Plant Physiol. (1983) 73, 262–267
0032-0889/83/73/0262/06/$00.50/0

Localization of Lipoxygenases 1 and 2 in Germinating Soybean Seeds by an Indirect Immunofluorescence Technique[1]

Received for publication March 8, 1983 and in revised form June 16, 1983

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ABSTRACT

Lipoxygenases 1 and 2 were localized in etiolated germinating soybean seeds (*Glycine max* [L.]. Merr. var. Williams) by an indirect immunofluorescence staining technique. Sections of paraffin-embedded seedlings were stained with affinity-purified antibodies directed against lipoxygenase 1 or 2. The specificity of the immunofluorescence technique was examined by use of nonimmune serum or immunoglobulin G preparations after total adsorption with the appropriate lipoxygenase coupled to Sepharose 4B.

After immunofluorescence staining with antilipoxygenase 1 or 2 IgG storage tissues of cotyledons fluoresce strongly the first days of germination. After 3 days, the abaxial hypodermis, the epidermis, and the vascular bundle sheaths show fluorescence, especially after incubation with antilipoxygenase 2 IgG. Fluorescence in cortex and pith of the hypocotyl migrates to the vascular cylinder during germination. In primary leaves, all tissues show fluorescence after 1 day of germination. In storage tissues of cotyledons, cytoplasm around the protein bodies fluoresces, whereas in other tissues protein bodies or other large cell organelles fluoresce.

It is reasonable to suggest that lipoxygenase exerts its function in cells at the time that rigorous changes in metabolism take place, namely at the start of mobilization of reserves in storage tissues and start of biosynthesis of chloroplastids in several tissues.

The enzyme lipoxygenase (linoleate:oxygen reductase, EC 1.13.11.12) catalyzes the dioxygenation of fatty acids containing a methylene-interrupted *cis,cis*-pentadiene structure. In 1947, Theorell *et al.* (23) isolated lipoxygenase 1 from soybeans. Christopher *et al.* (4, 5) reported the existence of two lipoxygenase isoenzymes in addition to lipoxygenase 1, designated lipoxygenases 2 and 3. In a standard polarographic assay with 1.8 mM linoleic acid, lipoxygenase 1 shows maximum activity at pH 9.0 and lipoxygenases 2 and 3 at pH 6.0 to 7.0. Product specificity of the dioxygenation of linoleic acid by both lipoxygenases 1 and 2 is highest at pH 9.0 (25). Considerable amounts of lipoxygenases 1 and 2 occur in soybean; one soybean seed (160 mg dry weight) contains approximately 70 mg protein comprising about 49 mg of the storage protein glycinin (24) and at least 0.23 mg lipoxygenase 1 and 0.45 mg lipoxygenase 2 (20). The total lipid fraction of a seed is 35 mg, and almost all of the lipoxygenase substrates linoleic acid (15 mg) and linolenic acid (2 mg) are present in triglycerides while there are only trace amounts of free fatty acids (17).

As yet the physiological role of lipoxygenase is far from clear. It appears that lipoxygenase activity is maximal during the early stages of seed germination. Although soybeans are epigeous, seedlings remain essentially heterotrophic during the first 10 d (18). Sugars, primarily sucrose and stachyose in the cotyledons and embryonic axis, are the principal carbon sources during the first 3 d of germination (1). Subsequently, fat utilization increases and continues for about 10 d. Protein utilization which starts almost immediately, accelerates during the first 10 d and continues up to cotyledonary senescence (12). Holman (11) studied the relationship between lipoxygenase activity and changes in fat composition during germination of soybean seeds. He found that lipoxygenase activity at pH 9.0 declines sharply after the 2nd d while from the 3rd d linoleic acid and linolenic acid contents begin to decrease. Both variations in lipoxygenase activity of different plant tissues during germination and data dealing with subcellular localization of lipoxygenases have been reviewed by Douillard (6, 7). Lipoxygenase usually is soluble and localized in storage tissues of the seeds of most plants. Knowledge on the exact cellular and subcellular localization in germinating seeds can throw new light on the physiological function of lipoxygenase. Hitherto, localization in subcellular fractions has been examined by differential centrifugation or centrifugation on density gradients. In soybean, nearly 100% of the enzyme activities at pH 6.6 and 9.0 is found in the supernatant after prolonged certrifugation at 100,000*g* of seed extracts. On the basis of subcellular fractionation, it is difficult to decide whether the enzyme activity present in the supernatant originates from broken cytoplasmic organelles or from the cytosol.

