The Lipoxygenase Isozymes in Soybean
[Glycine max (L.) Merr.] Leaves

Changes during Leaf Development, after Wounding, and following Reproductive Sink Removal

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The levels of individual lipoxygenase isoforms in soybean [Glycine max (L.) Merr.] leaves were assessed during leaf development, after mechanical wounding, and in response to reproductive sink removal. Native isoelectric focusing followed by immunoblotting was employed to examine individual lipoxygenase isoforms. In leaves of all ages, two distinct classes of lipoxygenase isoforms were detected. One class of lipoxygenase isoforms had nearly neutral isoelectric points (pIs) ranging from pH 6.8 to 7.2. The other class of lipoxygenase isoforms had acidic pIs ranging from pH 4.7 to 5.6. During leaf development, all of the neutral lipoxygenase isoforms and most of the acidic isoforms were present in greatest abundance in the youngest leaves examined and declined in amount as leaf age increased. However, four acidic lipoxygenase isoforms (pI = 4.70, 4.80, 4.90, 4.95) were more abundant in intermediate-age leaves than in either the youngest or oldest leaves examined. Following mechanical wounding of leaves, these same four acidic isoforms also increased in abundance both locally and systemically in leaves from wounded plants. Unlike the specific effects of wounding on the lipoxygenase isoforms in leaves, reproductive sink removal stimulated a general increase in most of the acidic lipoxygenase isoforms in leaves.

Multiple forms or isoforms of the enzyme lipoxygenase have been detected in both animal and plant species (Vick and Zimmerman, 1987; Siedow, 1991; Yamamoto, 1992). Although multiple isoforms of lipoxygenase have been identified in many plant species (Christopher et al., 1970; Ida et al., 1983; Yamamoto and Tani, 1986; Mulliez et al., 1987; Domoney et al., 1990; Todd et al., 1990), the physiological role of any specific plant lipoxygenase isoform has yet to be established. Because lipoxygenase is required in the biosynthesis of JA in plants (Vick and Zimmerman, 1987), one or more lipoxygenase isoforms present within a given plant species must be involved in JA biosynthesis. The jasmonates (JA and its naturally occurring, structural analogs) are known to influence a wide variety of physiological processes in plants (Parmthier, 1991; Koda, 1992). JA has also been proposed to serve as a mediator of plant defense responses to wounding and pathogen attack (Farmer and Ryan, 1992; Gundlach et al., 1992; Kauss et al., 1992). Thus, lipoxygenase may be involved in a variety of physiological processes due to its role in the synthesis of JA. Other proposed physiological roles of lipoxygenase, unrelated to JA biosynthesis, include membrane degradation during hypersensitive resistance responses (Croft et al., 1990), production of fatty acid-derived, anti-microbial molecules (Ohta et al., 1990, 1991; Kato et al., 1992a; Croft et al., 1993), and the synthesis of ABA (Creeelmann et al., 1992). In addition, lipoxygenase has been proposed to serve as a storage protein in both seeds (Peterman and Siedow, 1985b; Siedow, 1991) and leaves (Tranbarger et al., 1991). Given the presence of multiple isoforms of lipoxygenase in plants, it is possible that individual lipoxygenase isoforms within a plant may have distinct physiological roles.

The soybean [Glycine max (L.) Merr.] lipoxygenase isoforms have been the most intensively studied among plant lipoxygenases. In mature soybean seeds, three to four lipoxygenase isoforms are present, and during seed germination, three additional isoforms appear in soybean cotyledons (Christopher et al., 1970, 1972; Kato et al., 1992b). In addition, multiple isoforms have been identified in soybean hypocotyls. One major and two to three minor lipoxygenase isoforms appear in soybean hypocotyl/radicle axes during germination that have more acidic pIs than the well-characterized isoforms found in mature soybean seeds (Park and Polacco, 1989). Multiple lipoxygenase isoforms also occur in soybean leaves. Grayburn et al. (1991) used chromatofocusing to separate three distinct peaks of lipoxygenase activity in extracts from soybean leaves. On the basis of physical and kinetic characterizations of the three leaf lipoxygenases, Grayburn et al. (1991) concluded that the soybean leaf lipoxygenase isoforms are distinct from the isoforms found in seeds and that at least one of the leaf isoforms is different from the lipoxygenase isoforms found in hypocotyl/radicle axes. IEF followed by immunoblotting has been used to demonstrate that each of the three leaf lipoxygenase activity peaks separated by chromatofocusing contains several distinct lipoxygenase isoforms (Grayburn et al., 1991).

