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Differential Expression of Lipoxygenase Isoenzymes in Embryos of Germinating Barley[1]

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Expression of lipoxygenase was studied in barley (*Hordeum distichum* L.) embryos during germination. Total lipoxygenase activity was high in quiescent grains, dropped during the 1st d of germination, and subsequently increased to a level similar to that in quiescent grains. The contribution of two isoenzymes, lipoxygenases 1 (LOX-1) and 2 (LOX-2), was studied at the activity, protein, and mRNA levels. Activity ratios of the two isoforms were determined via the ratio of 9- and 13-hydroperoxides, which are formed from linoleic acid. Isoenzyme protein levels were determined using specific monoclonal antibodies. mRNA levels were studied using the specific cDNA probes LoxA and LoxC, which correspond to LOX-1 and LOX-2, respectively. The major difference in temporal expression of LOX-1 and LOX-2 was observed in quiescent grains. At this stage, LOX-1 contributed almost exclusively to total lipoxygenase activity. LOX-2 activity rapidly increased until d 2 of germination. From this time point onward, LOX-1 and LOX-2 showed similar patterns at both activity and protein levels. The tissue distribution of the two isoenzymes in the germinating embryo was closely similar, with the highest expression levels in leaves and roots. The levels of LOX-1 and LOX-2 may be regulated mainly pretranslationally, as suggested by the similarity of the protein and mRNA patterns corresponding to the two isoforms.

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are a class of non-heme, iron-containing dioxygenases that catalyze the oxygenation of polyunsaturated fatty acids with a 1,4-*cis*,*cis*-pentadiene structure to form conjugated diene hydroperoxides. The enzyme is widely distributed both in plants (reviewed by Gardner, 1991; Siedow, 1991; Vick, 1993) and in animals (reviewed by Schewe et al., 1986; Yamamoto, 1991).

Despite extensive studies, the physiological role of lipoxygenases in plants is poorly understood. It has been suggested that lipoxygenase is involved in plant growth and development (Hildebrand et al., 1991; Siedow, 1991). Highest levels of lipoxygenase activity were found in rapidly growing tissues, suggesting a correlation between lipoxygenase levels and the rate of cell elongation. Furthermore, lipoxygenase may play a role in senescence, wounding and infection, and pest resistance (Gardner, 1991; Siedow, 1991; Vick, 1993). In these processes, the relatively reactive products of the lipoxygenase reaction may cause the observed membrane damage. Furthermore, lipoxygenase may be involved in the biosynthesis of regulatory molecules. For instance, traumatin, or "wound hormone," which is formed via the lipoxygenase pathway from the 13-hydroperoxide of linolenic acid, may be involved in the responses of the plant to wounding (Gardner, 1991; Siedow, 1991; Vick, 1993). Another metabolite originating from the lipoxygenase-mediated oxidation of linoleic acid, jasmonic acid, is suggested to cause, among other effects, inhibition of growth, abscission, senescence, and responses to plant wounding and pathogen attack (Gardner, 1991; Creelman et al., 1992; Vick, 1993; Bell et al., 1995). Additionally, lipoxygenase may play a role in the mobilization of lipid reserves (Feussner and Kindl, 1992), and soybean leaf lipoxygenase has been suggested to function as a vegetative storage protein for temporary storage of nitrogen (Tranbarger et al., 1991).

For barley (*Hordeum distichum* L.), two lipoxygenase isoenzymes have been described: LOX-1, which exists in the quiescent as well as in the germinating grain (Yabuuchi, 1976; Baxter, 1982; van Aarle et al., 1991; Doderer et al., 1992; Yang et al., 1993; Hugues et al., 1994), and LOX-2, which generally appears only after germination (Yabuuchi, 1976; Baxter, 1982; Doderer et al., 1992; Yang et al., 1993; Hugues et al., 1994). The two isoenzymes clearly differ in properties. For example, LOX-1 produces mainly the 9-hydroperoxide from linoleic acid, whereas LOX-2 forms primarily the 13-hydroperoxide from this fatty acid (van Aarle et al., 1991; Doderer et al., 1992; Yang et al., 1993; Hugues et al., 1994). This demonstrates that each of the two isoenzymes yields a different subset of lipoxygenase pathway end products and possibly fulfills a distinct physiological role in the germinating barley kernel. However, what these roles could be is unclear at present.

