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Subcellular Localization Studies Indicate That Lipoxygenases 1 to 6 Are Not Involved in Lipid Mobilization during Soybean Germination[1]

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Soybean (*Glycine max*) lipoxygenase (LOX) has been proposed to be involved in reserve lipid mobilization during germination. Here, subcellular fractionation studies show that LOX1, -2, -3, -4, -5, and -6 isozymes were associated with the soluble fraction but not with purified oil bodies. The purified oil bodies contained small amounts of LOX1 (<0.01% total activity), which apparently is an artifact of the purification process. Immunogold labeling indicated that, in cotyledon parenchyma cells of LOX wild-type seeds that had soaked and germinated for 4 d, the majority of LOX protein was present in the cytoplasm. In 4-d-germinated cotyledons of a LOX1/2/3 triple null mutant (L0), a small amount of label was found in the cytoplasm. In epidermal cells, LOX appeared in vacuoles of both wild-type and L0 germinated seeds. No LOXs cross-reacting with seed LOX antibodies were found to be associated with the cell wall, plasma membrane, oil bodies, or mitochondria. Lipid analysis showed that degradation rates of total lipids and triacylglycerols between the wild type and L0 were not significantly different. These results suggest that LOX1, -2, -3, -4, -5, and -6 are not directly involved in reserve lipid mobilization during soybean germination.

LOX (EC 1.13.11.12) is a dioxygenase that catalyzes the peroxidation of fatty acids containing a *cis*,*cis*-1,4 pentadiene moiety. The principal substrates for LOX in plants are C18:2, ω6 (lineolate) and C18:3, ω3 (linolenate). LOXs have been reported to oxygenate isolated components of biomembranes (Maccarrone et al., 1994). The physiological function of LOX has been problematical, and various functions have been suggested. LOXs have been implicated in plant growth and development, senescence, and wound responses (Hildebrand, 1989), pest and disease resistance (Croft et al., 1993; Slusarenko et al., 1993), and the temporary storage of N_2 in vegetative tissue (Tranbarger et al., 1991). LOX also functions as one of the enzymes involved in the C18:2, ω6/C18:3, ω3 cascades, producing a variety of compounds that may function as signaling and/or defense substances (Anderson, 1989; Siedow, 1991; Slusarenko et al., 1991; Farmer and Ryan, 1992; Reinbothe et al., 1994; McConn et al., 1997; Vijayan et al., 1998).

Seed oils are packaged into discrete subcellular compartments referred to as lipid bodies or oil bodies (Herman, 1987), which are mobilized to nourish the developing seedling after seed germination. Oil bodies contain a matrix of TAGs surrounded by a half-unit membrane of one phospholipid layer embedded with abundant and unique proteins termed oleosins (Tzen et al., 1990; Murphy, 1993). During lipid mobilization in germinating seeds, the composition of mature oil bodies changes and a new set of proteins is synthesized and transported to the oil bodies (Sturm et al., 1985; Feussner and Kindl, 1992; Radetzky et al., 1993). Expression of LOX is differentially regulated during soybean (*Glycine max*) development and germination (Funk et al., 1986; Park and Polacco, 1989; Hildebrand et al., 1991; Kato et al., 1992; Park et al., 1994). The fact that preexisting LOX1, -2, and -3 disappear and new LOXs (LOX4, -5, and -6) appear in germinating soybean cotyledons (Kato et al., 1992) suggests that the newly synthesized LOX may play an important role in some aspects of seedling growth. Oil bodies start to disappear after 3 d of germination (Vernooy-Gerritsen et al., 1984; Song et al., 1990), and at this stage LOX4, -5, and -6 reach high levels in cotyledons. It is of great interest whether these new LOXs play a role in lipid mobilization.

Determination of the intracellular localization of a protein is important in the understanding of its function. Two methods have been used to localize LOX at the subcellular level. Fractionation studies indicated that LOX was associated with oil bodies of germinating soybean cotyledons; therefore, it has been suggested that LOX played a role in TAG metabolism in oil bodies (Feussner and Kindl, 1992). Similarly, Macri et al. (1994) reported that acidic LOX (pH optimum, 5.5–6.0) was associated with plasma membranes of hypocotyls of 4-d-germinated soybean. However, immunogold-labeling studies indicated that LOX was randomly distributed throughout the cytoplasm in storage parenchyma cells and that no LOXs were associated with oil bodies in germinating soybean (Vernooy-Gerritsen et al., 1984; Song et al., 1990). Tranbarger et al. (1991) reported that paraveinal mesophyll LOX accumulated in vacuoles in immunogold-labeling studies. Similarly, Grimes et al. (1992)

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Abbreviations: BHT, butylated hydroxytoluene; LOX, lipoxygenase; L0, a soybean seed (embryo) LOX1/2/3 triple null mutant; TAG, triacylglycerol; X:Y, a fatty acyl group containing X carbon atoms and Y *cis* double bonds.

confirmed that the majority of methyl jasmonate-responsive LOX appeared in the vacuoles of cotyledons, both before and after exposure of 5-d-germinated soybean seedlings to methyl jasmonate. These observations raise questions regarding whether LOXs (seedling or seed) are truly associated with oil bodies and where LOX is localized in germinating soybean seedlings. The objective of this study was to investigate whether LOX is involved in lipid mobilization during soybean germination. To achieve this objective, we used a number of strategies: (a) a soybean embryo LOX mutant was used to individually assess the effects of embryo and seedling LOXs; (b) subcellular fractionation and immunogold labeling were used to determine the localization of embryo and seedling LOXs; and (c) comparison of lipid degradation between the soybean LOX mutant and wild-type seeds during germination was investigated.

