 × 3.9 mm) from 80% hexane–20% ethyl acetate containing 1% acetic acid to 60% hexane–40% ethyl acetate containing 1% acetic acid in 15 min at a flow rate of 1.5 mL/min. The fractions corresponding to jasmonic acid (and its epimer) were collected, methylated, and analyzed according to Creelman et al. (1992).

RNase Protection Assays

RNase protection assays were performed following the manufacturer's standard protocol (RPA II kit; Ambion, Austin, TX) using 5 μg of total

RNA and 2 fmol of a gel-purified antisense RNA probe. The length of x-ray film exposures of the RNase protection assay gels was varied, depending on antisense *vlx* RNA probe–specific activity and length to facilitate comparison with *vlx* transcript levels. *vlxA*, *vlxC*, and *vlxD* antisense RNA probes were generated from HindIII-linearized pTK18-Ribo, BamHI-linearized pNRlox-Ribo, and BamHI-linearized pTK11-Ribo plasmids, respectively, and incorporated ^{32}P-CTP. Corresponding sense transcripts were generated from BamHI-linearized pTK18-Ribo, EcoRI- linearized pNRlox-Ribo, and EcoRI-linearized pTK11-Ribo, respectively, using either T3 or T7 bacteriophage RNA polymerases (MaxiScript; Ambion) and incorporated ^{3}H-CTP.

pTK18-Ribo was constructed by subcloning a 314-bp pTK18 EcoRI fragment into the polylinker EcoRI site of pBluescript SK– vector. pTK11-Ribo was constructed by subcloning a 395-bp pTK11 EcoRI fragment into the EcoRI site of pBluescript SK–. The plasmid pNRlox-Ribo was constructed by subcloning a 374-bp EcoRI fragment of pNRlox4A (which contains the *vlxC* cDNA) into pBluescript SK–. The orientation and identity of the insertion sequences were verified by double-stranded DNA sequencing across the junction boundaries.

The antisense vegetative storage protein *VspB* RNA probe was generated by in vitro transcription with T7 RNA polymerase from a 292-base polymerase chain reaction product generated by amplification from the pKSH3 plasmid (Mason et al., 1988) using the forward primer 5′-GATATCAAGCTTCCAATGGAGCGATCTGC-3′ and the reverse primer 5′-CGCGGATCCTAATACGACTCACTATAGGGAGAAGGTACGTGGAGTG-TCTTAGG-3′. The antisense *VspB* RNA probe is complementary to a 247-bp region of *VspB* mRNA and is not fully protected by *VspA* mRNA. The underlined regions of the primers are capable of hybridizing to the *VspB* cDNA insert contained in the pKSH3 plasmid.

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