Immunocytochemical staining is an efficacious technique, which has in the present paper been applied to the localization of lipoxygenases in soybean seedlings. We used indirect (two-layer) immunofluorescence to examine seedlings after a germination time of 2 h to 10 d.

MATERIALS AND METHODS

Plant Material. Seeds of *Glycine max* (L.) Merr. var. Williams were externally sterilized in ethanol for 10 s, rinsed with running tap water for 10 min, and soaked for 50 min in bidistilled H_2O flushed with O_2. At this stage, germination time was taken 1 h and seeds were then layered on wet filter paper on cotton wool in a plastic box covered with glass. The box was placed at 26°C in the dark.

Processing for Light Microscopy. Seeds or seedlings without testas were fixed for 24 h at room temperature in 1% (w/v) glutaraldehyde and 2% (w/v) formaldehyde in PBS[2] (0.05 M Na phosphate buffer [pH 7.4] containing 0.09 M NaCl). They were

[1] This investigation was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for the Advancement of Pure Research.

[2] Abbreviation: PBS, phosphate-buffered saline.

dehydrated in graded ethanol from 50% (v/v) to 100% and embedded in paraffin.

Antibodies. Lipoxygenases 1 and 2 were isolated from soybeans and antibodies directed against the isoenzymes were raised in rabbits as described previously (26). Before immunization with lipoxygenase 2, the enzyme was affinity-purified over a column with lipoxygenase 1 antibodies coupled to Sepharose 4B (Pharmacia Fine Chemicals). In an Ouchterlony double gel immunodiffusion test, antilipoxygenase 1 serum did not cross-react with lipoxygenase 2 and antilipoxygenase 2 serum did not precipitate with lipoxygenase 1. Crude extracts of soybean seeds precipitated in one sharp line in an Ouchterlony test with antilipoxygenase 1 or antilipoxygenase 2. IgG fractions of antisera were isolated, affinity-purified, and tested in an enzyme-linked immunosorbent assay (unpublished results).

Immunofluorescence Staining. Sections of 5 μm were put on microscope glasses coated with 1.5% (w/v) gelatin in 0.5% (w/v) $KCr(SO_4)_2$. The sections were deparaffinized in xylene, rehydrated in a graded alcohol series, and washed in PBS. Washing in 0.1 M lysine in PBS for 10 min was used to block the nonspecific binding sites of the tissue. Sections were incubated in affinity-purified rabbit antilipoxygenase IgG (0.1 mg/ml) in PBS for 60 min in a moist Petri dish. After three washings for 10 min each in PBS, the sections were incubated 60 min in sheep antirabbit IgG coupled with fluorescein isothiocyanate (1:100) diluted in PBS (Institut Pasteur, Paris). The sections were rinsed again for three periods of 10 min in PBS and mounted in 10% (v/v) glycerol in PBS (pH 8.0).

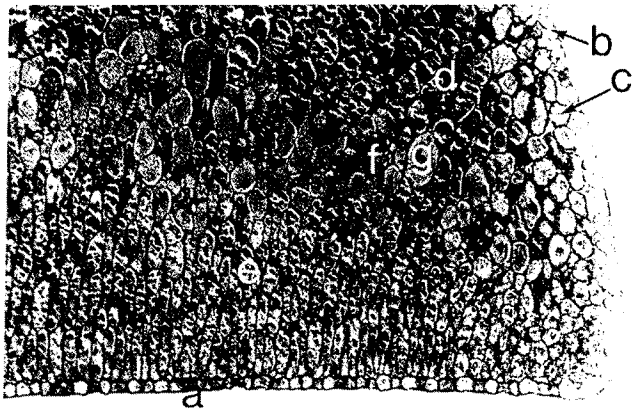
Histochemical Staining. Deparaffinized and rehydrated sections of 5 μm were stained with 0.1% (w/v) amido black in 7% (v/v) acetic acid for 5 min. After destaining for two periods of 10 min each in 7% (v/v) acetic acid, the sections were rinsed in water, dehydrated in graded ethanol from 70% (v/v) to 100% and mounted in DePex (Serva). Other sections were stained 5 min with a mixture of methylene blue and azure II in water, rinsed in water, and after dehydration in ethanol mounted in DePex.

