Expression, Activity, and Cellular Accumulation of Methyl Jasmonate-Responsive Lipoxygenase in Soybean Seedlings

Howard D. Grimes*, David S. Koetje, and Vincent R. Franceschi

Department of Botany, Washington State University, Pullman, Washington 99164–4238

ABSTRACT

Exposure of soybean (Glycine max) seedlings to low levels of atmospheric methyl jasmonate induced the expression and accumulation of one or more lipoxygenase(s) in the primary leaves, hypocotyls, epicotyls, and cotyledons. In the primary leaf, the major site of lipoxygenase accumulation in response to methyl jasmonate was in the vacuoles of paraveinal mesophyll cells. In the other organs, however, most of the methyl jasmonate-responsive lipoxygenase(s) were associated with both the epidermal and cortical cells and were present in both vacuoles and plastids. In plastids, the methyl jasmonate-responsive lipoxygenase was sequestered into protein inclusion bodies; no lipoxygenase was evident in either the thylakoids or the stroma. Both spectrophotometric measurement of conjugated diene formation and thin layer chromatography of lipoxygenase product formation indicated that methyl jasmonate caused an increase in the amount of lipoxygenase activity. Electron microscopy of the methyl jasmonate-responsive lipoxygenase protein in the vacuoles showed that it was arranged into a stellate, paracrystalline structure in various cell types other than the paraveinal mesophyll cells. The paracrystals appeared to be composed of tubular elements of between 5 and 8 nm in diameter, were of variable length, and were observed in most cell types of the seedling organs.

Jasmonic acid and its methyl ester, methyl jasmonate, are rapidly emerging as strong candidates for intracellular or intercellular messengers in higher plants. When applied directly to plants or suspension cultures, they produce a number of phytohormone-like effects (1, 19, 26, 31). The case for these compounds functioning as messengers was strengthened by the recent demonstration that exceptionally low levels (approximately $10^{-7}$ M maximum) of atmospheric methyl jasmonate would stimulate the expression of protease inhibitor proteins in tomato leaves (3) and the accumulation of VSPs2 in soybeans (7).

Jasmonic acid is structurally similar to eicosanoids such as the prostaglandins present in a wide range of animal species (27). In mammals, eicosanoids are synthesized following the release of arachidonic acid into the cytoplasm and function as intracellular stress messengers (27). Analogously, plant cells are able to utilize linolenic acid as a substrate for the lipoxygenase-dependent synthesis of jasmonic acid (30). Although it has yet to be definitively established that jasmonic acid or methyl jasmonate are intracellular messengers in plant systems, the results of Farmer and Ryan (3) suggest that they may be involved in a wound-responsive signal cascade.

The expression and accumulation of soybean VSPs is regulated by jasmonic acid and methyl jasmonate (1, 7, 10, 14, 22, 23). The VSPs consist of three proteins of 27, 29, and 94 kD (vsp27 or vspα, vsp29 or vspβ, and vsp94, respectively), which accumulate during vegetative growth in a specialized cell layer termed the PVM layer (5, 6, 8, 11, 13). The PVM layer apparently serves as a direct conduit from the palisade parenchyma to the phloem (4–6, 8). VSP transcripts and their proteins respond to the immediate need to store nitrogen or amino acids (22, 34) and to the removal of sink tissue or pods (20, 25, 32, 33). The VSPs decrease in abundance during seed development (21, 34). Apparently, the VSPs have been recruited for temporary storage of nitrogen during vegetative growth, and this nitrogen is later contributed to the developing seed.

In soybean (Glycine max) seedlings, atmospheric methyl jasmonate induces the accumulation of all three VSPs, including vsp94, in the shoot tips, primary leaves, stems, and cotyledons (7). These organs normally express the VSPs at very low levels, and, of these organs, only the primary leaf has PVM cells. In response to atmospheric methyl jasmonate, vsp94 accumulated in primary leaves and expanding first trifoliolates, whereas vspβ preferentially accumulated in the cotyledons (7). These results suggest that methyl jasmonate may alter the source-sink relationships by shifting the priority of nitrogen utilization or increasing the rate of nitrogen assimilation and amino acid storage. Hence, methyl jasmonate appears to affect nitrogen partitioning in soybean plants. We recently demonstrated that the 94-kD VSP present in mature soybean leaves is actually a member of the lipoxygenase gene family (25). Because of the strong localization of vsp94 in the PVM vacuoles, we termed this protein pvmLOX.