MATERIALS AND METHODS

Plant Materials

One wild-type soybean (*Glycine max* [L.] Merr. cv Century) and cv L0 were used in this study. The cvs Century and L0 both have normal expression of seedling LOXs after germination. Soybean seeds were germinated as described by Park and Polacco (1989). Imbibition of soybean seeds was in deionized water at 4°C for 12 h.

Lipid Analysis

Cotyledons of seeds or seedlings germinated for the indicated times in the dark were lyophilized. Thirty dried cotyledons were ground into a powder. Before extraction, TAG-17:0 (heptadecanoic acid) was added into cotyledon powder as an internal standard. About 100 to 200 mg of powder was extracted with 5 mL of chloroform:methanol: formic acid (10:10:1, v/v) plus 0.01% BHT (an antioxidant) followed by one additional extraction with 5 mL of chloroform:methanol (2:1, v/v) plus 0.01% BHT. The chloroform layer was collected after addition of 5 mL of a solution (0.2 M H_3PO_4 and 1 M KCl) and centrifugation. After residues were dried under N_2, they were dissolved in 0.5 mL of chloroform. TAG was separated from other lipids by TLC (silica gel, J.T. Baker), which was developed in hexane: ethyl ether:acetic acid (50:50:1, v/v) with 0.01% BHT. Lipids were visualized by spraying the TLC plates with a solution of 0.001% primulin in 80% acetone. The fatty acid compositions of TAG and total lipids were measured by GC (model 5890 with flame-ionization detection, Hewlett-Packard), after direct transmethylation, on an FFAP column (10 m × 0.2 mm; film thickness, 0.33 μm) at 120°C to 216°C at 12°C/min (Dahmer et al., 1989).

Isolation of Oil Bodies

Oil bodies were purified by the discontinuous Suc gradient method described by Sturm et al. (1985) with minor modifications. Fifteen milliliters of the homogenate was layered onto 15 mL of 20% Suc (w/v) in 20 mM Tricine buffer, pH 7.5. Five milliliters of 8% Suc (w/v) in 20 mM Tricine buffer, pH 7.5, was layered onto the homogenate in a 50-mL polypropylene tube. An alternative method for oil-body purification involving a high-salt wash described by Herman (1987) was also used for the purpose of comparison. All centrifugations were performed at 85,000*g* at 4°C (maximum gravity force was recommended for the ultracentrifuge used).

Purification of Oil Bodies from L0 in the Presence of Soluble LOX Protein

Oil bodies from the L0 mutant were purified in the presence of soluble LOX protein to determine whether LOX protein can artifactually associate with oil bodies. Approximately 10 g of dry, powdered cv Century seeds was extracted with a prechilled mortar and pestle on ice with 40 mL of extraction buffer (Sturm et al., 1985). The extracts were filtered through Miracloth (Calbiochem) and centrifuged at 22,740*g* for 30 min (model J2–21, Beckman). After the oil-body layer was removed, the soluble protein fraction (15% Suc fraction) was collected and subjected to one additional centrifugation as described above to further purify the soluble fraction away from oil bodies and cell debris. The final supernatant was adjusted to 30 mL with deionized water. The supernatant from cv Century soluble fractions (7.5 or 15 mL) was mixed with extraction buffer to a final volume of 20 mL, and this was then used to homogenize 5 g of powdered cv L0 seeds. The final homogenate was adjusted to 15 mL with deionized water. The modified discontinuous Suc gradient procedure was then used to purify oil bodies.

Enzyme Digestion

Twenty microliters of diluted oil bodies (diluted 1:1 [v/v] with water) was digested with 15 μg of trypsin or 15 μg of trypsin plus 15 μg of trypsin inhibitor in 30 μL of 0.2 M Tris buffer, pH 8.0, for 30 min. The reaction was stopped by adding SDS-PAGE loading buffer followed by boiling for 3 min. Results were analyzed by SDS-PAGE.

Protein Measurement, IEF- and SDS-PAGE, and Western Blotting

Purified oil bodies were diluted with deionized water to a final level of 1 mg lipid μL^{-1} in water. Proteins were extracted from oil bodies by the method described by Sturm et al. (1985) and quantified by a modified Lowry method (Bensadoum and Weinstein, 1976). IEF- and SDS-PAGE and western blotting were performed as described by Hildebrand et al. (1991).

LOX Activity Measurement

LOX activity was determined by the O_2-electrode polarography method (Kaplan, 1957; Siedow and Girvin, 1980). The reaction mixture (1 mL) contained 1.2 mM C18:2,0.08% (v/v) Tween 20, 40 mM phosphate, pH 6.83, or 40 mM borate, pH 9.0. Activity was defined as the quantity of enzyme catalyzing the consumption of 1 μmol O_2 s^{-1} at 25°C.