Abbreviations: JA, jasmonic acid; TBST, 10 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween-20.

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In soybean leaves, total lipoxygenase protein and activity have been reported to vary with respect to leaf size (Hildebrand et al., 1988; Altschuler et al., 1989; Grayburn et al., 1991). Because soybean leaves contain multiple lipoxygenase isozymes, variation in total lipoxygenase protein and activity during leaf development probably results from changes in individual lipoxygenase isozymes, but the relationship of individual lipoxygenase isozyme levels to leaf age has not been established.

Increases in host lipoxygenase activity and individual lipoxygenase isozymes after infection with bacterial and fungal pathogens have been observed for several plant species (Yamamoto and Tani, 1986; Keppler and Novacky, 1987; Croft et al., 1990; Kato et al., 1992b; Koch et al., 1992). Physical wounding also increases lipoxygenase expression in soybean leaves both locally and systemically (Hildebrand et al., 1989; Bell and Mullet, 1991). Increases in soybean lipoxygenase transcript levels have been detected in leaves within 8 h after wounding (Bell and Mullet, 1991). In Arabidopsis thaliana, differential expression of lipoxygenase genes was observed following wounding (Bell and Mullet, 1993). Local and systemic increases in Atlox2 transcript levels were detected in leaves following wounding, but Atlox1 transcript levels were unaffected by wounding (Bell and Mullet, 1993). To date, the effects of wounding on the levels of individual lipoxygenase isozymes in soybean or any other plant species have not been reported. Given the proposed role of lipoxygenase in the defense systems of plants against attacks by pests and pathogens (Farmer and Ryan, 1992; Gundlach et al., 1992; Kauss et al., 1992; Koch et al. 1992; Ryan, 1992; Croft et al., 1993), it is important to understand the effects of physical damage on the levels of individual lipoxygenase isozymes.

To understand more fully the role of lipoxygenase isozymes in soybean, the behavior of lipoxygenase isozymes in soybean leaves was examined during leaf development, after wounding, and in response to reproductive sink removal.

**MATERIALS AND METHODS**

**Plant Materials**

For studies of leaf development, wounding, and reproductive sink removal, soybean [Glycine max (L.) Merr. cv Ransom] seeds were sown directly in a mixture containing equal parts (v/v/v) of soil, sand, and perlite. Plants were grown in the Duke University greenhouses under natural photoperiods. For wounding experiments, approximately one-month-old plants with mature, fully expanded first and second trifoliate leaf were wounded once at the beginning of the experiment with a hemostat. Wounds were centrally located in each leaflet and were perpendicular to and through the midrib. After harvest, leaves were immediately frozen with liquid nitrogen and stored at −80°C. Frozen soybean cotyledon and hypocotyl/radicle axes originated from etiolated seedlings. Seedlings were grown in complete darkness for 3 d after imbibition at 28°C. Cotyledons and hypocotyl/radicle axes were excised from the seedlings and the plumules were discarded. Seedling tissues were immediately frozen with liquid nitrogen and stored at −80°C. Frozen plant tissues were powdered with a mortar and pestle that was precooled with liquid nitrogen. Powdered tissue samples were stored at −80°C for later use in preparation of tissue extracts.

In experiments to examine the effects of reproductive sink removal, soybean plants were grown as described above, and beginning at anthesis, flowers and flower buds were removed daily for a period of 2 weeks. Floral tissues were not removed from control plants and seed pods were allowed to develop normally. Leaves were harvested at anthesis and at 2 weeks postanthesis as described above.

**Preparation of Tissue Extracts for IEF**

Ice-cold 1.7-mL plastic microcentrifuge tubes were filled approximately three-fourths full with frozen, powdered plant tissue. While the tubes were held on ice, 300 µL of ice-cold extraction buffer (5.0 mM sodium phosphate, pH 7.0, 1.0% [w/v] polyvinylpolypyrrolidone) was added. After thawing on ice, each mixture was homogenized with a disposable pellet pestle while in the microcentrifuge tube (Kontes Scientific Glassware/Instruments, Vineland, NJ). All subsequent operations were conducted at 4°C. After homogenization, the tubes were centrifuged for 20 min at 13,000g. Each supernatant was collected to a new tube and centrifuged again for 10 min at 13,000g. Each supernatant was collected to a new tube and centrifuged again for 10 min at 13,000g. The final supernatant was collected, divided into 50-µL aliquots, frozen with liquid nitrogen, and stored at −80°C for later use. Soluble protein concentrations were determined as described by Lowry et al. (1951) using BSA as a standard.

