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The *LOX1* Gene of *Arabidopsis* Is Temporally and Spatially Regulated in Germinating Seedlings[1]

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We examined the temporal and spatial expression patterns of the *LOX1* gene during the development of *Arabidopsis thaliana* seedlings. Measurements of steady-state *LOX1* mRNA levels indicated that this gene is transiently expressed during germination. *LOX1* mRNA was not detected in seed that had imbibed (T_0) but reached a maximum level by 1 d in both light- and dark-grown seedlings. The induction of the *LOX1* gene was not light dependent; however, mRNA levels were 4-fold greater in light-grown seedlings. Immunoblot analysis of lipoxygenase protein levels and measurements of enzyme activity suggested that the induction of the *LOX1* gene resulted in the production of functional lipoxygenase enzyme. Lipoxygenase protein was not present in dry seed or seed that had imbibed, but was first detected by immunoblot analysis after 1 and 2 d of growth in the light and dark, respectively. In both cases, lipoxygenase protein levels remained high for 2 d and then declined. Lipoxygenase activity paralleled the changes in protein levels. In situ hybridization studies revealed that the *LOX1* gene is transiently expressed in the epidermis and the aleurone layer during germination. *LOX1* mRNA levels were particularly high in the epidermis of the radicle and the adaxial side of the cotyledons. These results suggest that the *LOX1* gene product is produced specifically during early germination and plays a role in the functioning of the epidermis.

Lipoxygenases catalyze the hydroperoxidation of unsaturated fatty acids such as linoleic and linolenic acids, which contain a pentadiene double bond system (see Mack et al., 1986; Vick and Zimmerman, 1987; Hildebrand et al., 1988; Siedow, 1991, for reviews). The primary products of the lipoxygenase reaction are typically metabolized into molecules of known or suspected regulatory activity. In mammals, lipoxygenase-derived fatty acid hydroperoxides are precursors for the synthesis of a number of eicosanoids (e.g. prostaglandins, prostacyclins, leukotrienes, lipoxins, and thromboxans) that regulate specific cellular responses (Anderson, 1989). In higher plants, two major pathways have been described for the metabolism of fatty acid hydroperoxides. They are known collectively as the "lipoxygenase pathway" (Vick and Zimmerman, 1987). One branch of the lipoxygenase pathway produces traumatic acid, a compound that may be involved in wound responses in plant cells (Zimmerman and Coudron, 1979), as well as C_6 volatiles such as *trans*-2-hexenal, which is antimicrobial and may play a role in pathogen defense (Croft et al., 1993). The second branch produces jasmonic acid, a molecule likely to serve a regulatory role in plant cells (Staswick, 1992; Sembdner and Parthier, 1993). At physiological concentrations jasmonates promote seed germination (Berestetzky et al., 1991; Ranjan and Lewak, 1992) and stimulate cell division (Ravnikar et al., 1992). In addition, it has also been suggested that jasmonates are involved in a wound- and pathogen-induced signal-transduction cascade (Farmer and Ryan, 1992).

The in vivo functions of lipoxygenase in higher plants are not clear. However, evidence has accumulated that indicates that plant lipoxygenases may play an important role in a variety of processes, including stress responses (Bell and Mullet, 1991, 1993), pathogen defense (Croft et al., 1993; Melan et al., 1993), senescence, and the regulation of growth and development (Hildebrand et al., 1988; Siedow, 1991). Unfortunately, it has been difficult to determine the exact function of lipoxygenase in these processes, in part because the plants examined to date have large lipoxygenase gene families that encode a number of lipoxygenase isozymes (Siedow, 1991). In the hope of finding a more simple system for studying the physiological functions of lipoxygenase in higher plants, we initiated a molecular genetic and biochemical analysis of the lipoxygenases of *Arabidopsis thaliana*. A similar approach has been undertaken by Bell and Mullet (1993). All studies to date indicate that *Arabidopsis* contains a very simple lipoxygenase gene family. Only two members have been identified. We previously identified the *LOX1* gene of *Arabidopsis* (Melan et al., 1993) and Bell and Mullet (1993) have identified a second lipoxygenase gene in *Arabidopsis*, *AtLox2*, which is distinct in sequence and expression patterns from *LOX1*.

We are interested in the function of lipoxygenases during germination and early development. Increases in lipoxygenase activity during germination have been reported for a number of plant species, including soybean (Holman, 1948; Hildebrand and Hymowitz, 1983; Park and Polacco, 1989; Kato et al., 1992), wheat (Guss et al., 1968), pea (Anstis and Friend, 1974), barley (Yabuuchi, 1976; Doderer et al., 1992), rice (Ohta et al., 1986), cucumber (Matsui et al., 1988, 1992), lupine (Beneytout et al., 1988), and *Vicia sativa* (Andrianarison and Beneytout, 1992). For rice (Ohta et al., 1986), cucumber (Matsui et al., 1992), and soybean (Park and Polacco, 1989; Kato et al., 1992; Park et al., 1994), evidence consistent with the de novo synthesis of lipoxygenases during germination has also been reported. In rice, the increase in lipoxygenase activity during germination is inhibited by cyclohexamide (Ohta et al., 1986), whereas in cucumber cotyledons the increase in activity has been correlated with an