RESULTS

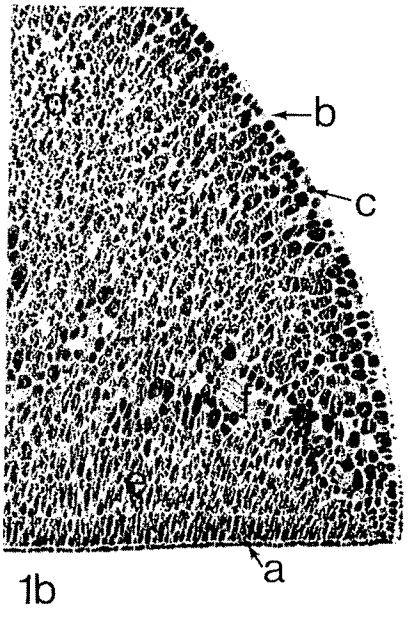
Morphology and Lipoxygenase Activity during Germination. During germination of soybeans at 26°C in the dark, the radicles emerge after 1 d and cotyledons open after 6 d, revealing the expanding unifoliate primary leaves. Secondary roots appear in 4 d.

Lipoxygenase activity at pH 6.6 decreased from the 1st d whereas activity at pH 9.0 decreased after 2 d of germination. After 6 d the activity of lipoxygenase is decreased to a low level, the activity at pH 6.6 being somewhat higher than the activity at pH 9.0 (11 and 6% of maximal activity, respectively). After 8 d a second activity maximum at pH 6.6 reaches at least 40% of the level at day 1.

Anatomy of Cotyledons. The flat face of a cotyledon of *G. max* corresponds to the adaxial face of a leaf. Parenchymatous storage tissue makes up most of the cotyledon and is surrounded by an epidermis. A distinct hypodermis is present only under the abaxial epidermis. The storage tissue appears to consist of two zones, an outer (abaxial) zone corresponding to the spongy parenchyma of a leaf, and an inner (adaxial) zone corresponding to the palisade tissue. Vascular bundles are situated more or less along the boundary of the inner and outer storage tissues. They are surrounded by a parenchymatous bundle sheath and consist of procambium which differentiates in xylem and phloem during the first 3 d of germination. The cotyledon has an extensive system of intercellular spaces, which can be observed as triangles at every cell wall junction of the abaxial parenchyma in transverse sections. Figure 1 shows cotyledons after 17 h of germination stained with amido black or methylene blue and azure II.



1a



1b

FIG. 1. Soybean cotyledon after 17 h of germination. 1a, stained with methylene blue and azure II. 1b, stained with amido black. The following abbreviations are used on Figures 1 to 4: a, adaxial epidermis; b, abaxial epidermis; c, abaxial hypodermis; d, outer "spongy" storage parenchyma; e, inner "palisade" storage parenchyma; f, vascular tissue; g, vascular bundle sheath; anti-1, immunofluorescence-stained section incubated with antilipoxygenase 1; anti-2, immunofluorescence-stained section incubated with antilipoxygenase 2.

All cells are filled with large protein bodies, those in the storage tissues staining most intensely with amido black.

Changes in cell structure in cotyledons of legume seeds during germination have been described for *Pisum* species by Bain and Mercer (3) and by Smith and Flinn (22) and for *Phaseolus vulgaris* by Öpik (15) and by Smith (21). In soybean, mobilization of reserves begins at the abaxial side of the cotyledons. During seedling growth, the reserve proteins in storage parenchyma cells are catabolized, the protein bodies fuse and form a central vacuole.

Immunofluorescence Staining. Fixation of seedlings in formaldehyde only or in combination with $CaCl_2$ resulted in material with poor sectioning properties. Glutaraldehyde solutions with various concentrations of picric acid and/or acetic acid proved to be also unsatisfactory as fixatives. Fixation in 1% (v/v) glutaraldehyde only or plus 1% (v/v) formaldehyde as well as fixation