These observations raise the obvious question of whether the 94-kD protein induced by methyl jasmonate in seedlings is also a lipoxygenase. This question takes on added importance when one considers that in the seedling only the primary leaves have a PVM layer (13). We have demonstrated that methyl jasmonate induces the expression of the 94-kD protein in all above-ground organs of the soybean seedling...
(7). If the 94-kD protein that is induced by methyl jasmonate in seedlings is a lipoxygenase, then its activity in mature soybean leaves was estimated to be a lipoygenase. This question is especially relevant in terms of the evidence indicating that jasmonic acid and methyl jasmonate are synthesized via a lipoygenase-dependent pathway (30).

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Soybean (*Glycine max* Merr. cv Wye) seeds were planted in potting compost in 3-inch pots and grown in a controlled environment chamber with 360 to 400 μmol m⁻² s⁻¹ PPFD at canopy height, 16 h photoperiod and 24/18°C day/night temperatures.

**Methyl Jasmonate Treatments**

Methyl jasmonate was prepared by diluting with 100% ethanol at a ratio of 1:9 (methyl jasmonate:ethanol). The methyl jasmonate was obtained from Bedoukian Research Co. (Danbury, CT) and consisted of 90.6% 1R,2R-enantiomer and 8.1% 1R,2S-enantiomer according to the manufacturer. Controls consisted of 100% ethanol.

Four seeds were sown in 3-inch pots and allowed to germinate in the growth chamber until the cotyledons were above ground (approximately 5 days after sowing). Just after the cotyledons emerged from the soil, the seedlings were exposed to methyl jasmonate vapor by placing methyl jasmonate onto the tip of a cotton swab anchored in a central location in the pot but not directly contacting the plants. The plants were placed inside an air-tight plexiglass box for the remainder of the experiment; these boxes were placed inside the growth chamber. The final concentration of methyl jasmonate was 120 μL/L of box volume, with the controls receiving 120 μL/L of ethanol. At the initiation of our experiments, the maximum amount of atmospheric methyl jasmonate (assuming a fully saturated atmosphere and 24°C) has been calculated to be about 2.4 × 10⁻⁷ mol/L of air. Exposure to methyl jasmonate was for 5 days, unless specified otherwise in the text.

**Protein Extraction and Electrophoresis**

Samples from various organs were weighed and quickly 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/g of tissue of homogenization buffer, which consisted of 25 mM Tris·HCl (hydroxymethyl) methylglycine (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 μg mL⁻¹ PMSF. The homogenate was centrifuged at 10,000g 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 instructions (Bio-Rad Laboratories). Extracts were mixed 1:1 (v/v) with 2X Laemmli sample buffer (12), heated at 90°C for 5 min, and centrifuged at 13,000g for 5 min, and the supernatants were loaded onto SDS-PAGE gels on an equal protein basis. Proteins were resolved according to Laemmli (12) 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. (24) at 100 mA for 12 h followed by 200 mA for 1 h. The blot was blocked for 2 to 3 h with 10% nonfat Carnation instant milk in Tris-buffered saline 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 (1:5000 dilution) in the blocking solution. This antibody was prepared against isoelectric-focused soybean leaf lipoxygenase peak 3 (9) and was kindly supplied by Dr. David Hildebrand (University of Kentucky). Secondary antibody was goat anti-rabbit immunoglobulin G-peroxidase (Pierce Chemical Co.) in the blocking solution at a concentration of 1:5000 for 1 h. Color was developed by immersing the blot in 60 mg of 4-chloro-1-napthol (first dissolved in 20 mL of cold methanol) and 60 μL of H₂O₂ in 100 mL of Tris-buffered saline.

**RNA Isolation and Northern Analysis**

RNA was extracted from seedling tissues according to Chomczynski and Sacchi (2). Tissue was ground in liquid N₂ to a powder in a mortar and pestle. All subsequent volumes are for 1.0 g fresh weight of tissue. A denaturing solution containing 4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 M β-mercaptoethanol was added to the powder. The resulting slurry was transferred to a Corex tube containing 10 mL of phenol (pH 8.0) and 2 mL of chloroform. The phenol/chloroform aqueous phase was thoroughly mixed and incubated on ice for 15 min. Samples were centrifuged at 15,000g for 20 min at 4°C. The aqueous phase was collected and precipitated with an equal volume of isopropanol for 1 h at −20°C. Precipitated nucleic acid was pelleted at 10,000g for 20 min at 4°C and resuspended in 500 μL of denaturing solution. The resuspended nucleic acids were extracted twice with an equal volume of phenol (pH 8.0):chloroform (1:1). The final aqueous phase was collected in a 1.5-mL microcentrifuge tube and precipitated with an equal volume of isopropanol for 1 h at −20°C. The 1.5-mL microcentrifuge tubes were centrifuged at maximum speed in a microcentrifuge at 4°C for 10 min, the pellets were rinsed with 80% ethanol, and were dried in a Speed-Vac until dry. The final pellets were resuspended in diethylpyrocarbonate-treated water for spectrophotometric analysis.