More insight may be obtained by studying the behavior of the two isoenzymes in embryos of germinating barley.

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Abbreviations: 9-HPOD, 9-hydroperoxy-10*E*,12*Z* octadecadienoic acid; 13-HPOD, 13-hydroperoxy-9*Z*,11*E* octadienoic acid; LOX-1 and -2, lipoxygenase-1 and -2; Mab, monoclonal antibody(ies).

Until now, such information has been rather scarce and has been based solely on activity measurements after separation of both isoenzymes by column chromatography (Yang et al., 1993). Therefore, we have performed a detailed study of the temporal and spatial expression of LOX-1 and LOX-2 during germination of barley at the activity, protein, and mRNA levels.

MATERIALS AND METHODS

Plant Materials

Barley grains (*Hordeum distichum* L. cv Caruso, harvest 1992) were germinated between two times three layers of moist filter paper in a plastic plant propagator (33 × 22 cm) at 25°C in the dark for 9 d. Each day, germinating barley grains were collected and dissected to obtain embryos. From 2 d after germination, embryos were divided into scutella, roots, and etiolated leaves including coleoptiles (henceforth simply called "leaves").

Preparation of Crude Extracts

All steps were performed at 0 to 4°C. Crude extracts of (parts of) embryos were prepared by homogenizing 10 grain parts in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM PMSF and 2 mM NaN_3. For extraction, the ratio of buffer (mL) to fresh weight (g) was 10:1, with a minimum volume of 3 mL. After homogenization using an Ultraturrax (Janke & Kunkel, Staufen, Germany) (five pulses at 24,000 rpm of 30 s each, with 30-s intervals), the resulting suspension was centrifuged for 5 min at 3,400*g*. Subsequently, the supernatant was centrifuged for 20 min at 15,800*g*. The supernatant was assayed for lipoxygenase activity immediately and stored at −20°C for further use.

Enzyme Assay and Protein Determination

Lipoxygenase activity was measured by a spectrophotometric assay as described by Doderer et al. (1992). The activity was expressed as μmol hydroperoxide formed per min (unit) at 25°C per 10 (parts of) embryos using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}. Protein concentrations were determined by the bicinchoninic acid protein assay, using BSA as a standard (Smith et al., 1985).

Northern Blotting

For each sample, 10 embryos were dissected from harvested grains, frozen immediately in liquid nitrogen, and stored at −80°C. RNA was isolated according to Slater (1984). From each sample, 7.5 μg of total RNA was denatured by glyoxal/DMSO and separated on a 0.8% agarose gel according to Maniatis et al. (1982). Northern blotting was performed using nylon membranes (GeneScreen Plus, DuPont) according to the manufacturer's instructions. Hybridization of the blots was performed with cDNA probes coding for one of the three barley lipoxygenase cDNAs and containing 142, 227, and 235 nucleotides of the 3′ untranslated part of LoxA, LoxB, and LoxC, respectively, as determined by van J.R. van Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp, and B.E. Valk (unpublished data). Nucleotide sequences of LoxA, LoxB, and LoxC are available in the EMBL, GenBank, and DNA Data Bank of Japan nucleotide sequence databases under accession numbers L35931 (LoxA), L37359 (LoxB), and L37358 (LoxC). The LoxA probe encompassed nucleotide positions 2659 to 2801, the LoxB probe encompassed positions 1028 to 1255, and the LoxC probe encompassed positions 1743 to 1978. One hundred nanograms of each cDNA probe were labeled with a random-priming kit (Pharmacia Biotech, Uppsala, Sweden) in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP and hybridized with an activity of 10^5 cpm/mL hybridization mixture. As a control for equal labeling of the probes, dot blots with 5 pg of cDNA of LoxA, LoxB, and LoxC were hybridized in the same experiment. The blots were rehybridized with a rRNA probe as a check for equal loading of the lanes.