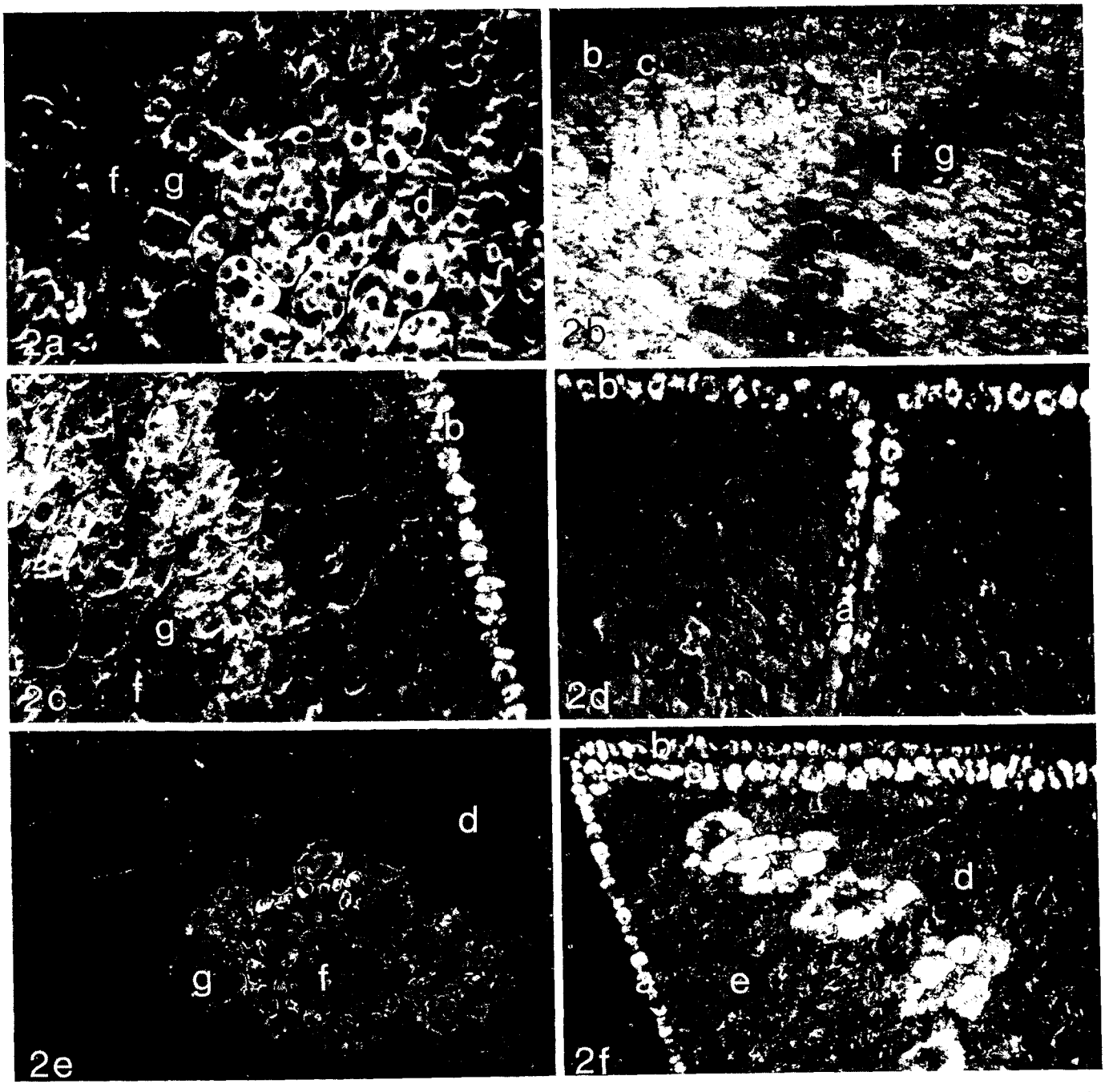


FIG. 2. Immunofluorescence-stained cotyledons of germinating soybean seedlings. 2a, cotyledon after 2 h of germination showing a vascular bundle and bundle sheath surrounded by fluorescing outer storage parenchyma (anti-1). 2b, cotyledon after 20 h of germination: only storage tissues are fluorescent (anti-1). 2, cotyledons after 40 h of germination with fluorescent abaxial epidermis and decreased fluorescence in parenchyma cells lying under the epidermis (anti-1). 2d, 40 h cotyledon with fluorescent adaxial and abaxial epidermis (anti-1). 2e, vascular bundle in cotyledon after 67 h of germination (anti-1). 2f, cotyledon after 67 h of germination showing high fluorescence in epidermis, hypodermis, and vascular bundle sheath (anti-2).

in a mixture of paraformaldehyde, glutaraldehyde, and acrolein according to Mollenhauer and Totten (13) resulted in good preservation of the structure. Nonspecific background fluorescence was the lowest in cotyledons fixed in 1% (v/v) glutaraldehyde plus 1% (v/v) formaldehyde.