Total RNA (10 μg) was resolved on formaldehyde gels according to Sambrook et al. (18) and transferred to Gene-screen Plus (DuPont) membranes via capillary action (18).
Hybridization to probe DNA (using 10–15 ng of probe/mL of hybridization solution) was performed according to the manufacturer’s recommendations. The probe used was a 1.4-kb EcoRI fragment of pTK11 (containing a cDNA to a hypocotyl/radicle-associated lipoxygenase, ref. 15). After hybridization overnight at 65°C, the membranes were washed twice for 5 min each with 2X SSC at room temperature, twice for 30 min each with 2X SSC and 1% SDS at 65°C, and twice for 30 min each with 0.1X SSC at room temperature. Membranes were then air-dried and subjected to autoradiography.

Lipoxygenase Activity Assays

Soluble protein was extracted from primary leaves and shoot apices as described above except that 1 volume of homogenization buffer/g fresh weight of tissue was used. Extracts were analyzed immediately or frozen in liquid N₂ and stored at −80°C. Two independent assays were used. The first was a spectrophotometric assay that measured the formation of conjugated dienes at A₂₃₅ using linolenic acid as the substrate at pH 6.8. All assay conditions were as described by Grayburn et al. (9). The second assay analyzed lipoxygenase product formation using TLC. Assay conditions were 40 mM Tris (pH 8.7), 1 mM CaCl₂, 0.1% Triton X-100, 0.5 mM linolenic acid, and approximately 50 μCi of [¹⁴C]linolenic acid. Final volume for the assays was 200 μL, and 2 μL of extract was added to initiate the reaction. The reaction was allowed to proceed for 5 min at 30°C and then was stopped with 360 μL of diethyl ether:methanol:1 mM citric acid (30:4:1), vortexed, and centrifuged for 1 min at 13,000g. The upper phase was collected and evaporated to near dryness in a Speed Vac. The volume was adjusted to 20 μL with ethyl acetate, and the entire volume was spotted onto TLC plates. Chromatography was done for approximately 1 h using chloroform:methanol:acetic acid (70:30:1). Standards were [¹⁴C]linolenic acid and [¹⁴C]J3HPOTrE, an initial product of lipoxygenase action on linolenic acid, and were obtained from Cayman Chemical Co. (Ann Arbor, MI).

Tissue Preparation and Immunolocalization

Soybean tissue was gently sliced into pieces approximately 2 mm² 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 CaCl₂. Tissue was allowed to fix for 6 h at room temperature with gentle agitation before being rinsed with 50 mM Pipes buffer (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 h. Sections (1 μm) were cut and dried onto gelatin-coated slides.

Sections were blocked with TTBS + BSA for 60 min, then incubated in soybean leaf lipoxygenase antisera at 1:150 dilution) at room temperature for 3 h. 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-conjugated gold (15 nm, Janssen, 1:50 in Tris-buffered saline) for 2 h. The sections were rinsed in the blocking solution three times for 5 min, then with TTBS + BSA three times for 5 min, followed by TTBS for 5 min. Sections were rinsed in water for 30 to 60 s and allowed to air dry and were then enhanced with silver (IntenseM, Amersham) twice for 5 min. Sections were then stained with 1% Safranin at room temperature and covered with immersion oil and a coverslip for light microscope examination.

Light microscopy was performed using a Leitz Aristoplan microscope. The Bioquant Meg IV image analysis programs were used for background correction and quantification of silver grains within cells. Quantification was as described in Tranbarger et al. (25).

Transmission EM 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 h at room temperature to block nonspecific protein-binding sites. The grids were incubated for 4 h in soybean leaf lipoxygenase antisera 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 h 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.

RESULTS

Atmospheric Methyl Jasmonate Induces the Expression and Accumulation of a Lipoxygenase in Soybean Seedlings

In previous work, we demonstrated that atmospheric methyl jasmonate induced the accumulation of the VSPs, including vsp94, in soybean seedlings (7). We subsequently demonstrated, in fully expanded trifoliolates from adult soybeans, that vsp94 was a member of the lipoxygenase gene family, was localized in the vacuoles of PVM cells, and was regulated by nitrogen status (25). Hence, the question was raised as to whether the 94-kD VSP induced by methyl jasmonate in seedlings was also a member of the lipoxygenase gene family.