Tissue Preparation and Immunolocalization

The seedling cotyledons and soaked seeds were cut with a razor blade into approximately 1-mm^3 blocks, placed immediately into a vessel containing a freshly prepared fixative (2% [v/v] paraformaldehyde and 1% [v/v] glutaraldehyde in 0.1 M phosphate buffer, pH 7.3), and fixed for 2 h in vacuo at room temperature. After being rinsed three times with 0.1 M sodium phosphate buffer for 5 min each, the tissue was dehydrated through an ethanol series of 30%, 50%, 70%, 90%, and 100% (all v/v) for 20 min each, infiltrated with London Resin White (London Resin Co. Ltd., London, UK), and polymerized at 55°C for 24 h. Sections 60 to 90 nm thick on nickel grids were blocked with 0.1% (w/v) BSA and 5% (v/v) heat-denatured normal goat serum in PBS buffer (0.148% Na_2HPO_4, 0.043% KH_2PO_4, 0.72% NaCl, and 0.13% NaN_3, pH 7.3) for 1 h and incubated in a mixture of soybean embryo LOX1/2/3 rabbit antisera, mp24 (containing antibody to an oil-body membrane oleosin protein; supplied by E.M. Herman) rabbit antiserum, or preimmune serum (dilution, 1:150) at room temperature for 3 h. After being washed with PBS plus 0.5% (v/v) Tween 20 four times for 10 min each, the sections were incubated with goat anti-rabbit immunoglobulin-gold conjugate (15 nm; Pelco, Ted Pella, Inc., Redding, CA; dilution, 1:40 in PBS) for 1 h. The sections were then rinsed with PBS plus 0.1% (w/v) BSA and 0.5% (w/v) Tween 20 three times for 5 min each and three times with deionized water, dried, and stained with uranyl acetate for 4 min and with lead citrate for 4 min. Sections were examined and photographed with a transmission electron microscope (model H 600, Hitachi, Tokyo, Japan).

RESULTS

Distribution of Soybean LOX Activity and Protein in Discontinuous Suc Gradients after Centrifugation

Originally, we wanted to determine whether LOX is associated with oil bodies in germinating soybean seeds, as suggested by the observation of Sturm et al. (1985) in cucumber. Developing and mature seeds of cv L0 are essentially devoid of LOX (Hajika, et al., 1991; Wang et al., 1994), but LOX4, -5, and -6 appear within 2 d after germination. Therefore, cv L0 and soybean wild type were used in this study to separate seedling from embryo LOXs. Table I shows the distribution of LOX activity in Suc density gradient fractions from germinated cv Century and cv L0 cotyledons. No activity in homogenates from cv L0 was detected at pH 9.0, so LOX activity in cv L0 at pH 9.0 was not measured in these experiments. Most of the LOX activity at pH 6.83 for both cultivars and at pH 9.0 for cv Century remained in the 15% Suc gradient fraction after centrifugation (Table I). No activity in purified oil bodies (washed oil bodies) from germinated cv Century and cv L0 was measurable at pH 6.83, which indicated that seedling LOX (LOX4, -5, and -6; optimal pH of approximately 6.5; Kato et al., 1992) and type II LOX (LOX2 and -3) were not present in the oil bodies. LOX activity at pH 9.0 was found in unwashed and washed oil bodies from cv Century.

A comparison of LOX activity between washed and unwashed oil bodies showed that washing largely removed the LOX activity seen in oil bodies. The amount of LOX activity at pH 9.0 that finally appeared in the unwashed and washed oil bodies of germinated cv Century cotyledons represented less than 0.1% and 0.01%, respectively, of the total activity found in the crude extracts. IEF gels also showed that most of embryo (cv Century) and seedling (cvs Century and L0) LOX proteins were localized in the soluble fraction of the Suc gradient (Fig. 1). Unwashed oil bodies from cv Century had a detectable weak band of LOX1, but this became much weaker in washed oil bodies. No other LOX isozymes were detected in washed oil bodies (Fig. 1). Comparison of each fraction by IEF gel showed that LOX2, -3, -4, and -6 were present in the soluble fraction but not in washed oil bodies. Although the presence of LOX5 was not seen in soluble or oil-body fractions because of high background, the fact that washed oil bodies from

Table I. *Distribution of LOX activity*

Oil bodies were purified from 4-d-germinated cotyledons by discontinuous Suc gradient centrifugation. After centrifugation, samples were taken from the bottom, middle, and top layers and served as 20%, 15%, and 8% fraction protein and enzyme sources, respectively. The white pad on top of the gradient was collected and used as unwashed oil bodies. Unwashed oil bodies were gently resuspended in 15% (w/v) Suc in 20 mM Tricine buffer at pH 7.5, subjected to one additional centrifugation, and used as washed oil bodies. LOX activity was assayed by measuring O_2 consumption. The activities of the crude samples were taken as the total activity. Relative LOX activity is equal to the activity in the fraction divided by the activity of the crude samples. Values are means ± SE of three independent experiments.

Fraction	cv Century		cv L0
	pH 6.83	pH 9.0	pH 6.83
	%		
Oil bodies			
Washed	ND[a]	$8.0 \times 10^{-3} \pm 6 \times 10^{-4}$	ND
Unwashed	$3.0 \times 10^{-2} \pm 1.6 \times 10^{-2}$	$9.4 \pm 10^{-2} \pm 6 \times 10^{-2}$	ND
8% Suc	1.8 ± 1.7	3.0 ± 1.3	7.3 ± 2.0
15% Suc	67.7 ± 3.5	79.6 ± 6.8	76.8 ± 9.2
20% Suc	26.0 ± 6.5	14.6 ± 2.2	18.0 ± 3.8

[a] ND, Not detectable.

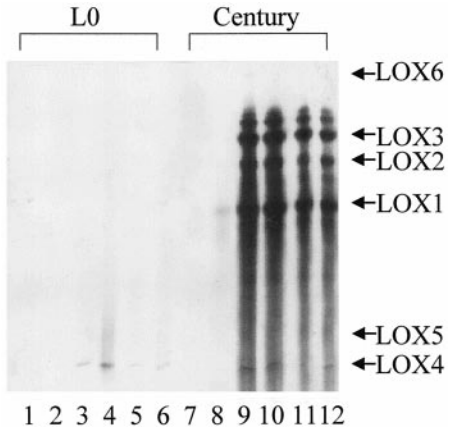

Figure 1. IEF-PAGE gel immunoblot illustrating distribution of LOX protein in Suc gradient fractions. Lanes 1 to 6, Four-day-germinated cv L0 cotyledons; lanes 7 to 12, 4-d-germinated cv Century cotyledons; lanes 1 and 7, washed oil bodies; lanes 2 and 8, unwashed oil bodies; lanes 3 and 9, 8% Suc fraction; lanes 4 and 10, 15% Suc fraction; lanes 5 and 11, 20% Suc fraction (see Table I); and lanes 6 and 12, crude extracts. Sixty micrograms of proteins was loaded for each lane. Soybean polyclonal LOX1, -2, and -3 antibodies were used to probe the membranes.