**IEF**

Native IEF was performed on vertical polyacrylamide gels as described by Robertson et al. (1987) with the following modifications. For IEF in the range pH 4.5 to 6.5, a 1:1 (v/v) mixture of pH 4 to 7 and pH 5 to 6 ampholytes was used. For IEF in the range pH 5.5 to 7.5, a 1:1 (v/v) mixture of pH 5 to 8 and pH 6 to 7 ampholytes was used. All ampholytes were purchased from Serva Biochemicals (Westbury, NY). Prior to IEF, the frozen tissue extracts were thawed and immediately placed on ice. Each sample was mixed with one-half volume of an aqueous solution of 60% (v/v) glycerol and 2% (v/v) of each of the component ampholytes used in the gel preparation as described above. Focusing was conducted for 1 h at 200 V and 4 h at 500 V. During IEF, the gel temperature was maintained at 24°C. PI standards were purchased from Sigma.

**Immunoblot Analysis of Lipoxygenase Isozymes**

After IEF was completed, the gels were incubated at 4°C for 45 min in 250 mL of transfer buffer (25 mM Tris, 192 mM Gly, 0.1% [w/v] SDS, pH 8.5). Proteins from gels were then electrotransferred at 300 mA for 12 to 15 h at 4°C to nitrocellulose membranes (BA-S, Schleicher & Schuell). After protein transfer, the nitrocellulose filters were rinsed...
in distilled H₂O and stained with Ponceau S to determine protein transfer uniformity (Harlow and Lane, 1988). Staining and all subsequent incubations of the nitrocellulose filters were conducted at 25°C. After destaining in distilled H₂O, the filters were incubated with agitation for 5 min in TBST, pH 8.0. This incubation was repeated twice with fresh TBST added each time. The filters were then incubated for 2 h in quench solution (5% [w/v] non-fat dry milk and 0.02% [w/v] sodium azide in TBST, pH 8.0). After two brief rinses in TBST, the filters were incubated in TBST for 15 min, followed by two additional washes of 5 min each. The filters were then incubated for 2 h with rabbit anti-soybean lipoxygenase-2 antibodies (Peterman and Siedow, 1985a) diluted 1:2000 in quench solution. Following incubation with primary antibodies, the filters were rinsed twice with TBST and washed once for 15 min and twice for 5 min each in TBST. Goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (Sigma) were diluted 1:5000 in quench solution and incubated with the filters for 1 h. The filters were then rinsed three times, washed once for 15 min and four times for 5 min in TBST. Bound, secondary antibodies were detected using the ECL chemiluminescence system according to the manufacturer’s directions (Amersham).

In-Gel Staining for Lipoxygenase Activity

After IEF was completed, the gels were rinsed in distilled H₂O and stained for lipoxygenase activity by the method of Funk et al. (1985) using the modifications employed by Park and Polacco (1989). Both linolenic and linoleic acids were used as substrates in separate experiments. Gels were incubated with gentle agitation in the presence of the reaction mixture for 2 to 3 h at 25°C. After staining was completed, the gels were rinsed several times with distilled H₂O and incubated in distilled H₂O with gentle agitation for 15 min at 25°C. After the gels were photographed, activity-stained bands were excised and stored at −80°C for later use in SDS-PAGE.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed on 82 × 60 × 1.5 mm slab gels according to Laemmli (1970) using 12% (w/v) acrylamide in the resolving gel. Following electrophoresis, immunoblotting was conducted as described above for lipoxygenase isozymes.

RESULTS

Developmental Patterns of Leaf Lipoxygenase Isozymes

Although multiple lipoxygenase isozymes have been demonstrated to exist in soybean leaves (Grayburn et al., 1991), the relationship between the levels of individual lipoxygenase isozymes and leaf age has not been determined. Developmental variation in total leaf lipoxygenase protein likely results from alterations in the levels of individual lipoxygenase isozymes. Therefore, an investigation of the developmental expression of the lipoxygenase isozymes in soybean leaves was initiated. First trifoliate leaves were harvested at four intervals (19, 23, 26, and 31 d after planting) spanning the complete cycle of leaf development. The initial harvest was made when the middle leaflet of the first trifoliate leaves had an average length of 16.8 mm. The final harvest consisted of mature, fully expanded leaves having an average middle leaflet length of 61.6 mm. Average leaf weights (in mg) in order of increasing leaf age for the four sets of leaves were 46.3, 199.3, 457.3, and 574.7, respectively.