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increase in lipoxygenase protein as determined by immunoblotting (Matsui et al., 1992). In soybean, Park and Polacco (1989) have shown that at least two lipoxygenase isozymes, with isoelectric points distinct from the well-characterized seed lipoxygenase isozymes, appear in the hypocotyl/radicle axis during germination. Results from protein-labeling experiments suggested that these axis lipoxygenase species are newly synthesized during germination. Park et al. (1994) have also recently characterized two soybean lipoxygenase cDNA clones that are expressed in the axis during germination and are likely to encode the germination-associated lipoxygenase isozymes. In the cotyledons of germinating soybeans, Kato et al. (1992) have identified three new lipoxygenase isozymes. All of these can be distinguished from the seed isozymes by ion-exchange chromatography, and the partial protein sequence of one (L-4) is distinct from the seed isozyme sequences.

In the studies presented here, we have examined the expression of the *LOX1* gene of *Arabidopsis* during the germination and early development of light- and dark-grown seedlings. These results indicate that the *LOX1* gene is under strict temporal and spatial control during germination. The *LOX1* gene is transiently expressed in the epidermis during the 1st d of development, the time during which the radicle emerges. We have also shown that the induction of the *LOX1* gene is correlated with the transient accumulation of functional lipoxygenase enzyme. These results suggest that the *LOX1* gene product is important for a function of the epidermis at a precise time during development and provide the background required for "antisense" experiments, which will directly test this hypothesis.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana ecotype Columbia seedlings were wet with 0.002% (v/v) Tween 20 and then surface sterilized by washing with 70% (v/v) ethanol for 1 min followed by two washes with sterile water and a 10-min treatment in 30% (v/v) bleach. The sterilized seeds were then extensively washed with sterile water, suspended in 1 mL of 0.1% (w/v) agarose/2500 seeds, and plated on defined mineral nutrient medium containing 0.6% (w/v) agarose (Estelle and Somerville, 1987) on 100 × 20 mm Petri plates. Approximately 2500 seeds were plated on each plate. The plates were covered and sealed with filter tape (Carolina Biological Supply, Burlington, NC) to allow for gas exchange and then incubated in the light at 4°C for 72 h to break dormancy and synchronize germination. T_0 samples were collected after the 4°C incubation. The plates were then transferred to a 24°C chamber and grown under continuous illumination (200–300 $\mu E\ m^{-2}\ s^{-1}$) with a combination of fluorescent and incandescent lamps or under continuous darkness. Samples were collected at T_0 and at various times thereafter and frozen in liquid N_2. Samples were ground to a powder in liquid N_2 with a mortar and pestle and stored at −80°C.

RNA Isolation and RNA Gel-Blot Analysis

Total RNA was isolated by phenol-SDS extraction and LiCl precipitation (Ausubel et al., 1987). RNA samples (10 μg) were separated by electrophoresis on formaldehyde-agarose gels (Ausubel et al., 1987), transferred to nylon filters (Biotrans, ICN, Irvine, CA) by capillary transfer in 25 mM sodium phosphate, pH 6.5, and cross-linked to the filter by UV irradiation (200 $\mu W\ cm^{-2}$ for 12–15 min). A parallel set of samples were run and stained with 1 μg/mL of ethidium bromide to verify even loading of samples. The filters were prehybridized for 1 to 2 h and then hybridized overnight at 65°C with the full-length *LOX1* cDNA clone (Melan et al., 1993) labeled with [α-^{32}P]dCTP by random priming (Multiprime Kit, Amersham, Arlington Heights, IL). This probe does not hybridize with the *AtLox2* gene under these conditions. The filters were washed twice at room temperature in 0.5% (w/v) BSA, 1 mM EDTA, 80 mM sodium phosphate (pH 7.2), and 5% (w/v) SDS followed by four 15-min washes at 65°C in 1 mM EDTA, 80 mM sodium phosphate (pH 7.2), and 1% (w/v) SDS (Church and Gilbert, 1984). The damp filters were exposed to Kodak X-AR5 film (Eastman Kodak, Rochester, NY) at −80°C with a Cronex intensifying screen (Dupont).

Protein Extraction and Immunoblot Analysis

Soluble protein extracts were prepared at 4°C by homogenization of frozen tissue powders in 0.1 M sodium phosphate (pH 6.5) and 1% (w/v) polyvinylpolypyrrolidone. The homogenates were clarified by centrifuging twice at 13,000*g* for 15 min each. An aliquot of the supernatant was used for determination of the total protein concentration using the method of Bradford (1976) with BSA as a standard. For the preparation of gel samples, aliquots of the resulting supernatant were immediately mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-Cl, pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 0.02% [w/v] bromphenol blue) and frozen in liquid N_2. The soluble proteins were resolved by SDS-PAGE (Laemmli, 1970) and then transferred electrophoretically from the gels to Immobilon-P polyvinylidene difluoride filters (Millipore, Bedford, MA). The blots were blocked by incubation for 1 to 16 h in 10% (w/v) powdered nonfat dry milk dissolved in TBS-T (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% [v/v] Tween 20). The filters were washed three times for 10 min each in TBS-T and then incubated for 1 h with a 1:10,000 dilution of an *Arabidopsis* lipoxygenase antibody in TBS-T containing 5% (w/v) powdered nonfat dry milk. The antibody had been raised against a lipoxygenase-maltose binding fusion protein produced in *Escherichia coli* (Peterman et al., 1994). The filters were then washed three times for 10 min each in TBS-T with 5% (w/v) powdered nonfat dry milk and incubated for 1 h with a 1:1000 dilution of a donkey anti-rabbit horseradish peroxidase secondary antibody. The filters were again washed three times for 10 min each in TBS-T with 5% (w/v) powdered nonfat dry milk. The resulting immune complexes were detected with the ECL immunodetection system (Amersham) according to the manufacturer's instructions.