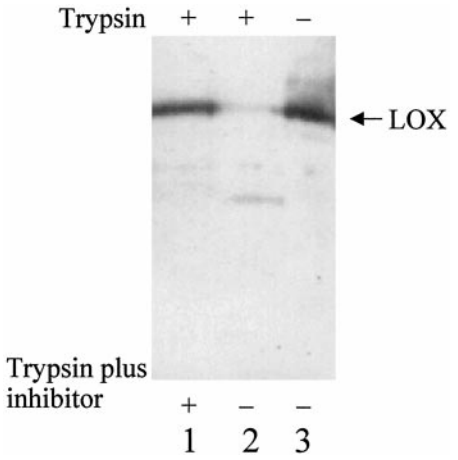

Figure 2. SDS gel immunoblot illustrating LOXs in oil bodies from 4-d-germinated cv Century cotyledons after trypsin treatment. Lane 1, Trypsin plus trypsin inhibitor; lane 2, trypsin alone; and lane 3, no treatment (control). Thirty micrograms of protein was loaded for each lane. Soybean polyclonal LOX1, -2, and -3 antibodies were used to probe the membranes.

germinated cv L0 had no LOX activity and protein supports the idea that soybean LOX5 is not associated with oil bodies. The purified oil bodies appeared to be intact, as indicated by light microscopy, and were uniformly approximately 0.3 to 0.5 μm in diameter (data not shown).

LOX Is Not Deeply Embedded in Oil-Body Membranes

After having obtained the data described above indicating that oil bodies purified by discontinuous Suc gradient centrifugation had little LOX activity at pH 9.0 and only a small amount of LOX1 protein, we decided to determine whether this LOX was localized on or in oil bodies. The purified oil bodies from 4-d-germinated cv Century cotyledons were incubated with trypsin or trypsin plus trypsin inhibitor for 30 min. LOX in purified oil bodies was cleaved by trypsin (Fig. 2, lane 2) but not by trypsin plus trypsin inhibitor (Fig. 2, lane 1). In addition, oil-body preparations washed with 0.5 M NaCl and 0.1 M $NaHCO_3$, according to the method of Herman (1987), had no detectable LOX in germinated cv Century cotyledons (data not shown). Taken together, the fractionation studies indicated that no embryo or seedling LOX was truly associated with oil bodies in germinating soybean seedlings.

Soluble LOX from cv Century Can Adhere to Oil Bodies from cv L0

Discontinuous Suc gradient fractions from cv Century dry seeds were made as described above, and the oil-body fraction was removed. The 15% Suc layer containing soluble LOX was saved. Five grams of cv L0 seeds was thoroughly ground in extraction buffer containing either 7.5 or 15 mL of the previously prepared cv Century Suc fraction containing high levels of soluble LOX (total LOX activity at pH 9.0 in 7.5 mL of 15% Suc supernatant from 30 mL per 10 g of cv Century dry seeds was the same as that from 15 mL per 15 g of 4-d-germinated cotyledon extracts). The result of mixing oil bodies from cv L0 and cv Century soluble LOX with subsequent purification of the oil bodies away from the soluble LOX (Fig. 3, lanes 2 and 3) indicated that LOX could artifactually adhere to the oil bodies. Purified oil bodies from 4-d-germinated cotyledons (Fig. 3, lane 4) or dry seed extracts (Fig. 3, lane 5) of cv L0 showed no detectable LOX. The proportion of LOX adhering to oil bodies depended on the total LOX amount added to the L0 preparation. The oil

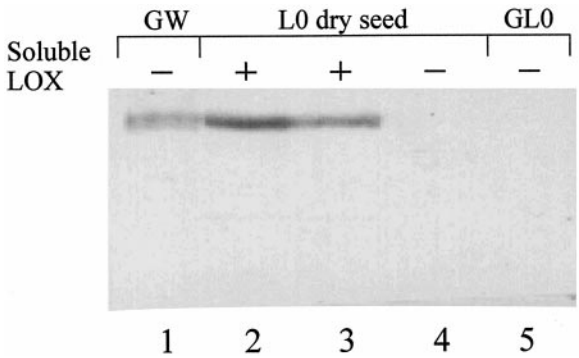

Figure 3. SDS-PAGE gel immunoblot illustrating that soluble LOXs from cv Century seeds can adhere to the oil bodies of cv L0. Oil bodies were removed from the cv Century dry seeds by centrifugation. An aliquot of the soluble LOX fraction from cv Century was added to cv L0 seed powder, and oil bodies were isolated. Lane 1, Protein of oil bodies purified from 4-d-germinated cv Century; lane 2, protein of oil bodies purified from cv L0 dry seeds plus 15 mL of the cv Century soluble fraction; lane 3, protein of oil bodies purified from cv L0 dry seeds plus 7.5 mL of the cv Century soluble fraction; lane 4, oil body protein from cv L0 dry seeds (no added soluble LOX); and lane 5, oil body protein from 4-d-germinated cv L0 seedling cotyledons. GW, Four-day-germinated cv Century; GL0, 4-d-germinated cv L0.

bodies from cv L0 dry seeds plus 15 mL of the cv Century soluble LOX fraction had more LOX protein than those from cv L0 plus 7.5 mL (Fig. 3, lanes 2 and 3).