To evaluate the levels of lipoxygenase isozymes, soluble protein extracts were prepared from the leaf tissues, subjected to IEF on polyacrylamide gels, electrotransferred to nitrocellulose membranes, and immunoblotted using anti-soybean lipoxygenase-2 antibodies. After initial experiments using IEF gels with a pH gradient from 4.0 to 7.0 (data not shown), it became clear that this pH gradient would not resolve all of the lipoxygenase isozymes in soybean leaves. To resolve the leaf isozymes, each sample was electrophoresed on two separate IEF gels optimized for more narrow pH ranges. Several lipoxygenase isozymes having pIs between 6.8 and 7.2 were detected in leaves of all ages (Fig. 1A). All the lipoxygenase isozymes in this region were most abundant in the youngest leaves examined and declined in amount as leaf age increased. In addition to the lipoxygenase isozymes with neutral pIs, lipoxygenase isozymes with more acidic pIs were also observed. The acidic lipoxygenases were observed to have pIs ranging from 4.7 to 5.6. Assuming that the anti-lipoxygenase-2 antibody employed has roughly equal avidity for

![Figure 1. Immunoblot analysis of the developmental variation in lipoxygenase isozymes from first trifoliate soybean leaves. Lipoxygenase isozymes resolved between pH 6.0 and 7.5 (A) and between pH 4.5 and 6.0 (B). Leaf extracts were prepared from first trifoliate leaves harvested 19 (lanes 1), 23 (lanes 2), 26 (lanes 3), and 31 (lanes 4) d after planting. Leaf extracts containing 100 µg of soluble protein were subjected to native IEF on polyacrylamide gels, and lipoxygenase isozymes were detected by immunoblotting using a polyclonal antibody prepared against soybean lipoxygenase-2. An extract from 3-d-old, etiolated hypocotyl/radicle axes was focused in lane H. Arrowheads in B indicate the position of the minor acidic lipoxygenase isozymes.](www.plantphysiol.org)
all the lipoxygenase isozymes, the relative abundance of the individual acidic isozymes is somewhat variable within leaves of any age class (Fig. 1B). At all leaf ages, the most abundant isozymes have pIs between 5.0 and 5.3. Many of the acidic isozymes were most abundant in the youngest leaves and declined with increasing leaf age. However, several minor lipoxygenase isozymes (pI = 4.70, 4.80, 4.90, 4.95) were more abundant in intermediate-age leaves than in either the youngest or oldest leaves (Fig. 1B). For comparison with previously identified lipoxygenase isoforms, extracts prepared using hypocotyl/radical axes from 3-d-old, etiolated seedlings were subjected to IEF. In hypocotyl/radical axes, two lipoxygenase isozymes with pIs of 5.1 and 5.3 were observed that co-migrated with the wound-responsive isozymes from leaves (Fig. 1B). In addition, an abundant lipoxygenase isozyme (lipoxygenase-1) with a pI of 5.9 was detected in hypocotyl/radical axes but not in leaves of any age (Fig. 1B).

Effects of Wounding on Leaf Lipoxygenase Isozymes

Increases after wounding in the expression of at least two different members of the soybean lipoxygenase gene family have been shown to occur at the transcriptional level (Bell and Mullet, 1991). To date, the effects of wounding on the lipoxygenase isoforms in soybean leaves have not been reported. Therefore, an investigation of the local and systemic effects of wounding on soybean leaf lipoxygenase isoforms was undertaken. For wounding experiments, approximately 1-month-old plants with mature, fully expanded first and second trifoliate leaves were used. At the start of the experiment, each leaflet of the first trifoliate leaves was wounded once by a single crush with a hemostat. To examine the local response of lipoxygenase isoforms to wounding, first trifoliate leaves were harvested from wounded and unwounded control plants at 12, 24, and 48 h after wounding. To examine the systemic response to wounding, second trifoliate leaves (unwounded) were harvested from plants with wounded first trifoliate leaves and from unwounded controls. Soluble protein extracts were prepared from the leaf tissues and subjected to IEF followed by immunoblotting as described above.