Lipoxygenase Activity Assays

Soluble protein extracts were prepared as described above except that the extraction buffer contained 0.1 M sodium

phosphate (pH 6.5), 1% (w/v) polyvinylpolypyrrolidone, 1% (v/v) Tween 20, and 10 mM EDTA, and the BCA protein assay (Pierce) was used to determine the total protein concentration. The extracts were not frozen but rather used immediately for enzyme-activity assays. Lipoxygenase activity was measured polarographically in a 2.0-mL reaction vessel fitted with a Clark-type O_2 electrode. The assays were performed at 25°C in 0.1 M sodium phosphate, pH 6.5, and initiated by the addition of 1.3 mM linoleic acid emulsified in Tween-20 according to the method of Surrey (1964).

Tissue Preparation and in Situ Hybridization

Seedlings were fixed for 24 h at room temperature with 4% (w/v) paraformaldehyde in 50 mM Pipes, pH 7.2, containing 2 mM $CaCl_2$, dehydrated through an ethanol series, cleared in xylenes, and embedded in paraffin (TissuePrep, Fisher Scientific). Sections (7 μm thick) were mounted onto slides coated with 100 μg/mL poly-L-Lys (Sigma). After deparaffinization and rehydration, the tissue sections were treated for 30 min with 1 μg/mL proteinase K (Boehringer-Mannheim) in 100 mM Tris-Cl, pH 7.5, 50 mM EDTA at 37°C and then acetylated (Hayashi, et al., 1978) with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine for 5 min at room temperature. Following dehydration through ethanol and air drying, the sections were hybridized overnight at 42°C with RNA probes prepared from a linearized Bluescript plasmid containing nucleotides 1258 to 2640 of the *LOX1* cDNA (λAtLox1–1; Melan et al., 1993). ^{35}S-labeled sense and antisense riboprobes were synthesized with either T3 or T7 RNA polymerase (RNA Transcription Kit, Stratagene). Probes were applied to the sections at an average concentration of 4×10^7 cpm/mL in a hybridization solution containing 50% (v/v) formamide, 0.3 M NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10% (w/v) dextran sulfate, 1× Denhardt's solution (0.02% [w/v] Ficoll, 0.02% [w/v] PVP, 0.02% [w/v] BSA), and 100 mM DTT. After hybridization, the slides were washed three times at room temperature in 4× SSPE (1× SSPE = 0.2 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), and 5 mM DTT, then treated for 30 min at 37°C with 25 μg/mL RNase in 500 mM NaCl, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA. The slides were subsequently washed three times at room temperature with 2× SSPE, 5 mM DTT and then washed three times at high stringency with 0.1× SSPE, 1 mM DTT at 57°C. Following dehydration through an ethanol series containing 300 mM ammonium acetate and air drying, the slides were coated with Kodak NTB2 autoradiography emulsion (Eastman Kodak) diluted 1:1 (v/v) with 600 mM ammonium acetate. The hybridized tissues were exposed to the emulsion for 17 d at 4°C and subsequently developed for 3 min in Kodak D-19 developer (Eastman Kodak). Tissue sections were stained with 0.025% (w/v) toluidine blue O and coverslips were mounted with Permount (Fisher Scientific). Bright- and dark-field microscopic observations were made using an Olympus Vanox microscope, and photographs were taken on Kodak TMax 400 film developed with Kodak D-76 developer (Eastman Kodak).

RESULTS

Induction of the *LOX1* Gene during Germination

The *LOX1* gene of *Arabidopsis* is induced upon bacterial pathogen attack and also by the phytohormones ABA and methyl jasmonate (Melan et al., 1993). To determine if this gene is also temporally regulated during early seedling development, *LOX1* steady-state mRNA levels were determined during development of light- and dark-grown plants. Seeds were plated on a defined mineral nutrient medium (Estelle and Somerville, 1987) without Suc and incubated at 4°C for 72 h to break dormancy. T_0 samples were collected after the 4°C incubation. The plants were then grown in either continuous light or dark at 24°C. *Arabidopsis* seedlings grown in this manner are shown in Figure 1. Under these conditions germination frequencies were approximately 100% and development of the majority of seedlings was synchronous.