Localization of LOX in Germinated Soybean Cotyledons by Immunocytochemistry

The fractionation studies indicating that LOXs are not normally directly associated with soybean oil bodies raise some interesting questions concerning the subcellular localization of the embryo LOXs (LOX1, -2, and -3) and the seedling LOXs (LOX4, -5, and -6). Although Vernooy-Gerritsen et al. (1984) and Song et al. (1990) both reported intracellular localization of LOX1 and -2 in germinating soybean seeds, little was known concerning seedling LOX4, -5, and -6. To address this issue, LOX wild-type cv Century and cv L0 soaked seeds and germinated seedling cotyledons were used for immunolocalization experiments. Germinating cv Century has at least six LOX isozymes (LOX1, -2, -3, -4, -5, and -6), but cv L0 has only seedling LOXs, of which LOX4 is the most abundant (Kato et al., 1992; Wang et al., 1995). Figure 4A shows a preimmune control of 4-d-germinated cv Century cotyledons with no label found in this treatment. Figure 4B demonstrates that an antibody to one of the oleosins (mp24, a 24-kD oil-body membrane protein; Herman, 1987) was clearly able to label oil-body membranes. These results functioned as negative (Fig. 4A) and positive (Fig. 4B) controls, confirming that the procedure was successful in the preservation of the antigenicity of cell proteins and the ultrastructure of soybean tissues. In cv Century cotyledons that had soaked for 12 h in which LOX1, -2, and -3 were present, the oil bodies and protein-storage vacuoles filled most of the cytoplasm in the cell. Using the LOX antibody, immunogold label mostly appeared in the cytoplasm of parenchyma cells, with some label present in protein-storage vacuoles (Fig. 4C). No spe-

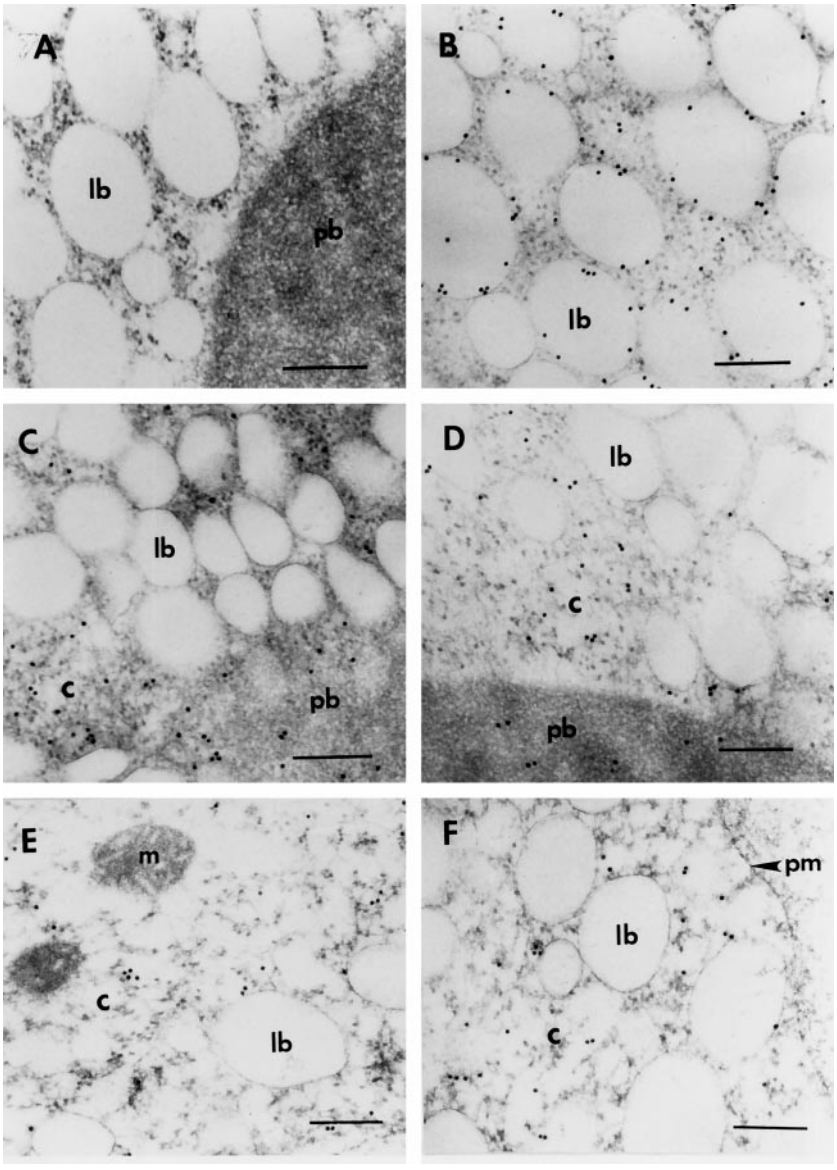

Figure 4. Electron micrographs showing immunogold labeling of LOX in a parenchyma cell of cv Century cotyledons. A, B, D, E, and F, Four-day-germinated cv Century cotyledons; C, 12-h-soaked cv Century cotyledons. A, Preimmune control; bar = 0.3 μm. B, Section cross-reacting with an mp24 antibody, which probes an oil-body membrane protein (Herman, 1987); bar = 0.25 μm. C, D, E, and F, Sections cross-reacting with soybean LOX1, -2, and -3 antibodies. C, ×60,000; bar = 0.25 μm. D, E, and F, ×55,000; bars = 0.25 μm. c, Cytoplasm; lb, oil body; pb, protein-storage vacuoles; m, mitochondrion; pm, plasma membrane.

cific label was found in oil bodies, mitochondria, or cell walls. In 4-d-germinated seedling cotyledons of cv Century, oil bodies and protein-storage vacuoles were less predominant in the storage parenchyma cells. Immunogold label was still found in the cytoplasm (Fig. 4, D–F) of parenchyma cells, but no specific label was seen in oil bodies, mitochondria (Fig. 4E), plasma membranes, or cell walls (Fig. 4F). In parenchyma cells of 4-d-germinated cv L0 cotyledons, LOX was observed in the cytoplasm at a lower concentration compared with parenchyma cells of cv Century (Figs. 4D and 5B). In epidermal cells of germinated cv Century and cv L0, LOX appeared in both the cytoplasm and the vacuoles (Fig. 5, C and D). Figure 5A shows a preimmune control in which no immunogold label was found.