The intensity of staining after incubation with various antiserum dilutions or affinity-purified IgG concentrations was examined. Antisera dilutions from 100 to 400 gave a good contrast between specific and background staining. Antilipoxygenase 1 IgG could be used in a range of 10 to 200 μg/ml and antilipoxygenase 2 IgG, 60 to 300 μg/ml. For localization of lipoxygenases in tissues of germinating soybean seeds, 100 μg/ml solutions of both affinity-purified IgG species were used.

The specificity of the immunofluorescence technique was checked with nonimmune rabbit serum, unadsorbed IgG fractions from lipoxygenase-Sepharose 4B immunoadsorbent columns, and with sera preincubated with lipoxygenase. No fluorescence could be detected after incubation with nonimmune sera or unadsorbed column fractions. Tissue sections incubated with antisera or IgG samples preincubated with the appropriate

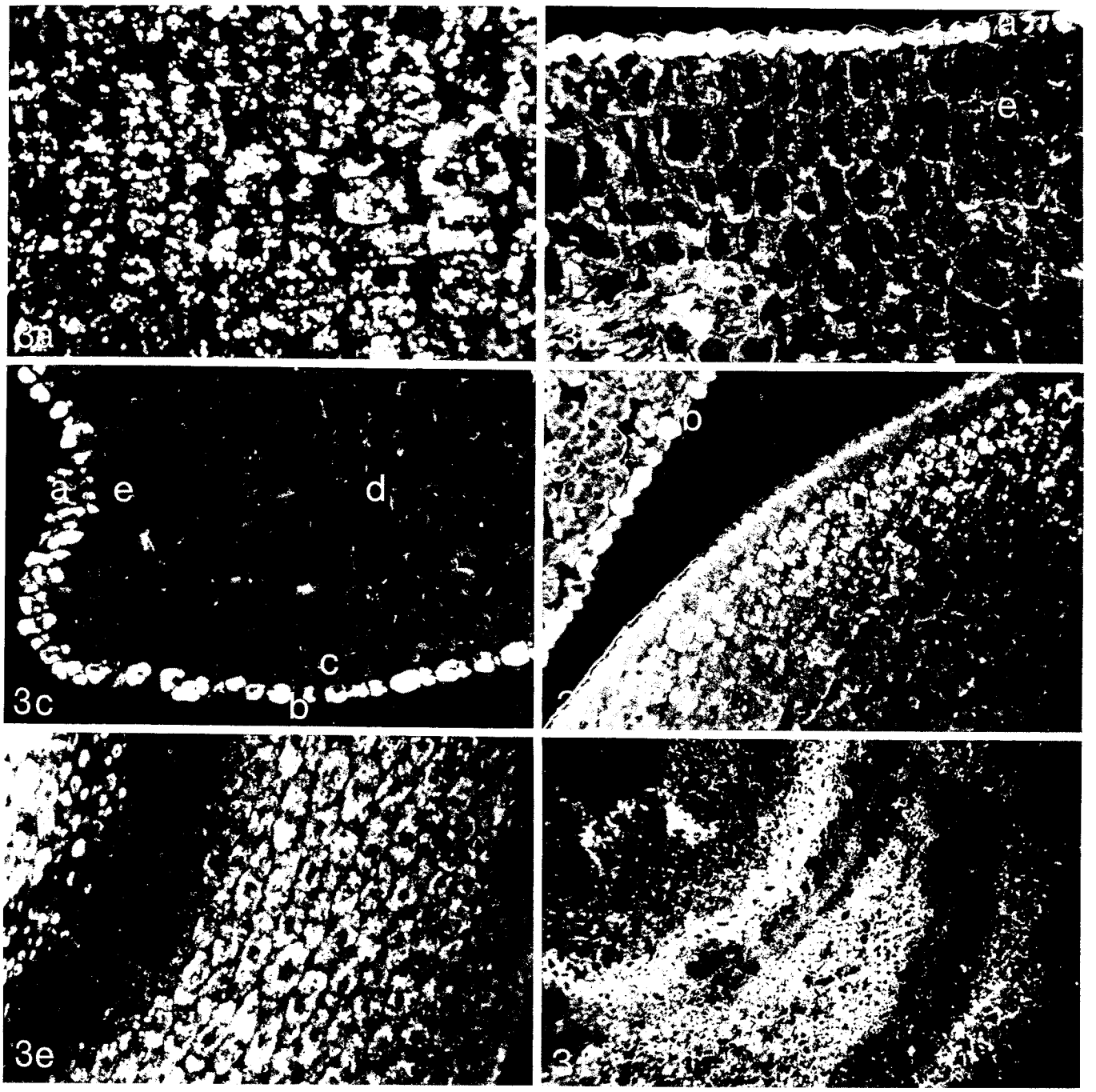


FIG. 3. Immunofluorescence staining of cotyledons and hypocotyl of germinating soybean seedlings. 3a, hypodermis of coyledon after 65 h of germination with large fluorescent particles (anti-2). 3b, cotyledon after 67 h of germination showing inner storage parenchyma with fused and swollen protein bodies or a central vacuole; one of the two vascular bundles, the adaxial epidermis, and cytoplasm in the parenchyma cells fluoresce (anti-2). 3c, 10-d-old cotyledon with collapsing parenchyma cells and fluorescent epidermis (anti-2). 