LOX1 steady-state mRNA levels were determined by RNA gel-blot hybridization analysis of total RNA isolated from samples collected at T_0 and during the following 5 d (Fig. 2). Equivalent amounts of total RNA were loaded in each lane as determined by the ethidium bromide staining intensity of the rRNA bands. *LOX1* mRNA was not detected at T_0. *LOX1* steady-state mRNA levels increased dramatically during the 1st d of development of both light- and dark-grown plants. In light-grown plants *LOX1* mRNA was first detected at 12 h, whereas in dark-grown plants it was not detected until 1 d. In both light- and dark-grown seedlings *LOX1* mRNA levels were maximal at 1 d and then declined during subsequent days of development. Maximal *LOX1* mRNA levels in light-grown plants were approximately 4-fold greater than

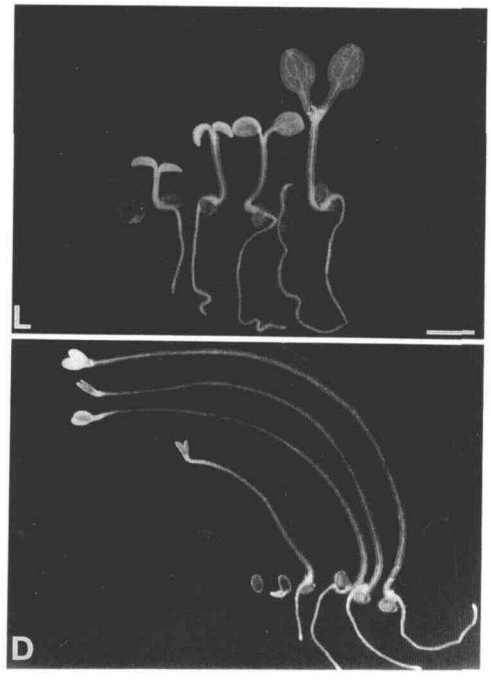

Figure 1. Early development of *A. thaliana* ecotype Columbia seedlings. Light- (L) and dark-grown (D) seedlings collected at T_0, 1, 2, 3, 4, and 5 d are shown from left to right. Scale bar = 3 mm.

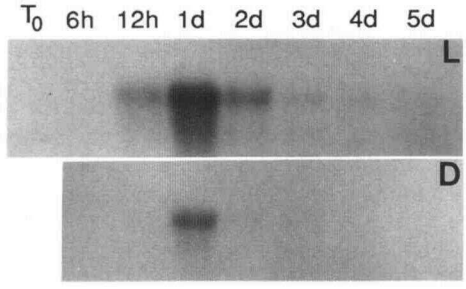

Figure 2. *LOX1* gene induction during *Arabidopsis* seedling development. Steady-state *LOX1* mRNA levels were determined by RNA gel-blot hybridization analysis. Total RNA was extracted, separated on formaldehyde gels, and blotted onto a nylon membrane. Equivalent amounts of RNA (10 μg), as determined by ethidium bromide staining of the ribosomal RNAs, were loaded for each time point. The membranes were probed with a *LOX1* cDNA fragment ^{32}P labeled by the hexanucleotide random-priming procedure.

those observed in dark-grown plants. The appearance of light- and dark-grown plants was identical for the 1st d (Fig. 1). After 12 h the seed appeared swollen by comparison to the T_0 seed, and within 1 d the radicles had emerged.

Lipoxygenase Protein and Enzymic Activity Levels Increase during Germination

Lipoxygenase protein (Fig. 3) and enzyme activity (Fig. 4) levels were measured to determine if the observed induction of the *LOX1* gene results in the increased production of functional lipoxygenase protein early in development and is thus physiologically relevant.

Lipoxygenase protein levels were measured by immunoblot analysis of total protein extracts. Protein extracts were prepared from light- and dark-grown seedlings collected at intervals over a 6-d time course. Protein samples were separated by 10% SDS-PAGE and then transferred electrophoretically from the gels to polyvinylidene difluoride membranes. The blots were probed with an *Arabidopsis* lipoxygenase antibody that had been raised against a lipoxygenase-maltose binding fusion protein produced in *E. coli*. The *Arabidopsis* lipoxygenase detected by this antibody is 98 kD (Peterman et al., 1994). This is in agreement with the size

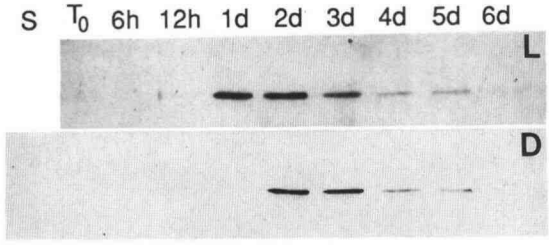

Figure 3. Accumulation of lipoxygenase protein during early development of light- and dark-grown *Arabidopsis* seedlings. Lipoxygenase protein levels, as a function of seedling age, were determined by immunoblot analysis of total protein extracts (5 μg/lane) from light- (L) and dark-grown (D) seedlings.