The fact that no label was found in the oil bodies of 12-h-soaked and 4-d-germinated cotyledons in cv Century and cv L0 indicated that embryo and seedling LOXs were not directly associated with oil bodies. These findings are consistent with our subcellular fractionation results.

Our investigation was focused mainly on whether embryo or seedling LOX was associated with oil bodies. It is impossible to determine the location of each LOX isozyme with our experimental design because we used the mixture of soybean seed LOX1, -2, and -3 antibodies, and the individual LOX antibodies show some cross-reaction with other LOX isozymes. The multiple localizations of embryo and seedling LOXs are probably attributable to the different targeting of the individual LOX isozymes. Monospecific antibodies would be needed to resolve this issue.

Changes of Lipid Levels during Germination

Our investigation showed that none of the LOXs (LOX1 to LOX6) were associated with soybean oil bodies. Failure to find LOXs in oil bodies does not exclude the possibility that a LOX plays a direct role in lipid degradation during germination. Therefore, changes in lipids during germination were also investigated (Fig. 6). The levels of total lipids and TAG in cvs Century and L0 decreased rapidly d 3 after germination. About 80% of TAG and total lipids in cvs Century and L0 was consumed by d 9 after germination. There were no differences in the rates of degradation of total lipids and TAG between cvs Century and L0 during germination. Feussner et al. (1997) observed that about 15% of polyunsaturated fatty acids in the storage lipids in 4-d-germinated cucumber were present as their corresponding hydroperoxy derivatives as a result of the storage lipids being deoxygenated by LOX. Assuming that this is also true in germinating soybean, the fatty acid mole composition in TAG, such as 18:2 and 18:3 (LOX substrates; 18:2 is the most abundant fatty acid in TAG), would be expected to change during soybean seed germination. The fatty acid mole composition is shown in Table II. The results show that the percentages of 16:0 (palmitate), 18:1 (oleate), and 18:3 (linolenate) in TAG decreased slightly but that the percentages of 18:0 (stearate) and 18:2 increased slightly in cvs Century and L0 during germination. These results indicate that significant oxygenation of polyunsaturated fatty acids in storage lipids of germinating cucumber does not occur in germinating soybean seeds. The changes in fatty acid mole percentages in TAG are similar to those of total lipids in cvs Century and L0 during germination, except

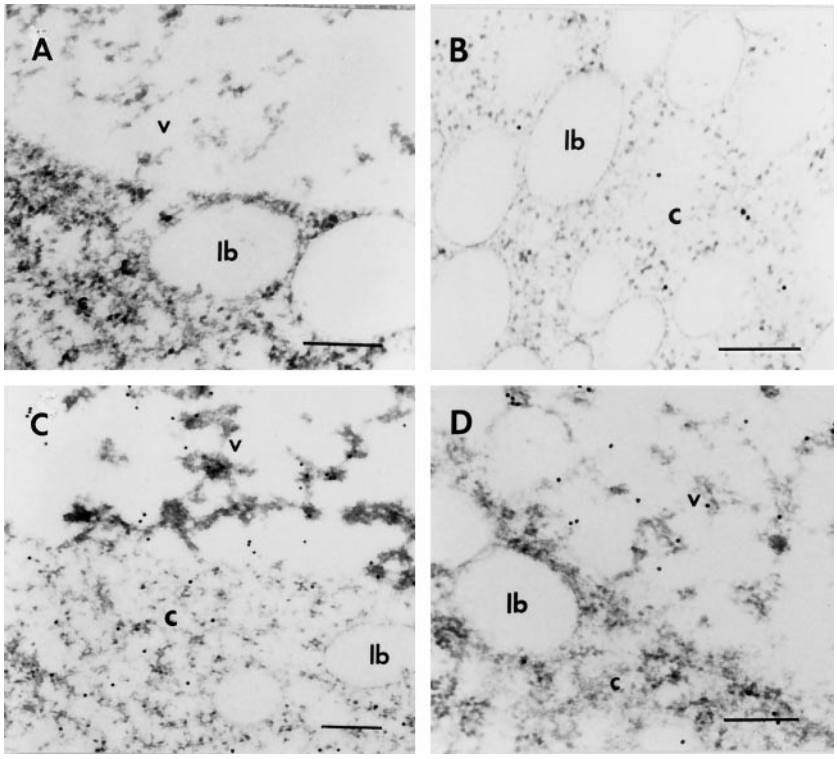

Figure 5. Electron micrographs showing LOX in epidermal and parenchyma cells of cvs L0 and Century. A, Four-day-germinated cv Century, preimmune control; bar = 0.25 μm. B, Section of a parenchyma cell from 4-d-germinated cv L0 cotyledons cross-reacting with soybean LOX antibodies; bar = 0.25 μm. C and D, Sections of epidermal cells from 4-d-germinated soybean cotyledons cross-reacting with soybean LOX antibodies. C, cv Century; bar = 0.25 μm. D, cv L0; bar = 0.25 μm. c, Cytoplasm; lb, oil body; v, vacuole.