Identification of 9- and 13-Hydroperoxides of Linoleic Acid

Crude extracts (100 μL) of whole embryos, prepared as described above, were incubated in 10 mL of 100 mM sodium phosphate buffer (pH 6.5) in the presence of linoleic acid (200 μM) for 40 min with agitation at room temperature. The lipoxygenase reaction was stopped by lowering the pH to 3.5, and 5 μg of prostaglandin B_2 were added to the incubation medium as an internal standard. As a control for auto-oxidation, 100 μL of buffer only were incubated with linoleic acid in the same experiment. The products of the lipoxygenase reaction were purified using an octadecyl solid-phase column. The column was washed with 3 column volumes of ice-cold methanol and 2 column volumes of ice-cold water. The incubation medium was then passed through the column followed by washing with 2 volumes of water. Hydroperoxides were eluted with 2 mL of cold methanol.

The products were analyzed by reverse-phase HPLC as described by van Aarle et al. (1991) using a ChromSpher C_{18} column (5 μM, 4.6 × 250 mm, Chrompack [Raritan, NJ]), a UV detector (L-4000, Merck-Hitachi [Tokyo, Japan]) set at 234 nm, and an integrator (D-2500, Merck-Hitachi). The isocratic solvent was tetrahydrofuran:methanol:water: acetic acid (25:30:44.9:0.1, v/v) adjusted to pH 5.5 with ammonia and was delivered at a flow rate of 0.5 mL min^{-1}. The pump used was an Intelligent Inert Pump (L-6210, Merck-Hitachi).

Generation of Mab

Female Balb/c mice were immunized intraperitoneally with 30 μg of antigen (highly purified LOX-1 or LOX-2 isolated from germinated barley embryos as described by Doderer et al. [1992], and kindly supplied by them) in 200 μL of an emulsion consisting of equal volumes of PBS and complete Freund's adjuvant. With time intervals of 3 weeks, two successive boosters were given with 30 μg of antigen each. Three days after the last booster injection, the spleen was removed and the lymphocytes were used for fusion. For this cell fusion, the mouse myeloma cell line

NS-1 was used. The myeloma cells were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, catalog no. 42430–025) supplemented with Gln (2 mM), penicillin (10 units mL^{-1}), streptomycin (10 $\mu g\ mL^{-1}$), kanamycin (50 $\mu g\ mL^{-1}$), 5×10^{-5} M β-mercaptoethanol, and 10% (v/v) heat-inactivated bovine calf serum. PEG-induced cell fusion was performed as described by van Duijn et al. (1989). Following fusion, the cell population was resuspended in a selective medium (containing 0.1 mM hypoxanthine and 5.8 μM azaserine) in which only hybrid cells can survive and was distributed over the wells of 96-well tissue culture plates. About 10 d after this fusion, the antibody production of the hybridomas was tested using an ELISA system as described by de Boer et al. (1988). Next, the anti-lipoxygenase-producing hybridoma cells were diluted and seeded over another 96-well tissue culture plate with a cellular density of approximately one cell per well. This so-called "subcloning procedure" was repeated four times with intervals of 2 weeks. At this stage the cell lines were considered to be monoclonal. To obtain sufficient amounts of Mab, the cloned cell lines were cultured in protein-free hybridoma medium (GIBCO). Lipoxygenase isoenzyme specificity of the Mab was determined using PAGE and western blotting analysis. The Mab secreted by the hybridoma cells were purified from culture medium using protein G affinity chromatography.

Gel Electrophoresis and Western Blotting

SDS-PAGE was performed on 12.5% homogeneous gels using the Multiphor II System (Pharmacia Biotech).