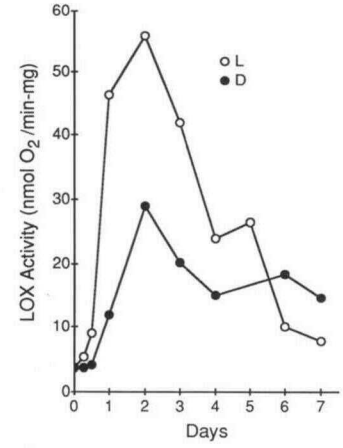

Figure 4. Lipoxygenase activity levels during early development of light- and dark-grown *Arabidopsis* seedlings. Lipoxygenase activity levels, as a function of seedling age, were measured using a Clark-type oxygen electrode in 0.1 M sodium phosphate, pH 6.5, with linoleic acid as substrate. The data shown are the average of three to four measurements.

of the *LOX1* gene product predicted from the full-length cDNA sequence (Melan et al., 1993). In the resulting immunoblots lipoxygenase protein was not detected in dry seed or at T_0 (Fig. 3). Lipoxygenase protein was first detected after 1 and 2 d of growth in the light and dark, respectively. In both light- and dark-grown seedlings the lipoxygenase protein levels remained high for 2 d and then declined during subsequent days of development.

Lipoxygenase enzyme activity levels were also measured during the early development of light- and dark-grown *Arabidopsis* seedlings. The results are shown in Figure 4. The lipoxygenase activity levels paralleled the levels of lipoxygenase protein detected by immunoblot analysis. In light-grown plants an approximately 13-fold increase in lipoxygenase activity, relative to that of T_0 seed, was observed on the 1st d of growth. Within 2 d a 15-fold increase in activity was observed. In dark-grown plants the increase in lipoxygenase activity was only 3-fold on the 1st d. Within 2 d of growth in the dark an 8-fold increase in activity was observed. In both light- and dark-grown plants, the maximal level of activity was observed after 2 d of growth. Lipoxygenase activity levels then declined during subsequent days of development.

After 2 d of growth, the time of maximal lipoxygenase enzyme activity, there were pronounced morphological differences between the light- and dark-grown seedlings (Fig. 1). In the light-grown seedlings the cotyledons were green and expanding and root growth and root-hair formation had begun. The dark-grown seedlings exhibited extensive hypocotyl elongation, with no cotyledon expansion or root-hair formation.

Spatial Expression of the *LOX1* Gene during Germination

In situ hybridization analysis of sectioned plant material was used to elucidate the spatial expression patterns of the

LOX1 gene during germination. Light- and dark-grown plant material, collected at T_0 and 1, 2, and 3 d, was fixed, embedded in paraffin, and cut into 7-μm sections. The sections were hybridized with either sense or antisense ^{35}S-labeled *LOX1* RNA probes and examined by bright- and dark-field microscopy.

Longitudinal (Figs. 5, E and F, and 6C) and cross-sections (Fig. 5, G and H) from seed collected at T_0 were hybridized with a *LOX1* antisense probe. No *LOX1* mRNA was detected in these sections, in agreement with the RNA gel-blot hybridization analysis (Fig. 2). Identical results were obtained with sections from seed collected at T_0 hybridized with a *LOX1* sense probe (data not shown).

Hybridization of longitudinal (Figs. 5, A and B, and 6A) and cross-sections (Figs. 5, C and D, and 6B) from 1-d-old light-grown seedlings, with an antisense probe, revealed that the *LOX1* gene is highly expressed in epidermal cells within the 1st d of development. In dark-field micrographs of both longitudinal (Fig. 5A) and cross-section (Fig. 5C) hybridization, signal was evident throughout the embryos but was most concentrated in the epidermal cells of the radicle and the adaxial side of the cotyledons. Hybridization was also seen in the aleurone layer, which is closely associated with the innermost cell layers of the testa.

The location of the *LOX1* mRNA in specific cell layers is most evident in the high-magnification bright-field micrographs shown in Figure 6. The hybridization signal in the epidermal cells of the adaxial surface of the cotyledons (c) is shown in Figure 6A. Hybridization of the probe with the epidermal cells of the radicle (r) and the abaxial surface of a cotyledon (c) is seen in Figure 6B. Hybridization signal over the aleurone layer of cells (a), a remnant of the endosperm (Vaughn and Whitehouse, 1971), is also shown in Figure 6B. Similar results were obtained with sections from 1-d-old dark-grown seedlings, with the exception that the concentration of hybridization signal was not as great as in the light-grown seedlings (data not shown). *LOX1* mRNA was not detected in sections from 2- and 3-d-old seedlings hybridized with the antisense probe (data not shown).

No hybridization signal was detected in sections from 1-d light-grown plants hybridized with the *LOX1* sense probe (Fig. 5, I–L). These results provide strong evidence that the hybridization signal detected in sections hybridized with the antisense probe (Figs. 5, A–D, and 6, A and B) was due to specific hybridization with the *LOX1* mRNA.

DISCUSSION

The *LOX1* gene was previously shown to be regulated by pathogen attack and the phytohormones ABA and jasmonic acid (Melan et al., 1993). The experiments presented here have demonstrated that the *LOX1* gene is also under precise temporal and spatial control during seed germination. Taken together, these results suggest that the *LOX1* gene product functions not only in plant stress responses but also in seed germination.