Several lipoxygenase isoforms were found to increase in abundance in first trifoliate leaves after wounding (Fig. 2). No consistent changes in the levels of the neutral lipoxygenase isoforms were observed following wounding (Fig. 2A). Among the acidic, leaf lipoxygenase isoforms, however, the four relatively minor isozymes having the lowest pIs (4.70, 4.80, 4.90, 4.95) increased after wounding (Fig. 2B, arrowheads). In wounded leaves, the levels of all four acidic isozymes were higher than in unwounded controls at 12, 24, and 48 h after wounding. No changes were observed in the levels of the most abundant, acidic lipoxygenase isoforms (pI = 5.0-5.3) after wounding (Fig. 2B).

In addition, systemic increases in the levels of specific lipoxygenase isoforms were detected in second trifoliate leaves following wounding (Fig. 3). Examination of the neutral soybean leaf isoforms present in second trifoliate leaves from both wounded and unwounded plants did not reveal any consistent increases (Fig. 3A). The four acidic lipoxygenase isoforms that increased locally after wounding (Fig. 2B) also increased in unwounded second trifoliate leaves from wounded plants, relative to controls (Fig. 3B, arrowheads). At 12 h after wounding, increased levels of the two least acidic isozymes (pl = 4.90 and 4.95) of the group of four were detected in leaves from wounded plants (Fig. 3B, upper two arrowheads). At 24 h postwounding, higher levels were detected in second trifoliate leaves from wounded plants for all four of the acidic lipoxygenase isoforms, compared to similar leaves from unwounded plants (Fig. 3B, arrowheads). By 48 h after wounding, only the two least acidic isozymes (pl = 4.90 and 4.95) of the group of four were detected in leaves from wounded plants (Fig. 3B). As was the case with the wounded, first trifoliate leaves, changes were not observed in the levels of the most abundant, acidic lipoxygenase isoforms (pl = 5.0-5.3) after wounding (Fig. 3B).

In Figures 2B and 3B, the four acidic wound-responsive lipoxygenase isoforms are not present in any lanes containing extracts from unwounded leaves. However, low levels of material that cross-reacted with the anti-lipoxygenase-2 antibody and co-migrated with the four acidic wound-responsive isoforms were routinely observed when the immunoblots were overexposed (data not shown).

Effects of Reproductive Sink Removal on Lipoxygenase Isozymes in Leaves

Recently, the 94-kD vegetative storage protein of soybean leaves was demonstrated to be a lipoxygenase
Soybean Leaf Lipoxygenase Isozymes

Figure 3. Immunoblot analysis of lipoxygenase isozymes in second trifoliate leaves (unwounded) from wounded (+) and unwounded (−) soybean plants. Lipoxygenase isozymes resolved between pH 6.0 and 7.5 (A) and between pH 4.5 and 6.0 (B). For wounded plants, each leaflet of the first trifoliate leaves was wounded once with a hemostat at the start of the experiment (h 0). Leaf extracts containing 100 μg of soluble protein were subjected to native IEF and immunoblotting as described in Figure 1. Arrowheads indicate the location of lipoxygenase isozymes that increase after wounding. Ps appear on the left.

Identification of Active Lipoxygenase Isozymes in Soybean Leaves

In the experiments described in the preceding sections, immunoblotting was used to assess the levels of leaf lipoxygenase isozymes. However, this procedure does not determine whether these lipoxygenase isozymes possess enzyme activity. Some of the lipoxygenase isoforms detected immunologically could be pI variants resulting from denaturation or partial degradation of native isozymes. Isozymic variation was probably not due to proteolytic degradation, however, because all leaf extracts used were analyzed by immunoblotting following SDS-PAGE and found to possess only a single molecular mass species that co-migrated with purified soybean seed lipoxygenase-2 (data not shown). To identify active lipoxygenase isozymes, IEF followed by in-gel activity staining has been employed previously (Verhue and Francke, 1972; Funk et al., 1985; Park and Polacco, 1989).