After electrophoresis, proteins were transferred onto nitrocellulose membrane by semidry blotting, essentially as described by Towbin et al. (1979) and Kyhse-Andersen (1984). After blotting, the nitrocellulose membranes were soaked in PBS containing 0.05% Tween 20 plus 1% BSA for 30 min, followed by incubation overnight with LOX-1- or LOX-2-specific Mab (Mab 33.3 and 4.2, respectively) diluted in the same buffer. Subsequently, the membranes were washed with PBS containing 0.05% Tween 20 and incubated for 1 h with goat anti-mouse IgG antibodies conjugated to alkaline phosphatase. Finally, the bound alkaline phosphatase was visualized by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium as the substrate. Color development was stopped by washing the nitrocellulose membranes with distilled water.

RESULTS

Temporal Expression of Lipoxygenase in Embryos of Germinating Barley

Lipoxygenase Activity

Activities of lipoxygenase were measured in embryos during germination and expressed as total activity per 10 embryos. Total lipoxygenase activity showed a sharp decrease during the 1st d of germination (Fig. 1). Subsequently, activity gradually increased, reaching a maximal value at d 7, that was comparable with the activity in the quiescent grain. Nine days after germination, activity decreased again. The activities of lipoxygenase in the different parts of the embryo will be described below.

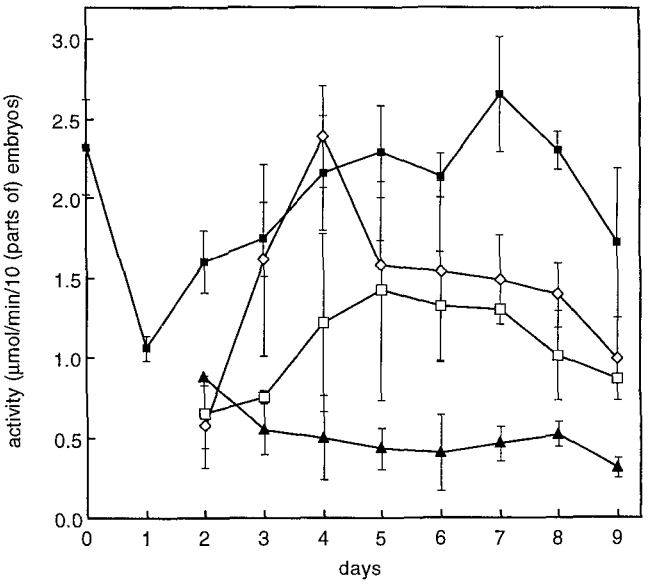


Figure 1. Time course of lipoxygenase activity in total embryo (■), scutellum (▲), leaf (◇), and root (□) of dissected barley embryos upon germination. Activity is expressed as μmol hydroperoxides formed per min per 10 (parts of) embryos. Values are the means ± SE of three independent experiments.

To study the contribution of LOX-1 and LOX-2 isoenzymes to total activity, we have determined the ratio of 9- and 13-hydroperoxides of linoleic acid formed by extracts of barley embryos. The ratio of 9- to 13-HPOD formed is an indication of the contribution of LOX-1 and LOX-2, respectively, to total activity during germination. Linoleic acid was used as a substrate in these experiments because it is the most abundant fatty acid in barley (Morrison, 1978) and has been shown to be the preferred substrate for both isoenzymes (Yabuuchi, 1976; Doderer et al., 1992; Yang et al., 1993).

Figure 2 shows that upon germination, the ratio of 9- to 13-HPOD decreased from 96:4 in embryos from quiescent grains to 62:38 in embryos from 2-d-old seedlings, remaining more or less constant after this time point. These data suggest that the contribution of LOX-1 to total activity decreased during the first days of germination to about 65%, whereas relative LOX-2 activity increased over this time. After 2 d of germination, their relative contributions remained constant.

Generation of Mab

The ratio of products formed by lipoxygenase provides an indication of the relative but not of the absolute content of LOX-1 and LOX-2 in embryo extracts. One of the ways to study the absolute level of the two isoenzymes is to determine the LOX-1 and LOX-2 protein levels with monospecific antibodies.