This question was addressed by first examining the expression and accumulation of the 94-kD VSP in various organs of soybean seedlings. Figure 1A shows a northern assay using total RNA probed with an EcoRI fragment from a plasmid (pTK11) containing a cDNA to a lipoxygenase expressed in soybean seedling stems (hypocotyls and epicotyls), primary leaf, and cotyledons. This cDNA is to a highly conserved base sequence in the lipoxygenase gene family. The results clearly demonstrate that a higher level of lipoxygenase mRNA accumulates in response to methyl jasmonate (Fig. 1A). Very high levels of lipoxygenase mRNA are found in cotyledons, primary leaves, and stem tissue after exposure to methyl jasmonate, although it appears that the cotyledons may be the most responsive tissue to methyl jasmonate (Fig. 1A).

Two possibilities exist to explain the increase in lipoxygenase mRNA levels after exposure to methyl jasmonate. First,
the methyl jasmonate may stabilize the lipoxygenase mRNA during seedling growth from normal degradation. This possibility is a concern because lipoxygenase activity and protein level is known to decrease in cotyledonary tissue as the tissue ages (16, 17). Second, the methyl jasmonate may induce the expression of lipoxygenase transcript. To differentiate between these two possibilities, a time-course experiment was performed. Seeds were germinated and the time course was initiated after the cotyledons were above ground. Time zero was prior to any treatment or enclosure of the seedlings in the plexiglass boxes. Seedlings were placed into the plexiglass boxes and whole seedlings were removed after 1, 3, and 5 d of exposure to ethanol (control) or methyl jasmonate. Total RNA was isolated from entire seedlings and was subjected to northern analysis (Fig. 1B). This experiment demonstrates that methyl jasmonate induces the expression of lipoxygenase transcript.

Because the expression of lipoxygenase was induced by methyl jasmonate treatment, we wanted to determine how much lipoxygenase protein accumulated in response to methyl jasmonate. Figure 2A shows that vsp94 accumulates in the cotyledons, shoot tip plus first expanding trifoliolate leaf, epicotyl, and the hypocotyl region. The other two VSPs, vsp-α and vsp-β, also accumulate in these organs (Fig. 2A). In Figure 2A, vsp-α is referred to as “A” and vsp-β as “B.” A western assay was performed using a lipoxygenase antiserum. The results of this assay are shown in Figure 2B and indicate that the methyl jasmonate-responsive 94-kD protein is cross-reactive with the lipoxygenase antiserum. This antiserum is quite specific for 94-kD lipoxygenase (25) and, hence, only a portion of the immunoblot is shown in Figure 2B. Densitometric measurements of these bands indicate that lipoxygenase expression is stimulated approximately 3.1-fold in the cotyledon, 2.5-fold in the shoot tip, and 2.4-fold in stem tissue (epicotyl and hypocotyl). This accumulation correlates with the increases observed in the lipoxygenase mRNA levels (Fig. 1A). These results show that methyl jasmonate causes the accumulation of lipoxygenase in soybean seedlings.

**Atmospheric Methyl Jasmonate Causes an Increase in the Amount of Lipoxygenase Activity in Primary Leaves and Shoot Apices**

Because methyl jasmonate induced the expression and accumulation of a lipoxygenase in various organs in the soybean seedling, it was of interest to determine if this methyl jasmonate-responsive lipoxygenase caused an increase in lipoxygenase activity. This issue is important in light of evidence indicating that jasmonic acid and methyl jasmonate are probably synthesized via a lipoxygenase-dependent pathway (30). To address this problem, two independent assays were performed to measure lipoxygenase activity before and after exposure to methyl jasmonate.

The first assay measures lipoxygenase product [13(s)HPOTrE] formation using TLC. Figure 3A presents the results of control and methyl jasmonate-treated extracts examined by this method. It is clear from this figure that methyl jasmonate induced more 13(s)HPOTrE formation when samples were loaded on an equal leaf volume basis. The protein contents of these two samples were very close (88 μg/μL for the control and 92 μg/μL for methyl jasmonate-treated). Hence, the lipoxygenase activity is increased when expressed as either total activity per leaf area or as specific activity. The second product visible just under the 13(s)HPOTrE is probably another lipoxygenase product [9(s)HPOTrE], but this has not been confirmed at this time.

A second assay was also used to quantitate the increase in lipoxygenase activity after methyl jasmonate treatment. This assay measured conjugated diene formation at 235 nm using linolenic acid as a substrate, and the results are presented in Figure 3B. Both of these assay methods indicate that methyl jasmonate causes an increase in lipoxygenase activity.