Expression of the *LOX1* Gene Is under Precise Developmental Control in Germinating *Arabidopsis* Seedlings

We have measured *LOX1* steady-state mRNA levels during the development of both light- and dark-grown *Arabidopsis* seedlings (Fig. 2). The *LOX1* gene was transiently expressed early in development. *LOX1* mRNA levels were maximal within the 1st d of development and then rapidly declined. These results indicate that expression of the *LOX1* gene is under very precise temporal control. Furthermore, these data suggest that the product of the *LOX1* gene functions during a precise time in development. Similarly in soybean, two seedling axis lipoxygenase transcripts were not detected in the seed but accumulated during germination. Unlike the *LOX1* mRNA, however, these transcripts continue to accumulate during early seedling development (Park et al., 1994).

The Induction of the *LOX1* Gene Is followed by an Increase in Functional Lipoxygenase Protein

The transient accumulation of the *LOX1* mRNA is correlated with an increase in enzymically active lipoxygenase protein. Measurements of lipoxygenase protein (Fig. 3) and enzyme activity (Fig. 4) levels revealed an increase in functional lipoxygenase protein in both light- and dark-grown *Arabidopsis* seedlings. It is likely that this increase in active lipoxygenase is due to the production of the *LOX1*-encoded protein. Of the two lipoxygenase genes identified in *Arabidopsis*, only the *LOX1* gene is highly expressed during germination. The *AtLox2* gene is expressed in seedlings following germination (Bell and Mullet, 1993) at levels at least 1 order of magnitude lower than the *LOX1* transcript (data not shown). These results suggest that the increase in *LOX1* mRNA results in the production of functional lipoxygenase enzyme and is thus physiologically significant.

Biochemical studies of the *LOX1*-encoded lipoxygenase will be essential for determining the role of this enzyme in germination. In mammalian cells lipoxygenase reaction products are precursors to a number of molecules that regulate specific cellular responses, and this is likely to be the case for plant cells as well (Siedow, 1991). Determination of the regiospecificity for hydroperoxidation of the germination-specific *Arabidopsis* lipoxygenase will provide information needed to determine the type of lipoxygenase metabolites that can possibly be produced. It will also be important to directly identify the lipoxygenase-derived metabolites that are produced in young seedlings. Such studies have provided a starting point for the characterization of the the germination-associated cotyledon lipoxygenases of soybean (Kato et al., 1992) and should provide valuable insights into the role of the *LOX1*-encoded lipoxygenase in germination.

Induction of the *LOX1* Gene Is Not Light Dependent

LOX1 mRNA and lipoxygenase protein and activity levels were determined in both light- and dark-grown plants because in *Sinapis alba*, a close relative of *Arabidopsis*, lipoxygenase synthesis was reported to be down-regulated by light and controlled by phytochrome in the cotyledons of young seedlings (Oelze-Karow and Mohr, 1976). In addition, there is evidence that the phytochrome system controls the expression of soybean lipoxygenase genes (Maccarrone et al., 1991). The induction of the *LOX1* gene and production of functional lipoxygenase protein was not down-regulated in the light. Rather, light-grown seedling *LOX1* mRNA levels were ap-