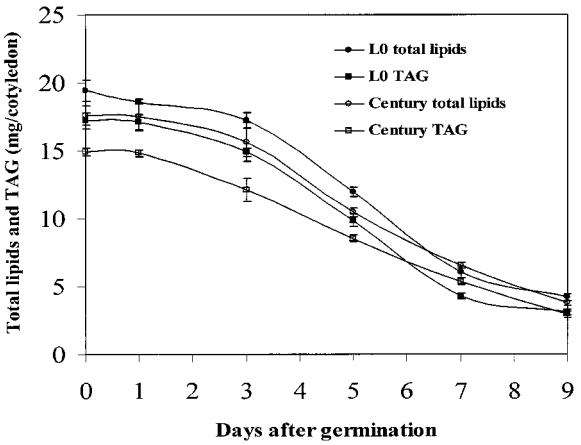

Figure 6. Changes in lipids in soybean cotyledons during germination. Total lipids were extracted with chloroform:methanol:formic acid (10:10:1, v/v) with 0.01% BHT. TAGs were separated by a TLC plate, which was developed in hexane:ethyl ether:acetic acid (50:50:1, v/v) with 0.01% BHT. TAG-17:0 was used as an internal standard. Values are means ± SE. Data were collected from at least three separate experiments, each having at least three duplicates ($n \geq 9$).

that 18:3 in total lipids increased after 7 d of germination (data not shown). These results are consistent with previous observations by Joshi et al. (1973) and Harwood (1975) in germinating soybean LOX wild-type seeds.

DISCUSSION

Soybean LOXs (LOX1 to LOX6) Are Vacuolar and Cytosolic but Not Associated with Oil Bodies

Evidence from fractionation and immunogold labeling demonstrated that embryo and seedling LOXs were not present in oil bodies of soaked soybean seeds or 4-d-germinated LOX wild-type and mutant soybean seedlings. The susceptibility of oil-body LOX to proteinase digestion and the absorption of LOX from cv Century to oil bodies of a cv L0 mutant indicate that the LOX present in oil bodies separated by discontinuous Suc gradients could be an artifact of the purification process. The facts that seedling oil bodies washed with 0.5 M NaCl and 0.1 M $NaHCO_3$ had no detectable LOX and that oil bodies washed by discontinuous Suc gradients had very little activity (<0.01% of total LOX activity) further support these observations. Early research by Siedow and Girvin (1980) also indicated that mitochondrial membranes could adsorb LOX during purification of mitochondria.

Comparison of gold label between 12-h-soaked and 4-d-germinated cv Century cotyledons (Fig. 4, C and D) showed that soybean seed LOX1, -2, and -3 were localized mainly in the cytoplasm and in the protein-storage vacuoles. Furthermore, we observed that the density of gold label in vacuoles of 4-d-germinated cv L0 was similar to that in 4-d-germinated cv Century cotyledons (Fig. 5, C and D). In contrast, much less gold label was found in the cytoplasm of 4-d-germinated cv L0 cotyledons compared with those of cv Century. Those observations indicate that vacuole-localized LOX in cvs Century and L0 is a seedling LOX(s), because only seedling LOXs are expressed in cv L0 cotyledons. In addition, label particles found in the cytoplasm of 4-d-germinated cv L0 indicate that at least one of the seedling LOXs is localized in the cytoplasm. A higher density of gold label in the cytoplasm of 4-d-germinated cv Century was attributable to the presence of both embryo and seedling LOXs, with the embryo LOXs being cytosolic.

Oil-body membrane proteins have been well studied in soybean. These so-called oleosins (18, 24, and 34 kD) are major oil-body membrane proteins in developing and mature seeds. That LOX or proteins of the molecular mass of LOX (approximately 95 kD) are not associated with oil-body membranes was also observed by Herman (1987) and Tzen et al. (1990). It is possible that amphipathic oleosins in the oil bodies bind the LOX. However, immunolabeling

Table II. *Changes of fatty acid mole composition in TAG during germination*

TAGs were separated by TLC after the lipids were extracted; values are means ± SE of three independent experiments.

Cultivar and Days after Germination	Fatty Acid				
	16:0	18:0	18:1	18:2	18:3
			%		
L0					
0	16.8 ± 1.8	4.9 ± 0.2	23.4 ± 0.4	46.0 ± 1.0	8.9 ± 0.6
1	17.1 ± 1.7	4.5 ± 0.2	22.5 ± 0.6	46.8 ± 1.1	9.0 ± 0.9
3	16.5 ± 1.5	4.8 ± 0.2	22.0 ± 1.4	47.7 ± 1.6	9.1 ± 0.7
5	16.1 ± 1.4	4.8 ± 0.4	21.9 ± 0.8	48.3 ± 1.6	8.9 ± 1.2
7	15.9 ± 1.4	5.4 ± 0.2	21.7 ± 0.5	48.5 ± 1.3	8.6 ± 0.5
9	15.4 ± 1.6	5.5 ± 0.5	21.9 ± 0.9	48.9 ± 1.6	8.3 ± 0.8
Century					
0	14.8 ± 1.0	4.7 ± 0.1	25.3 ± 0.5	49.0 ± 0.2	6.2 ± 0.5
1	14.7 ± 1.0	4.6 ± 0.1	25.3 ± 0.8	48.9 ± 1.6	6.6 ± 0.3
3	14.5 ± 1.3	4.8 ± 0.2	26.3 ± 1.7	48.1 ± 0.9	6.2 ± 0.2
5	14.6 ± 1.2	4.9 ± 0.2	25.0 ± 1.1	49.0 ± 1.5	6.4 ± 1.0
7	14.2 ± 1.3	5.2 ± 0.1	25.7 ± 0.4	49.3 ± 1.0	5.6 ± 0.4
9	13.9 ± 1.5	5.2 ± 0.1	24.9 ± 0.6	50.2 ± 1.3	5.8 ± 0.7

indicated that no LOX was associated with or surrounded the oil bodies.