**Atmospheric Methyl Jasmonate-Responsive Lipoxygenase Is Found in Several Types of Cells in Soybean Seedlings**

In mature soybean leaves, the vsp94/pvmLOX is localized primarily in the vacuoles of bundle sheath and PVM cells. The PVM cell layer, however, is found only in primary leaves and trifoliolates in soybeans; it is not present in the hypocotyl,

**Figure 1.** Northern analysis of lipoxygenase mRNA response to methyl jasmonate exposure. A, Comparison of lipoxygenase mRNA levels in various organs after a 5-d exposure to atmospheric methyl jasmonate (MJ) or ethanol (C). B, Time course of lipoxygenase mRNA induction upon exposure to atmospheric methyl jasmonate. In B, total RNA was isolated from entire seedlings for analysis. In both A and B, 15 μg of total RNA was electrophoresed. The size of the transcript was 2.6 kb.

---

*Plant Physiol. Vol. 100, 1992*
jasmonate. A leaf primary whether the epicotyl, or organs before and after a 5-d exposure to atmospheric methyl jasmonate. A, SDS-PAGE gel of soluble proteins extracted from various organs in control and atmospheric methyl jasmonate-exposed seedlings. Primary leaf includes the first expanding primary leaf as well as the shoot apex. The markers point to the 94 kD lipoxygenase, vspa (A, 27 kD) and vspb (B, 29 kD). B, Immunoblot of A using a leaf lipoxygenase antiserum. Only the cross-reactive region of the blot is shown.

epicotyl, or the cotyledon. Because of this, it was determined whether the methyl jasmonate-responsive lipoxygenase was expressed in all cell types or whether it was specifically associated with a subset of cell types in seedlings. Immunolocalization experiments were performed on thick-sectioned material using the lipoxygenase antibody.

Figure 4 shows the location of lipoxygenase within primary leaves before and after exposure to atmospheric methyl jasmonate. Preimmune controls (Fig. 4A) were unalabeled in primary leaf and all other organs. In the primary leaf, lipoxygenase is found predominantly in the PVM cell layers and the associated bundle sheath (Fig. 4B). The primary leaf characteristically has two PVM layers (13), and both show accumulation of the pvmLOX. It is also present in the epidermis but to a much lesser extent than in the PVM cells. By comparing Figure 4, B and C, it is apparent that methyl jasmonate causes a significant increase in the level of lipoxygenase protein accumulation in bundle sheath, PVM, and epidermal cells.

Figure 5 shows lipoxygenase accumulation before (A) and after (B) exposure to methyl jasmonate in hypocotyl tissue. Lipoxygenase was found in the vacuoles of most cell types in the hypocotyl (epidermis, cortex, pith, vascular, parenchyma) but was most abundant in the epidermal and cortical cells. The epidermal and cortical cells in the hypocotyl accounted for about 90% of the total lipoxygenase present in this organ (data not shown). Comparison of Figure 5, A and B demonstrates that lipoxygenase protein levels increase significantly after exposure to methyl jasmonate.

Figure 6 shows lipoxygenase protein immunolocalization before (A) and after (B) methyl jasmonate treatment in cotyledonary tissue. In the cotyledons after methyl jasmonate exposure, lipoxygenase was concentrated in the vacuoles of the epidermal cells as large protein aggregates (Fig. 6B). Most of the remainder of the cotyledon was made up of chlorenchymatous palisade cells with no noticeable protein labeling.

In all methyl jasmonate-exposed seedling tissues, with the exception of the PVM layer, the lipoxygenase protein often appeared as clumps composed of large branched or spiked clusters. This is in contrast with our earlier observations of the pvmLOX in fully expanded leaves, where it appears as a flocculent mass distributed fairly uniformly throughout the vacuoles (25). It is also evident from these immunocytochemical studies that methyl jasmonate consistently causes lipox-

Figure 2. SDS-PAGE and immunoblot analysis of soybean seedling organs before and after a 5-d exposure to atmospheric methyl jasmonate. A, SDS-PAGE gel of soluble proteins extracted from various organs in control and atmospheric methyl jasmonate-exposed seedlings. Primary leaf includes the first expanding primary leaf as well as the shoot apex. The markers point to the 94 kD lipoxygenase, vspa (A, 27 kD) and vspb (B, 29 kD). B, Immunoblot of A using a leaf lipoxygenase antiserum. Only the cross-reactive region of the blot is shown.

Figure 3. Lipoygenase activity after exposure to methyl jasmonate. A, Two microliters of primary leaf and shoot apex extract was incubated with [14C]linolenic acid. The amount of 13(s)HPOTrE and putative 9(s)HPOTrE produced is shown in controls and methyl jasmonate-treated seedlings. B, Results of a spectrophotometric assay measuring conjugated diene formation in controls and methyl jasmonate-treated seedlings (± s.e.).
lipoxygenase protein to accumulate in various tissues and cell types in the soybean seedling and are thus methyl jasmonate-responsive lipoxygenases.