After repeated screening in ELISAs and subcloning of the hybridoma cells, six anti-lipoxygenase Mab-producing hybridomas were obtained. Of these six types of Mab, three

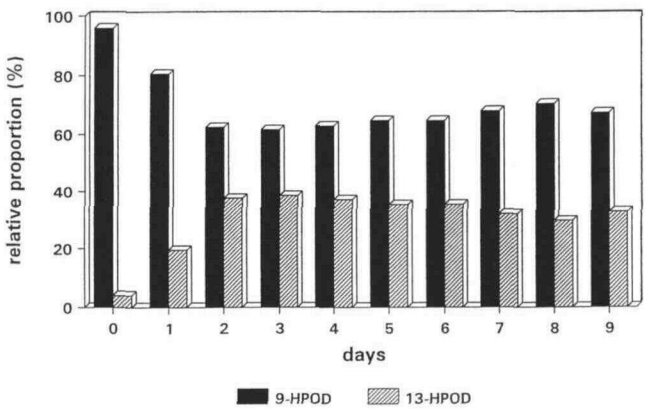


Figure 2. Relative proportion of 9- and 13-HPOD formed from linoleic acid by extracts from embryos from germinating barley.

(2G4, 33.3, and 5D2) showed a pronounced specificity toward barley LOX-1 as determined by ELISA (data not shown). Two Mab (Mab 4.2 and 5.8) recognized exclusively LOX-2 in ELISA, and one Mab (4.9) recognized both lipoxygenase isoenzymes (data not shown).

These six Mab were purified and further tested with respect to specificity in western blotting experiments. For these immunoblotting experiments, various antibody concentrations were used. Figure 3 shows the reactivity of five different purified Mab with LOX-1 and LOX-2 on western blots. Mab 33.3 showed exclusive specificity toward LOX-1 at higher dilutions (lane 3), whereas for Mab 4.2 and 5.8 an opposite specificity was observed (lanes 4–9). In agreement with the data obtained from ELISA experiments, Mab 4.9 showed a pronounced reactivity with both LOX-1 and LOX-2 (Fig. 3, a and b, lanes 10–12). Mab 5D2 showed a minor cross-reactivity with LOX-2 at high antibody concentrations. Dilution of this antibody by a factor of 1000 resulted in a complete loss of signal on the western blot of LOX-2, whereas at this concentration the antibody showed a very strong reactivity with LOX-1 (Fig. 3a, lane 15). Finally, the exclusive reactivity of Mab 2G4 toward LOX-1, as observed in ELISA, was confirmed in western blotting experiments (data not shown).

The antibodies recognized a double or even a triple band of proteins with approximate molecular masses of about 90 kD. In the purified LOX-1 and LOX-2 preparations used for western blotting experiments, the polypeptides with lower molecular mass have been suggested to be degradation products of the largest protein (Doderer et al., 1992).

Lipoxygenase Isoenzyme Protein Levels

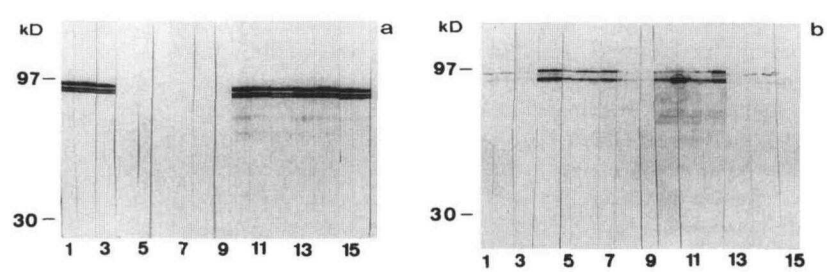
Levels of LOX-1 and LOX-2 protein in embryos of germinating barley were determined by western blotting experiments with use of the specific Mab 33.3 (reacting with LOX-1) and 4.2 (reacting with LOX-2), respectively, generated and characterized as described above.