3d, cotyledon (left) and hypocotyl of a 2-h-old seedling showing fluorescent particles in cortex parenchyma cells lying under the epidermis of the hypocotyl (anti-2). 3e, hypocotyl after 20 h of germination with fluorescence in cortex and pith parenchyma; note the migration of fluorescence towards the vascular cylinder (anti-2), when compared to 3d. 3f, hypocotyl after 67 h of germination with fluorescing parenchyma around the vascular cylinder (anti-1).

lipoxygenase isoenzyme stained very weakly. Preincubation of antilipoxygenase 2 IgG with lipoxygenase 1 resulted in a slight diminution of fluorescence.

Lipoxygenase in Cotyledons. Immunofluorescence staining with lipoxygenase 1 or 2 yields highly fluorescent cytoplasm of storage tissues from 2-h-old cotyledons, while protein bodies do not fluoresce at all (Fig. 2a). No fluorescence is found in tissues like vascular bundles with their parenchymatous sheaths, epidermis, and hypodermis. After 20 h of germination, the situation is unaltered (Fig. 2b). After 40 h, fluorescence has disappeared from parenchyma layers directly under the abaxial epidermis (Fig. 2c), but interestingly the epidermis fluoresces, starting with the abaxial epidermis, the most remote from the axis (Figs. 2c, 2d, 2f, 3b, and 3c). Hypodermis and vascular bundle sheath cells fluoresce after 60 to 65 h of germination (Fig. 2f). In the epidermis, hypodermis, and vascular sheath parenchyma, either