Image analysis techniques were used to quantify the distribution of the methyl jasmonate-responsive lipoxygenase in various cell types of different organs. The silver grains in the micrographs were quantitated by converting individual pixels that lie above a specific gray level to “marked” pixels (25). Figure 7 shows the data from quantifying the relative amount of silver grains present in different cell types in various organs. These data demonstrate that the PVM layer in primary leaves is a significant site of methyl jasmonate-responsive lipoxygenase accumulation. Epidermal and cortical cells in cotyledons and hypocotyls, respectively, also contain large amounts of methyl jasmonate-responsive lipoxygenase. These data, coupled with the densitometry on the immunoblot in Figure 1B, indicate that the hypocotyl and cotyledons are a major site for accumulation of methyl jasmonate-responsive lipoxygenase in soybean seedlings.

Ultrastructural Studies Indicate that the Methyl Jasmonate-Responsive Lipoxygenase in Hypocotyls and Cotyledons Is Present as a Paracrystalline Structure

The pvmLOX in the vacuoles of PVM and bundle sheath cells of primary leaves and trifoliolates appears as a flocculant material (25). In contrast with this, pvmLOX deposits in the vacuoles of the hypocotyl and cotyledon tissues are highly aggregated in discrete clumps. Because of these observations at the light microscope level, we performed transmission EM analysis of thin-sectioned material to probe the structural nature of the methyl jasmonate-responsive lipoxygenases.

Figure 8A shows a preimmune control for the lipoxygenase antibody in the hypocotyl. The protein cluster appears as a conglomerate of elongate and amorphous elements. Figure 8B shows the stellate, paracrystalline arrangement that these “protein bodies” often acquire. The inset in Figure 8B shows an enlargement of part of the paracrystal. Distinct tubular elements of variable length appear to be arranged as packets of tubules. The surrounding material seems to be amorphous, but this material may actually consist of tubules cut tangentially and in cross-section. One observation consistent with this interpretation is the abundance of circular profiles in these areas, which may be individual tubules. These tubular elements appear to be highly regular and to have an outer diameter of about 5 to 8 nm. As is clearly evidenced in Figure 7B, both of these regions are strongly labeled with the lipoxygenase antibody, indicating that the methyl jasmonate-responsive lipoxygenase(s) is organized in this manner.

The subepidermal cells and some cortical cells of the hypocotyl have plastids with thylakoids and protein inclusions (Fig. 5C). The lipoxygenase antibody cross-reacted specifically with the protein inclusions of the plastids, whereas no labeling was observed in the stroma or associated with thylakoids (Fig. 8D). These protein inclusions did not label when antibody to vspa/β was used (data not shown).

The methyl jasmonate-responsive lipoxygenase protein was present in the epidermal cell vacuoles of cotyledons and also demonstrated a paracrystalline structure (Fig. 9A). At higher magnification, the tubular elements can be visualized
in the cotyledon protein clusters (Fig. 9B). These regions of tubular elements contained methyl jasmonate-responsive lipoxygenase, as indicated by the distribution of labeling along them (Fig. 9B).

DISCUSSION

The results presented here demonstrate that low levels of atmospheric methyl jasmonate induce the expression, accumulation, and activity of lipoxygenase in soybean seedlings. Upon exposure to atmospheric methyl jasmonate, lipoxygenase was fairly uniformly stimulated in shoot tips, primary leaves, epicotyls, hypocotyls, and cotyledons. The regulation of this increase is at the transcriptional level since methyl jasmonate induces the expression of a lipoxygenase mRNA in intact seedlings. Previous work in our laboratory has indicated that these responses are due to atmospheric methyl jasmonate and not to increased ethylene levels in the chambers (7). Collectively, these results strongly indicate that there is a methyl jasmonate-responsive lipoxygenase present in

Figure 5. Immunolocalization of lipoxygenase in hypocotyl tissue. A, Control hypocotyl of a 5-d-old seedling immunostained with lipoxygenase antiserum. B, Methyl jasmonate-treated hypocotyl of a 5-d-old seedling immunostained with lipoxygenase antiserum.

Figure 6. Immunolocalization of lipoxygenase in cotyledonary tissue. A, Control cotyledon of a 5-d-old seedling immunostained with lipoxygenase antiserum. B, Methyl jasmonate-treated cotyledon of a 5-d-old seedling immunostained with lipoxygenase antiserum.