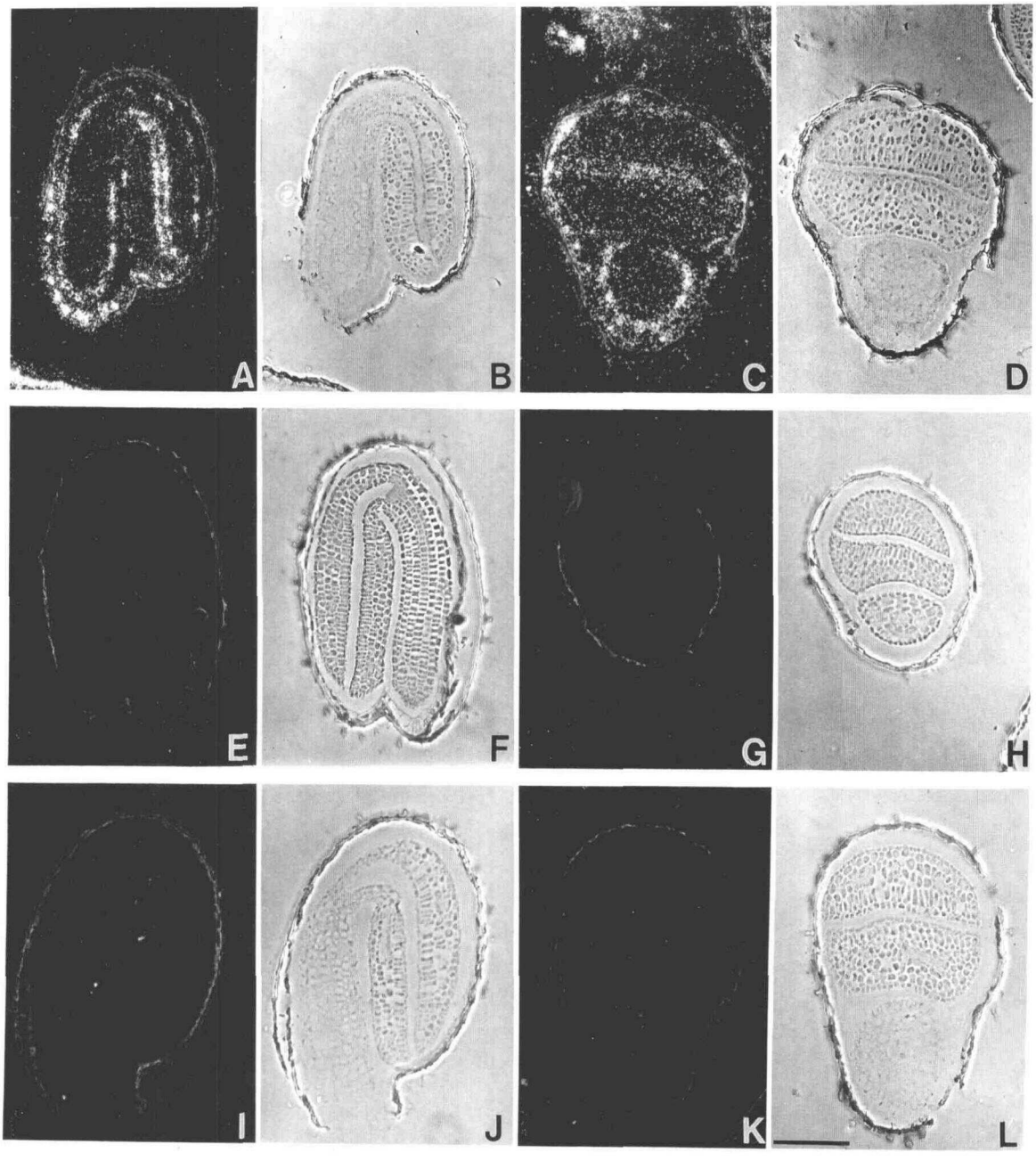

Figure 5. Localization of *LOX1* transcripts. A, Dark-field micrograph of a longitudinal section of a 1-d light-grown seedling after in situ hybridization with a *LOX1* antisense probe. B, Bright-field micrograph of the same section shown in A. C, Dark-field micrograph of a cross-section of a 1-d light-grown seedling after in situ hybridization with a *LOX1* antisense probe. D, Bright-field micrograph of the same section shown in C. E, Dark-field micrograph of a longitudinal section of a T_0 seed after in situ hybridization with a *LOX1* antisense probe. F, Bright-field micrograph of the same section shown in E. G, Dark-field micrograph of a cross-section of a T_0 seed after in situ hybridization with a *LOX1* antisense probe. H, Bright-field micrograph of the same section shown in G. I, Dark-field micrograph of a longitudinal section of a 1-d light-grown seedling after in situ hybridization with a *LOX1* sense probe. J, Bright-field micrograph of the same section shown in I. K, Dark-field micrograph of a cross-section of a 1-d light-grown seedling after in situ hybridization with a *LOX1* sense probe. L, Bright-field micrograph of the same section shown in K. Scale bar in L = 100 μM for all sections.

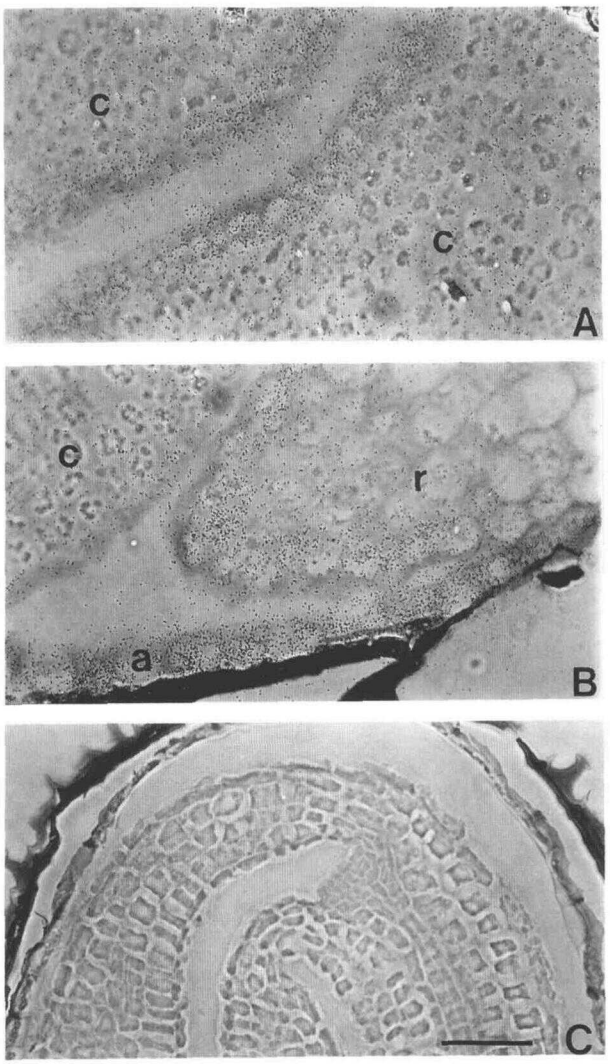

Figure 6. Localization of *LOX1* transcripts to specific cell layers. Bright-field micrographs of sections after in situ hybridization with a *LOX1* antisense probe. A, Longitudinal section of a 1-d light-grown seedling. Silver grains are most concentrated over the adaxial epidermis of the cotyledons (c). B, Cross-section of a 1-d light-grown seedling. The radicle (r), aleurone layer (a), and abaxial surface of a cotyledon (c) are shown. Silver grains are most concentrated over the radicle (r) epidermis and the aleurone cell layer (a). C, Longitudinal section of a T_0 seed. No silver grains are seen in seedlings at this stage of germination. Scale bar in C = 20 μM for all sections.