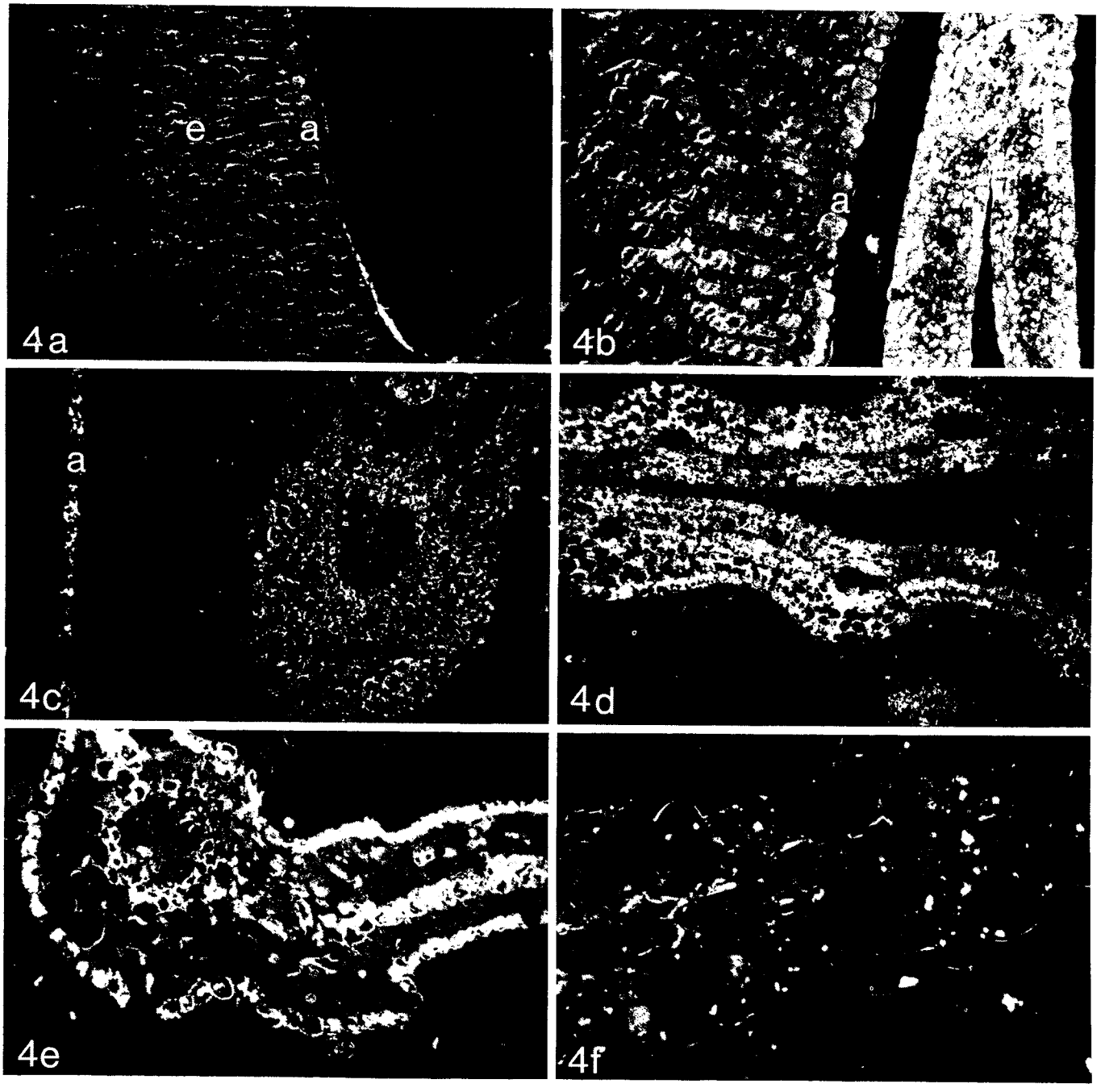


FIG. 4. Primary leaves of germinating soybean seeds after immunofluorescence staining. 4a, leaf surrounded by cotyledons of a 20-h-old seedling, showing no fluorescence at all (anti-1). 4b, more developed fluorescent part of the 20-h leaf (anti-2). 4c and 4d, leaves after 65 h of germination with fluorescent parenchyma and epidermis (anti-1). 4e, leaf tissue after 67 h of germination showing a fluorescent epidermis (adaxial and abaxial) and a fluorescent zone with vascular bundles between spongy and palisade parenchyma (anti-2). 4f, transverse section of a young leaf from a 2-month-old soybean plant growing in the light. Only a weak fluorescence around the chloroplastids in parenchyma cells is observed (anti-2).

the protein bodies or other large cell organelles fluoresce (Fig. 3a). After 10 d storage parenchyma cells collapse and only the epidermis shows a weak fluorescence (Fig. 3c).

Incubation with antilipoxygenase 2 IgG gave results which are similar to incubations with antilipoxygenase 1 IgG, although fluorescence of hypodermis and epidermis is more intense. Fluorescence of the vascular sheath was much higher after incubation with antilipoxygenase 2 IgG than with antilipoxygenase 1 IgG (Fig. 2, e and f).

Hypocotyls. A soybean hypocotyl comprises an epidermis, cortex parenchyma, pith parenchyma, and vascular cylinder between cortex and pith. Fluorescence staining after incubation with antilipoxygenase 1 or antilipoxygenase 2 IgG samples was identical. After 2 h of germination, parenchyma cells lying under the epidermis and facing the cotyledon contain fluorescent granula (Fig. 3d). After 20 h of germination, pith and cortex parenchyma fluoresce (Fig. 3e). During aging of the hypocotyls, fluorescent zones in pith and cortex move towards the vascular cylinder (Fig. 3, e and f).

Roots. Roots, comprising epidermis, cortex parenchyma, and a vascular cylinder without pith, did not fluoresce specifically after incubation with one of the antilipoxygenase IgG samples.