Figure 7. Quantification of lipoxygenase in different cell types and organs in methyl jasmonate-exposed seedlings. Data were quantitated on an individual cell basis. Error bars indicate the SE of measurement, and at least 20 cells were quantitated for each cell type.
Figure 8. Transmission electron micrographs of hypocotyl tissue in 5-d-old seedlings. All of the panels in this figure show tissue sections after a 5-d exposure to atmospheric methyl jasmonate. A, Vacuolar region of hypocotyl tissue stained with preimmune serum (x19,900; bar = 0.5 μm). B, Vacuolar region of hypocotyl tissue stained with lipoxygenase antiserum. Note the paracrystalline structure (x10,500; bar = 1 μm). Inset shows an enlargement (x74,000) of the paracrystalline structure. C, Section showing cell wall, plastid, and vacuole after staining with lipoxygenase antiserum. Note that there is reactive material in all of these subcellular compartments (x13,800; bar = 1 μm). D, Plastid present in the hypocotyl after methyl jasmonate exposure stained with lipoxygenase antiserum. The plastid shows a significant amount of lipoxygenase present inside of a protein inclusion body (x49,500; bar = 0.2 μm). V, Vacuole; P, plastid; CW, cell wall.
soybean seedlings. This is an intriguing observation considering that (a) jasmonic acid and methyl jasmonate are potentially derived from a lipoxygenase-dependent pathway (30) and (b) methyl jasmonate is a strong candidate as an intracellular or intercellular regulator of VSP gene expression and nitrogen partitioning (7, 22).

The potential activity of this methyl jasmonate-responsive lipoxygenase is a difficult area to assess. In our in vitro experiments, we have been able to demonstrate that lipoxygenase activity is stimulated somewhat (approximately 25%) after methyl jasmonate exposure. This increase in activity correlates with, but does not closely parallel, the increase that we observe at transcript level or lipoxygenase protein level. The issue of lipoxygenase activity is further complicated by the fact that the major site of accumulation for the methyl jasmonate-responsive lipoxygenase(s) is in the vacuole. Our in vitro assay conditions may not closely simulate the in vivo conditions. At this time, then, all we can conclude is that the methyl jasmonate-responsive lipoxygenase(s) is potentially active. We are currently assaying for in vivo lipoxygenase product formation to resolve this uncertainty.

In mature soybean leaves, the nitrogen-responsive lipoxygenase is primarily associated with the PVM layer, and we have named this protein the pvmLOX to reflect this fact (25). Although a small amount of VSP, including the lipoxygenase, is expressed in epidermal cells, quantitative immunochrometry indicates that this amount is at least two orders of magnitude less than the level associated with the PVM layer (25).

In seedlings, however, only the primary leaf contains PVM cells, where they exist as a double layer rather than the single layer present in mature trifoliolate leaves (13). Because methyl jasmonate induced the expression of a lipoxygenase in organs that do not contain a PVM layer, we wanted to determine where in these organs the lipoxygenase was accumulating. The results of immunochromatolocalization experiments indicated that several different types of cells have material that is cross-reactive with the lipoxygenase antiserum. In the primary leaf, both layers of the PVM show high amounts of the lipoxygenase, and the epidermal cells also contain a small amount of lipoxygenase protein. Palisade parenchyma and spongy mesophyll do not contain detectable amounts of lipoxygenase. This pattern is very similar to the pattern seen in mature soybean leaves after removal of the sink tissue (25). In hypocotyl tissue, small amounts of the lipoxygenase were found in most cell types including the epidermis, cortex, pith, and vascular parenchyma, but it was most abundant in the epidermis and underlying cortex regions. Cambial cells did not appear to have any label associated with them. In cotyledonary tissue, the lipoxygenase was primarily associated with epidermal cells. The remaining chlorenchymatous palisade cells had no detectable label.

Huang et al. (10) presented evidence that in mature soybean leaves from depodded plants, methyl jasmonate caused an increase in vspα/β levels in many cell types. Apparently, the methyl jasmonate eliminated the normal cell-specific expression of vspα/β and caused these proteins to be expressed in numerous cell types (10). In seedlings, we also observe that methyl jasmonate-responsive lipoxygenase is found in numerous cell types. We are currently performing a developmental study of lipoxygenase localization to deter-

Figure 9. Transmission electron micrographs of cotyledonary tissue after a 5-d exposure to atmospheric methyl jasmonate showing the paracrystalline nature of the methyl jasmonate-responsive lipoxygenase. A. A region of the cotyledonary vacuole immunostained with the lipoxygenase antiserum (×17,800; bar = 0.5 μm). B. Higher magnification (×74,000) of vacular region immunostained with the lipoxygenase antiserum showing the tubular arrangement of these paracrystalline structures (bar = 2 μm).
mine if the expression pattern is altered by methyl jasmonate exposure or if the methyl jasmonate-responsive lipoxygenase is regulated differently than vplo/B.