Figure 4a shows that LOX-1 was already present in embryos of quiescent grains. On the 1st d of germination, the level of LOX-1 protein in embryos showed a decrease. At d 4 and 5, clearly higher amounts of LOX-1 protein were detected, after which levels decreased again.

Levels of LOX-2 protein were relatively low in embryos of quiescent grain. The increase in LOX-2 protein detected during germination, resulting in highest levels from d 2 to 5 (Fig. 5a), is in agreement with the increasing contribution of LOX-2 to total lipoxygenase activity, as shown in Figure 2. After d 5, LOX-2 protein levels decreased.

These results demonstrate that the decrease in total lipoxygenase activity observed at d 1 of germination was a result of the decreased level of immunoreactive LOX-1

Figure 3. Western blotting analysis showing the reactivity of five different purified Mab with LOX-1 (a) and LOX-2 (b). The various Mab were diluted as indicated in the table below the blots.



Lane	Mab	Dilution factor	Lane	Mab	Dilution factor
1	33.3	40	9	5.8	50
2	33.3	100	10	4.9	10
3	33.3	1000	11	4.9	100
4	4.2	10	12	4.9	1000
5	4.2	100	13	5D2	10
6	4.2	50	14	5D2	100
7	5.8	10	15	5D2	1000
8	5.8	100			

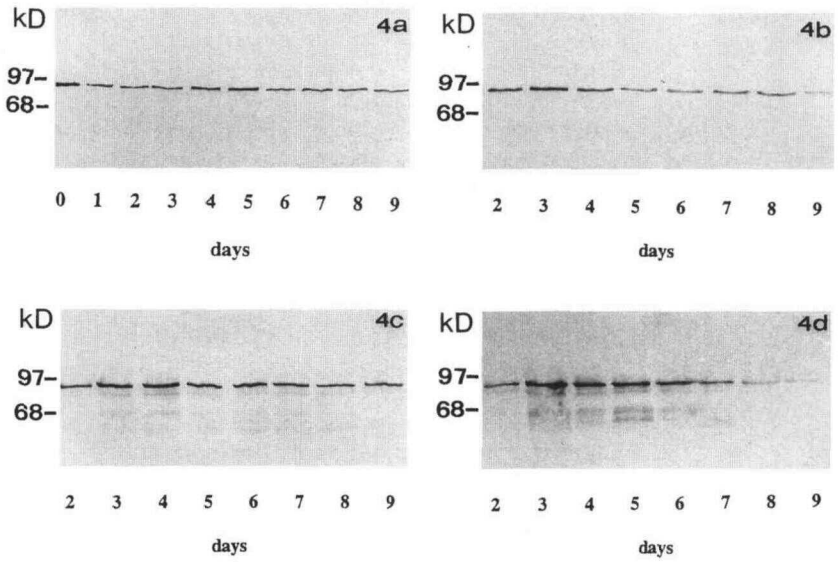


Figure 4. Patterns of LOX-1 protein in total embryo (a), scutellum (b), leaf (c), and root (d) of barley in the course of germination as determined by western blotting using the LOX-1-specific Mab 33.3. The same volume content was loaded on the gel, i.e. 0.006 embryos in each lane in a and 0.011 scutella, 0.011 leaves, and 0.011 roots in b, c, and d, respectively. Molecular mass markers are indicated on the left.

protein in the embryo extracts. The subsequent activity increase was paralleled by increased protein levels of both isoenzymes. Finally, during the last phase of germination, the decrease in total lipoxygenase activity appeared to be preceded by a decrease in both LOX-1 and LOX-2 proteins.