Leaves. In early stages of growth, leaves do not fluoresce after immunofluorescence staining with antilipoxygenase 1 or 2 IgG (Fig. 4a). In the course of germination, all leaf tissues fluoresce after treatment with one of the antibody species (Fig. 4, b–d). Almost the whole cell content except the nucleus and vacuole fluoresces, especially after staining with antilipoxygenase 2 IgG.

After 10 d, parenchyma cells around the vascular bundles and the abaxial and adaxial epidermis still fluoresce very strongly (Fig. 4e). Mature leaves of 2-month-old plants growing at 25°C in a glasshouse show a very weak fluorescence around chloroplastids in cells of spongy and palisade parenchyma (Fig. 4f).

DISCUSSION

The profile of lipoxygenase activity at pH 9.0 in etiolated germinating soybean seeds of the Williams variety resembles the results of Holman (11) who used Agat soybean seeds, planted in soil and growing in normal daylight. Activity at pH 6.6 also decreased during the first days of germination but, in contrast to pH 9.0 activity, showed a second activity maximum after 8 d. Anstis and Friend (2) also reported an increase of lipoxygenase activity at pH 6.2 between days 7 and 15 in dwarf pea seedlings germinating at 21°C in the dark.

Immunofluorescence staining of the storage tissues in cotyledons of germinating soybean seeds is paralleled by the lipoxygenase activity as can be determined in extracts of the seedlings: initially very high and decreasing after 2 d. Lipoxygenases 1 and 2 disappear from storage tissues before the actual mobilization of fat reserves has started. Holman (11) suggested that lipoxygenase is inactivated as a consequence of its action and that the enzyme has its function only in initiating the oxidation of the unsaturated fatty acids.

During germination, O_2 consumption by cotyledons increases rapidly, reaching higher rates than in immature seeds at any stage (14). The initial burst of respiratory activity of soybean seed tissue is cyanide-insensitive as is lipoxygenase which has been held responsible in part for that activity (16). However, Fritz *et al.* [10] showed that, whereas lipoxygenase activity accounted for a large portion of the O_2 absorption in crude extracts of corn seedlings, only 0.4% of O_2 was actually chemically incorporated into the tissue during rapid respiration of the seedlings *in vivo*. Recently, Shingles *et al.* (19) reported that lipoxygenase contamination of preparations of potato mitochondria does not contribute significantly to the cyanide-insensitive O_2 consumption. Simultaneously, Dupont *et al.* (9) arrived at the same conclusion for mitochondria from potato, soybean, and arum. Dupont *et al.* also described the oxidative conversion of linoleic acid hydroperoxide by mitochondria, during which heme compounds in mitochondria are destroyed. The same phenomenon was observed with Cyt *c*.

A possible physiological function for lipoxygenase *in vivo* may be regulating or triggering various processes in the cell during early stages of germination rather than being primarily involved in respiration or in mobilization of unsaturated fatty acids. The location of lipoxygenases (mainly lipoxygenase 2) in hypodermis, epidermis, and vascular bundle sheaths of cotyledons after 2 to 3 d of germination could favor such a regulatory function. Cells of the abaxial hypodermis and epidermis, cells of adaxial epidermis, and those surrounding the vascular bundles become photosynthetic after 8 to 10 d of germination in the light. It thus appears that lipoxygenase is present in the cell at the time at which intracellular reorganization is about to start. Both lipoxygenases are also present in young developing hypocotyls and young leaves not yet photosynthesizing.

Subcellular location of soybean lipoxygenases 1 and 2 in parenchyma cells of storage tissues of the cotyledons could be in the cytosol or small organelles not visible at 1000-fold magnification. In other tissues of the seedling, large cell organelles like protein bodies fluoresce clearly after immunofluorescence staining (Figs. 3a, 3d, 3e, and 4b). Wardale and Galliard (27) also found that the location of lipoxygenase activity was different for each tissue in cauliflower, potato, and pea and Douillard and Bergeron (8) found lipoxygenase activity in developing plastids of peas and wheat during initial stages of leaf growth. Because precise subcellular localization of lipoxygenase is not possible with light microscopic techniques, we are developing an electron microscopic method for more detailed investigations.

Acknowledgments—We wish to thank Dr. H. J. T. Goos for providing equipment and Mr. H. van Kooten, Mr. F. Kindt, Mr. M. Kramer, and Mr. J. L. den Boesterd for taking and printing the photographs.

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