proximately 4-fold greater than those in dark-grown seedlings. In addition, the *LOX1* mRNA was detected 12 h earlier in the light-grown seedlings. Similarly, lipoxygenase protein and activity levels increased earlier and to higher levels in light-grown plants. It will be interesting to determine the reasons for these differences. It is possible that light enhances expression of the *LOX1* gene directly. Alternatively, light may exert an influence in a more indirect way by its photomorphogenetic effects. Although 1-d-old light- and dark-grown seedlings were indistinguishable, by 2 d the morphogenetic effects of light were pronounced.

The *LOX1* Gene Is Transiently Expressed in the Epidermis during Germination

The spatial distribution of the *LOX1* mRNA was examined by in situ hybridization. The results demonstrated that the *LOX1* gene is expressed transiently in the epidermis and in the aleurone layer during germination. No *LOX1* mRNA was detected in seeds that had imbibed (T_0). However, within 1 d the *LOX1* mRNA was found at high levels in the epidermis, especially of the radicle and the adaxial side of the cotyledons. The *LOX1* gene was also highly expressed in the aleurone layer, which is intimately associated with the testa (Figs. 5 and 6). A low level of expression was detected throughout the embryo. In sections of 2- and 3-d-old seedlings no *LOX1* mRNA was detected. This is the first localization of a specific plant lipoxygenase mRNA.

Several immunolocalization studies of soybean lipoxygenase proteins have been reported (Vernooy-Gerritsen et al., 1983; Tranbarger et al., 1991; Grimes et al., 1992). However, multiple lipoxygenase isozymes are present in soybeans and it is likely that the antibodies used for all of these studies cross-reacted with a number of these species. Vernooy-Gerritsen et al. (1983) immunolocalized lipoxygenase proteins in germinating soybean seeds. They found a high signal throughout the cotyledons during the 1st d of germination due to the seed lipoxygenases. Within 3 d lipoxygenase protein was detected in the abaxial hypodermis, the vascular bundle sheaths, and the epidermis. Tranbarger et al. (1991) showed that in soybean leaves lipoxygenase proteins were localized primarily in the vacuoles of paraveinal mesophyll cells. A much lower level of lipoxygenase was also detected in the epidermal cells. In methyl jasmonate-treated soybean seedlings lipoxygenase proteins accumulated in the shoot tips, primary leaves, epicotyls, hypocotyls, and cotyledons (Grimes et al., 1992). Especially high levels of lipoxygenase protein were seen in the epidermal cells of the cotyledons and the cortical cells of the hypocotyl.

The expression of the *LOX1* gene in the aleurone layer is intriguing. This cell layer, which is derived from the endosperm during embryogenesis, is typical of seeds of the Brassicaceae (Vaughn and Whitehouse, 1971). Its function during seed germination, however, is unknown.

A number of genes that are expressed in the epidermis at some point in the life cycle of the plant, including members of the phenylammonia lyase and chalcone synthase gene families (Schmelzer et al., 1989), lipid transfer protein genes (Sossountzov et al., 1991; Sterk et al., 1991), genes involved in flower pigmentation (Jackson et al., 1991; Goodrich et al., 1992), and soybean Pro-rich cell-wall proteins (Wyatt et al., 1992), or genes that are epidermis specific (Clark et al., 1992) have been identified. It will be interesting to determine if the regulatory sequences of the *LOX1* gene share homology with other germination-specific and/or epidermally expressed genes. From such an analysis it may be possible to identify putative regulatory sequences required for gene expression, specifically in the epidermis and/or during germination.

The epidermis controls water loss and gas exchange and provides mechanical and chemical defense against pathogen and predator attack. In addition, it has been postulated that plant organ growth is controlled by the epidermis (Nick et

al., 1990). The epidermal location of the *LOX1* mRNA and its transient accumulation in young seedlings suggest that lipoxygenase contributes to one of these functions of the epidermis during germination. One possibility is that the *LOX1* gene product is required for the production of molecules that function prophylactically in the protection of young seedlings from pathogens. It is interesting to note that the mRNAs of several other defense-related genes, which encode enzymes of the phenylpropanoid pathway, are localized to the epidermis in uninfected plants (Schmelzer et al., 1989) and accumulate transiently during germination (Kubasek et al., 1992). It has been postulated that the phenylpropanoid pathway is required for the production of flavanoids, which protect the young seedlings from UV light or pathogens (Kubasek et al., 1992). Another possibility is that the *LOX1* gene product generates fatty acid hydroperoxide precursors to molecules that regulate the growth or expansion of the epidermis. Interestingly, jasmonic acid, a lipoxygenase-derived metabolite, stimulates germination in some species (Berestetzky et al., 1991; Ranjan and Lewak, 1992).

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