Figure 4. Immunoblot analysis of lipoxygenase isozymes in trifoliate leaves from plants with and without reproductive sinks. Lipoxygenase isozymes resolved between pH 6.0 and 7.5 (A) and between pH 4.5 and 6.0 (B). For plants lacking reproductive sinks, floral tissues were removed daily beginning at anthesis. Leaves were harvested at anthesis (lanes 1) and 2 weeks later from plants with (lanes 2) and without reproductive sinks (lanes 3). Leaf extracts containing 100 μg of soluble protein were subjected to native IEF and immunoblotting as described in Figure 1. Arrowheads indicate the position of wound-inducible lipoxygenase isozymes as they appear on the left.
Extracts were prepared from immature leaves (from 19 d after planting) and from both unwounded and wounded mature leaves and focused as before, although the amount of soluble protein per lane was increased to 400 µg. After focusing, the gels were stained for lipoxygenase activity at pH 7.0 using linolenic acid as the substrate. In general, more intense lipoxygenase activity staining was observed with extracts from immature leaves. Six activity-stained bands were observed with an extract from immature leaves in the same region of the IEF gels where the neutral lipoxygenase isozymes were observed by immunoblotting (Fig. 5A). These same bands were also observed in mature leaves, and no significant differences in the intensity of any band were observed when wounded and unwounded leaves were compared (data not shown).

In the region of the major acidic isozymes (pI = 5.0–5.3) detected by immunoblotting, strong lipoxygenase activity staining was observed using extracts from both unwounded and wounded leaves (Fig. 6, A and B, bands 2–4). In the regions that corresponded to the locations of the four acidic, wound-inducible lipoxygenase isozymes identified by immunoblotting, weak staining was observed (Fig. 6, A and B, bands 5–8). However, the activity staining in these regions was more intense with an extract from wounded leaves (Fig. 6B, bands 5–8) than with an extract from unwounded leaves (Fig. 6A, bands 5–8). In several independent experiments, low levels of activity staining in the region corresponding to the four acidic, wound-inducible lipoxygenases were always detected when extracts from wounded leaves were utilized. Unfortunately, these faint bands are not readily apparent following photographic reproduction (Fig. 6, A and B, bands 5–8). In other experiments, linoleic acid was used as the substrate, but the activity staining of all bands was more rapid and intense with linolenic acid as the substrate (data not shown). Lipoxygenase activity staining was also conducted at pH 5.0 (100 mM citrate), 6.0 (100 mM Mes), 8.0 (100 mM Tris), and 9.0 (100 mM borate). For all activity-stained bands from leaf extracts, the staining was most intense at pH 7.0 with linolenic acid as the substrate, and no additional bands appeared under any other conditions (data not shown). Varying the pH and/or substrate did not appear to affect differentially the activity staining of individual leaf lipoxygenase isozymes (data not shown).

To be certain that the activity-stained bands represented lipoxygenase isozymes, the stained bands were excised from IEF gels, denatured, and subjected to immunoblotting. All activity-stained bands contained protein that cross-reacted with anti-lipoxygenase-2 and co-migrated with purified soybean seed lipoxygenase-2 (Figs. 5B and 6C). In some lanes (Fig. 6C, lanes 2–8), two bands of very similar molecular mass were observed after immunoblotting. This may represent some partial degradation of the lipoxygenase isozymes during the 3-h activity-staining procedure.

**DISCUSSION**

IEF on polyacrylamide gels followed by immunoblotting has been utilized to resolve the lipoxygenase isozymes in soybean leaves and to examine the levels of the different isozymes in soybean leaves during leaf development (Fig. 1), after wounding (Figs. 2 and 3), and in response to reproductive sink removal (Fig. 4). As leaf age increased, most lipoxygenase isozymes decreased in relative abundance. However, the four most acidic isozymes (pI = 4.70, 4.80, 4.90, 4.95) were more abundant in intermediate-age leaves than in either the youngest or the oldest leaves examined (Fig. 1B, arrowheads). After wounding, these same four lipoxygenase isozymes were found to increase both in wounded leaves (Fig. 2B, arrowheads) and in unwounded leaves from wounded plants (Fig. 3B, arrowheads). The removal of reproductive sinks resulted in increased levels of most all of the acidic lipoxygenase isozymes in soybean leaves (Fig. 4B). Finally, neither...
wounding nor reproductive sink removal resulted in the appearance of any new lipoxygenase isozymes that were not detected in leaves from untreated plants.