Ultrastructural studies were initiated to determine the subcellular localization of the methyl jasmonate-responsive lipoxyngease in these various cell types and to investigate the structural nature of the methyl jasmonate-responsive lipoxyngease. In our earlier studies with mature leaves, the vpmlox was shown to accumulate within the cell vacuoles (25). Although cytoplasmic labeling was observed, we consider most of this to be due to synthesis and transport of the vacuolar lipoxyngease from the cytoplasm to the vacuoles (25). In seedling primary leaves and cotyledons, the majority of the methyl jasmonate-responsive lipoxyngease was associated with the vacuoles. In the hypocotyl, however, other cellular compartments, such as the cytosol and plastids, contained appreciable levels of the methyl jasmonate-responsive lipoxygenase. Specifically, the subepidermal and cortical cells have plastids with both thylakoids and protein inclusions. The lipoxyngease antibody specifically cross-reacted with the protein inclusions but not with the stroma or thylakoids. Vernoo-y-Gerritsen et al. (28, 29) have examined the subcellular distribution of soybean seed lipoxyngeases and suggest that during germination they are initially present in the storage tissues of etiolated cotyledons and later appear in the cytoplasm and protein bodies. Our results, on the other hand, show that the vast majority of lipoxyngease that cross-reacts with our antiserum is present in the vacuoles, both before and after exposure to methyl jasmonate. This difference is probably due to the fact that our seedling tissue (5-d-old) was older than theirs (1-d-old). In our data, we detect some activity associated with the cell wall in the hypocotyls, but this is probably due to the presence of lignin in this tissue and antiserum binding nonspecifically to the lignin fraction.

The morphology of the vacuolar-deposited methyl jasmonate-responsive seedling lipoxyngease is very different than the morphology of these proteins in mature leaves. The vpmlox is present in the vacuoles of mature leaf PVM cells as a flocculant material. In seedlings, the proteins are present as paracrystalline structures in the vacuoles. When sections are taken through these paracrystals, tubular elements in both tangential sections and cross sections are observed. The material in cross-section appears to be composed of circular profiles representing individual tubules, and the diameter of these tubules is between 5 and 8 nm. Lipoxyngease antiserum strongly cross-reacts with the tubular elements that comprise the paracrystals. This paracrystalline arrangement and tubular substructure of the methyl jasmonate-responsive lipoxyngease is unprecedented for any vacuolar protein of which we are aware.

From our immunocytochemical and ultrastructural studies, it would appear that methyl jasmonate is inducing the expression and accumulation of more than one member of the lipoxyngease gene family. In the primary leaves, atmospheric methyl jasmonate clearly induces the vpmlox as well as a lipoxyngease present in the epidermal cells. All of the VSPs, including the vpmlox, are present primarily in the PVM layer and, to a much lesser extent, in the epidermal cells.

Hence, this lipoxyngease is likely the product of a single gene that is expressed in two cell types. In other tissues, however, numerous types of cells express a lipoxyngease in response to methyl jasmonate exposure. In hypocotyls, we found distinct evidence indicating that methyl jasmonate induced the expression of a lipoxyngease that was associated with protein inclusion bodies in the plastids present in epidermal and cortex cells. These same cells also contained a vacuolar lipoxyngease. Because it is difficult to imagine a single gene product being targeted to both vacuoles and plastids, we hypothesize that in the hypocotyl, methyl jasmonate induces the expression of at least two lipoxyngease genes. Determination of the exact number of lipoxyngease genes that respond to methyl jasmonate will have to await the availability of gene-specific probes to the members of the lipoxyngease gene family.

In summary, we have demonstrated that low levels of atmospheric methyl jasmonate induce the expression and accumulation of lipoxyngeases in various cell types of soybean seedlings. This increase in the lipoxyngease protein level is correlated with an increase in the amount of lipoxyngease activity after exposure to methyl jasmonate. Ultrastructural studies indicate that most of the methyl jasmonate-responsive lipoxyngeases are associated with vacuoles where the lipoxyngeases are present as paracrystalline structures of apparently tubular elements.

ACKNOWLEDGMENTS

We thank Tae Kyung Park and Dr. Joseph Polacco (Biochemistry Department, University of Missouri-Columbia) for the lipoxyngease cDNA (pTK11) and Dr. David Hildebrand (Department of Agronomy, University of Kentucky) for the lipoxyngease antiserum. The assistance of Dr. Ted Farmer with the lipoxyngease TLC assay is gratefully acknowledged. Lastly, we thank Dr. Chris Davitt and the staff of the Electron Microscopy Center for their assistance.

LITERATURE CITED