Lipoxygenase mRNA Levels

A study on the level of the mRNAs corresponding to the two lipoxygenase isoenzymes may indicate at which levels the expression of LOX-1 and LOX-2 are regulated. In barley, three cDNAs, namely LoxA, LoxB, and LoxC, have been identified; LoxA and LoxC probably encode LOX-1 and LOX-2, respectively, whereas LoxB encodes an as-yet-unidentified lipoxygenase isoform (J.R. von Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp, and B.E. Valk, unpublished data). However, it should be noted that the presence of a third isoenzyme could not be demonstrated in germinating barley (Doderer at al., 1992).

cDNA probes containing 142 to 235 nucleotides of the 3′ untranslated region of LoxA, LoxB, and LoxC (J.R. von Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp, and B.E. Valk, unpublished data) were used to study the corresponding mRNA levels in embryos of germinating barley. Dot-blot experiments showed that these LoxA, LoxB, and LoxC cDNA probes specifically hybridized with LoxA, LoxB, and LoxC cDNAs, respectively (data not shown).

Figure 6 shows that each of the three probes hybridized with mRNA isolated from embryos from germinating barley. However, there is a remarkable difference in expression patterns of LoxA, LoxB, and LoxC. LoxA mRNA was already present in the quiescent grain (Fig. 6a). Transcripts of LoxA were present throughout germination, with the highest levels from d 1 to 5. LoxB mRNA could be detected

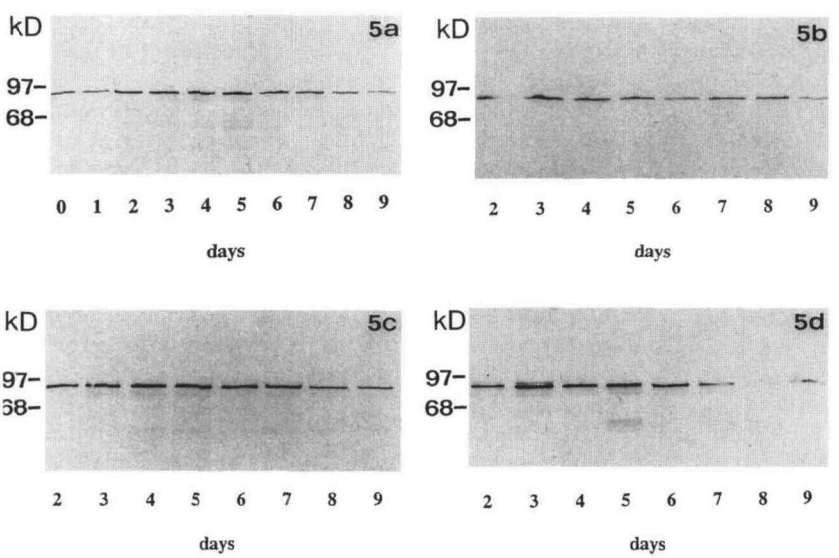


Figure 5. Patterns of LOX-2 protein in the embryo (a), scutellum (b), leaf (c), and root (d) of barley in the course of germination as determined by western blotting using the LOX-2-specific Mab 4.2. Loading of the gel was performed as described for Figure 4. Molecular mass markers are indicated on the left.

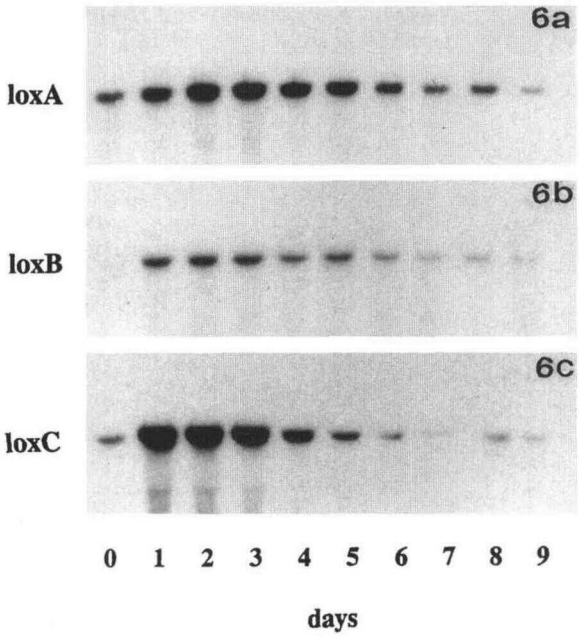


Figure 6. Patterns of LoxA (a), LoxB (b), and LoxC mRNA (c) in barley embryos in the course of germination as determined by northern blotting. Each lane contains 7.5 μg of total RNA.