To determine whether the leaf lipoxygenase isozymes revealed by immunoblotting have enzyme activity, leaf extracts were subjected to IEF on polyacrylamide gels followed by in-gel staining for lipoxygenase activity. Activity-stained bands were detected in the same regions of the IEF gels as the lipoxygenase isozymes detected immunologically (Figs. 5A and 6, A and B). Furthermore, when the activity-stained bands were excised from the gels and subjected to SDS-PAGE, all activity-stained bands contained material that cross-reacted with anti-lipoxygenase-2 antibodies and had molecular masses similar to soybean lipoxygenase-2 (Figs. 5B and 6C). Given that the wound-responsive, acidic isozymes are present in relatively minor amounts (Figs. 2B and 3B), it is not surprising that their activity staining was relatively weak (Fig. 6B, bands 5–8). In several similar experiments, low levels of activity staining were consistently detected in this same region of IEF gels when extracts from wounded leaves were analyzed (not shown). These results indicate that the lipoxygenase isozymes identified by immunoblotting following IEF are active lipoxygenases that do not result from degradation. In addition, the relative levels of lipoxygenase isozymes detected by immunoblotting correlate well with the relative intensity of bands detected by in-gel staining for lipoxygenase activity (Figs. 1A, 2B, 5A, and 6, A and B). This correlation implies that the anti-lipoxygenase-2 antibodies utilized have roughly equal avidity for the individual lipoxygenase isozymes in soybean leaves.

The lipoxygenase isozymes found in soybean seeds have been characterized by IEF, and pl values of 5.68, 6.25, and 6.15 have been observed for lipoxygenases-1, -2, and -3, respectively (Christopher et al., 1972). In the experiments presented here, the soybean seed lipoxygenase isozymes were not detected in extracts from leaves under any circumstance. In a previous investigation of lipoxygenase isozymes found in unfractionated leaf extracts, lipoxygenase isozymes corresponding to the seed isozymes were not observed, but after the leaf extracts were fractionated by chromatofocusing, a lipoxygenase isozyme with a pl nearly identical to that of lipoxygenase-1 from soybean seeds was detected by immunoblotting (Grayburn et al., 1991). Whether this isozyme is present but undetectable in unfractionated leaf extracts or is an artifact of the purification procedure is unclear. Park and Polacco (1989) have identified active lipoxygenase isozymes in hypocotyl/radicle axes with pIs more acidic than the three seed isozymes. Although they were not examined extensively in the current investigation, two lipoxygenase isozymes were found to be common between leaves and hypocotyl/radicle axes (Fig. 1B). The observation that soybean leaves and hypocotyls possess some common acidic lipoxygenase isozymes was reported previously (Grayburn et al., 1991).

Tranbarger et al. (1991) have identified the 94-kD vegetative storage protein as a lipoxygenase. Following the removal of reproductive sink tissues, lipoxygenase accumulates primarily in the vacuoles of paraveinal mesophyll cells in soybean leaves (Tranbarger et al., 1991). Grimes et al. (1992) suggested that the paraveinal mesophyll lipoxygenase is most likely the product of a single member of the soybean lipoxygenase gene family. More recently, however, Grimes et al. (1993) have suggested that the paraveinal mesophyll lipoxygenase is probably composed of several lipoxygenase isoforms. Kato et al. (1993) have demonstrated that soybean lipoxygenase-4 is elevated in leaves of depodded plants relative to control leaves from podded plants. Because the predicted amino acid sequence for lipoxygenase-4 was not identical to the partial amino acid sequence of the paraveinal mesophyll lipoxygenase reported by Tranbarger et al. (1991), Kato et al. (1993) suggested that paraveinal mesophyll lipoxygenase may be composed of several closely related proteins.

To identify other lipoxygenase isozymes that respond to reproductive sink removal, the lipoxygenase isozymes in leaves of deflowered plants were compared to leaves from podded control plants. After daily removal of floral tissues for 2 weeks, higher levels of all acidic lipoxygenase isozymes were observed in leaves, relative to podded controls (Fig. 4B). An increase in the level of a single neutral lipoxygenase isozyme (pl = 6.9) also occurred in leaves in response to reproductive sink removal (Fig. 4A). The results presented here clearly indicate that the accumulation of lipoxygenase in soybean leaves following reproductive sink removal involves increases in the levels of multiple, primarily acidic, lipoxygenase isozymes and suggest that the paraveinal mesophyll lipoxygenase is not a single lipoxygenase isozyme. In addition, both the results of Kato et al. (1993) and the current study call into question the partial amino acid sequence reported for the paraveinal mesophyll lipoxygenase, which was obtained by sequencing peptides derived from cyanogen bromide cleavage of a 94-kD protein resolved by SDS-PAGE (Tranbarger et al., 1991). Because the individual lipoxygenase isozymes present in soybean leaves were not resolved prior to cyanogen bromide cleavage, it is unlikely that the peptide sequence obtained was from a single lipoxygenase polypeptide.