LOX1 to LOX6 Are Not Directly Involved in TAG Mobilization during Soybean Germination

There are a number of early reports of lipid mobilization during soybean germination (Lin et al., 1982; Yoshida, 1984). Although β-oxidation and the glyoxylate cycle are known to be involved in energy supply during soybean germination, the mobilization of storage TAG has not been well documented. Early studies by Lin et al. (1982) showed that glyoxysomal lipase is involved in the hydrolysis of storage TAG during soybean germination. Furthermore, the soybean glyoxysomal lipase was active toward trilinolein, dilinolein, and monolinolein but could not hydrolyze triolein, tristearin, or tripalmitin. Yoshida (1984) reported that TAG containing one or more saturated fatty acids was hydrolyzed slightly faster than other species. These data suggested that the mechanism of initial TAG hydrolysis might be different for different molecular species.

The recent suggestion that cucumber LOX is involved in the mobilization of storage TAG (Feussner et al., 1995, 1997) opens the possibility that soybean LOX may be active in the initial hydrolysis of storage TAG. Analysis of LOX substrates in vitro indicated that soybean seed LOXs could oxygenate biomembranes (Maccarrone et al., 1994) and trilinolein, dilinolein, and monolinolein (Zhuang et al., 1991). Similarly, Matsui and Kajiwara (1995) also observed that cucumber cotyledon LOX can oxygenate trilinolein in vitro. These data suggest that, although soybean embryo LOXs are localized in the cytoplasm, they might be involved in 18:2 degradation in vivo at an early stage of germination (about d 1–3). However, the facts that storage lipid degradation in the mutant cv L0 is similar to that in cv Century and that the soybean glyoxysomal lipase is active toward trilinolein, dilinolein, and monolinolein indicate that embryo LOXs are not essential in lipid mobilization during germination. That cv L0 is normal in development and growth further supports this finding.

This is the first report, to our knowledge, to compare lipid degradation between a soybean wild type and a triple-LOX mutant during germination. Grimes et al. (1992) reported that vacuole-localized seedling LOX(s) is responsive to methyl jasmonate and may serve as a temporary storage protein during soybean germination. In addition, our results do not support the cytoplasm-localized seedling LOX being involved in lipid degradation. First, the cytoplasm-localized seedling LOX was at its lowest concentration in the vicinity of oil bodies (Fig. 5, B–D). Second, the mole percentages of 18:2 and 18:3 (LOX substrates) in TAG did not show a decrease up to d 3 after germination, whereas TAG decreased dramatically (Table II; Fig. 6). In another study, Feussner et al. (1995) observed that the hydroxylinoleic acid:linoleic acid ratios in neutral lipids and phospholipids increased significantly during cucumber germination as a result of the lipids being oxygenated by LOX. The ratios can reach about 0.17 in neutral lipids and 0.55 in phospholipids in 4-d-germinated cucumber. However, the ratio in the oil bodies from soybean dry seeds was only 0.055 and decreased to 0.028 at d 4 after germination, at which time soybean seedling LOX protein and activity had reached high levels. Furthermore, high levels of a hydroperoxy derivative (LOX product) of linoleic acid were seen in oil bodies in cucumber (Feussner et al., 1997) but not in soybean (Feussner et al., 1995). These data are consistent with our observations. Vick and Zimmerman (1982) observed that when plant tissues were homogenized in a solvent without previous enzyme inactivation, much higher concentrations of oxygenated fatty acids could be detected. It is important that during lipid analysis lipid autooxidation and enzyme activity such as LOX activity should be inhibited or prevented. It is not known if soybean or other seedling storage lipids would contain hydroperoxylinoleic acid when such precautions were taken.

Possible Roles of LOXs in Seedlings

The physiological role of seed (embryo) and seedling LOXs is still unclear. A partial reason for this is the ambiguous or conflicting results of LOX subcellular localization studies. Our results clearly show that soybean embryo and seedling LOXs are localized in the cytosol and the vacuoles. These data further support the finding that LOX1 to LOX6 are soluble proteins. Previous studies (Vernooy-Gerristen et al., 1984; Grimes et al., 1992) also found LOXs in soybean seedling cotyledons to exist in both the cytosol and the vacuoles. Labeling was much higher in epidermal cells than in storage-parenchyma cells, which is inconsistent with a role in TAG mobilization. The observation that embryo LOXs are not involved in lipid mobilization, coupled with cv L0 being normal in all developmental stages, including germination, further supports earlier speculation that soybean embryo LOXs may function as seed storage proteins (Siedow, 1991). On the other hand, it cannot be ruled out that seed LOXs might be involved in pathogen and/or insect defense through jasmonic acid and/or other oxylipin biosynthesis during seed development. Soybean seedling LOXs are present in the cotyledons and hypocotyls of wild type and all seed LOX mutants, including LOX single, double, and triple null mutants (Park and Polacco, 1989; Kato et al., 1992; C. Wang and D.F. Hildebrand, unpublished data). What role(s) seedling LOX might play in the germinating seedlings is still not understood. The presence of seedling LOX in vacuoles supports a hypothesis that LOX is sequestered from its substrate until cells are damaged, at which time a fatty acid peroxidation cascade is initiated that can lead to enhanced pest defense.

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