Although reproductive sink removal may alter leaf nitrogen status, it is possible that some of the lipoxygenase isozymes that increase after flower removal responded to influences other than leaf nitrogen status. Hildebrand et al. (1989) demonstrated that repetitive wounding of soybean leaves for 7 or more days causes local and systemic increases in lipoxygenase protein and activity. Because the removal of reproductive tissues cannot be accomplished without wounding, some of the increases in leaf lipoxygenase isozymes after reproductive sink removal may be part of a systemic response to wounding. Two of the acidic lipoxygenase isozymes (pl = 4.90 and 4.95) that increase locally and systemically after wounding (Figs. 2B and 3B, upper two arrowheads) also increased following reproductive sink removal (Figs. 4B, arrowheads). In addition, ABA has been shown to increase in soybean leaves after reproductive sink removal (Setter et al., 1980), and ABA has been reported to elevate the expression of Arabidopsis lipoxygenase-1 mRNA (Melan et al., 1993). Additional studies must
be conducted to determine the effects of plant growth substances, such as ABA and JA, on the levels of lipoxyn-
genase isozymes in soybean leaves.

The neutral lipoxynagenase isozymes detected in leaves from young vegetative plants (about 30 d after planting) (Figs. 2A and 3A) were not detected in leaves from plants at anthesis (75 d after planting) or from podded plants 2 weeks after anthesis (Fig. 4A). Although the physiological roles of the neutral lipoxynagenase isozymes in soybean leaves are unknown, this observation indicates that these lipoxynagenes are not involved in the physiological processes that occur within the leaves of unwounded, re-

ductive plants.

Wounding stimulated increases both locally and system-
ically in the levels of the four most acidic lipoxynagenase

isozymes. In wounded leaves, increases in all four isozymes occurred within 12 h after wounding (Fig. 2B). In unwounded second trifoliate leaves from plants with wounded first trifoliate leaves, increases in only two of the four acidic lipoxynagenase isozymes were observed at 12 h after wounding (Fig. 3B, upper two arrowheads), but by 24 h postwounding increased levels of all four isozymes were detected compared to controls (Fig. 3B, arrowheads). In addition, all four of the acidic, wound-responsive li-

poxynagenase isozymes were observed in intermediate-age leaves from vegetative plants (Fig. 1B), and two (pI = 4.90 and 4.95) of the four isozymes were observed in mature leaves from reproductive plants (Fig. 4B). These observa-
tions coupled with the wound inducibility of the four acidic lipoxynagenase isozymes suggest that these isozymes may be important in physiological processes that are common to both wounded and unwounded plants. Alterna-
tively, the physiological functions of these four minor lipoxynagenase isozymes may be exclusively for plant de-
fense. Their presence prior to wounding may be necessary to aid in plant defenses before any wound-induced gene expression can occur. Subsequent increases in these four acidic lipoxynagenase isozymes may be part of an amplifica-
tion of the initial response to physical damage. Although the results presented here clearly indicate which lipoxyn-

agenes isozymes increase in abundance following physical damage to leaves, the lack of increases in other lipoxyn-

agenase isozymes in leaves does not exclude their participa-
tion in plant defense responses.

The presence of multiple isozymes will make the task of defining the physiological roles of plant lipoxynagenase chal-
lenging. However, given the proposed roles of lipoxy-

genase in plant defense against pests and pathogens (Farmer and Ryan, 1992; Gundlach et al., 1992; Kauss et al., 1992; Koch et al., 1992; Ryan, 1992; Croft et al., 1993), understanding the function of lipoxynagen is important. Defining both the expression of individual lipoxynagen genes and the physiological roles of the specific lipoxynagen iso-

zymes that they encode may be useful in developing strat-
ges to genetically engineer crop plants that have en-
hanced resistance to pests and pathogens. To understand fully the physiological functions of lipoxynagen in plants, the function of individual lipoxynagen isozymes must ultimately be determined.

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