after d 1 only, and, as for LoxA, its highest mRNA levels were found from d 1 to 5 (Fig. 6b). As for LoxB, transcript levels of LoxC rapidly increased during the 1st d of germination (Fig. 6c). The expression of LoxC was most abundant from d 1 to 3, after which it decreased. Increasing levels of LoxA and LoxC mRNA correlated with increases in LOX-1 and LOX-2 protein during early germination, suggesting that the expression of LOX-1 and LOX-2 are regulated at the pretranslational level.

It should be emphasized that for detection of LoxB mRNA (Fig. 6b), a longer exposure time (i.e. 2 d) was needed than for detection of LoxA and LoxC mRNA (Fig. 6, a and c, respectively; exposure time 6 h). Because we have checked for equal labeling of the probes and equal loading of the lanes (data not shown), it seems likely that in embryos of germinating barley, transcript levels of LoxB are less abundant than those of LoxA and LoxC.

Spatial Expression of Lipoxygenase in Embryos of Germinating Barley

Lipoxygenase Activity

From 2 d after germination, embryos were divided into scutella, leaves, and roots and assayed for lipoxygenase activity and protein levels of both isoenzymes.

In leaves, a distinct optimum in activity of lipoxygenase was detected at 4 d after germination, whereas activity in roots showed a broad optimum between d 4 and 7 (Fig. 1). Lipoxygenase activity in scutella was clearly lower than in leaves and roots and stayed more or less constant over time. Division of (total) leaves of 4-d-old seedlings into coleoptile and first leaf revealed that two-thirds of leaf-associated activity resides in the coleoptile (data not shown).

Currently, we have no explanation for the fact that the sum of activities of the different parts of the embryo often exceeds the activity of the whole embryo. For example, protein determinations showed no discrepancy in recovery, and no action of an activity-stimulating or -inhibiting component in any part of the embryo could be detected.

Lipoxygenase Isoenzyme Protein Levels

The presence of LOX-1 and LOX-2 protein was demonstrated in each seedling part investigated and displayed a similar pattern. In leaves the highest levels are present around d 4 (Figs. 4c and 5c), whereas in roots the highest amount was found from d 3 to 6 (Figs. 4d and 5d). In the scutellum levels seemed to decrease gradually after d 3 (Figs. 4b and 5b). Furthermore, western blotting experiments showed that, in agreement with activity measurements, the highest protein levels of both LOX-1 and LOX-2 within leaves from 4-d-old seedlings were present in coleoptiles (data not shown).

DISCUSSION

The expression of two lipoxygenase isoenzymes during germination of barley embryos was described at the levels of activity, protein, and mRNA. To our knowledge, this is the first example of such an extensive study in any cereal.

Total lipoxygenase activity in dry barley grains is present at a high level, which rapidly decreases 2-fold during the 1st d of germination and subsequently increases, reaching the same level as dry grain. The rapid drop of activity during the 1st d of germination was observed when activity was expressed both as units/10 embryos and as units/gram tissue (data not shown). An up to 2-fold decrease in activity during early germination was also observed by Lulai et al. (1981) and Schwarz and Pyler (1984) and was attributed to lack of oxygen during steeping. This explanation seems unlikely on the basis of the results presented here, since steeping did not form part of the germination procedure. Baxter (1982) and Martel et al. (1993) observed that lipoxygenase activity remained constant in the early germination phase. The subsequent increase in activity observed by several authors (Lulai et al., 1981; Baxter, 1982; Schwarz and Pyler, 1984; Martel et al., 1993) varied from 3- to 6-fold, reaching a lipoxygenase activity that was considerably higher than that in the quiescent grain. The differences in lipoxygenase activity patterns might be explained by the use of different barley varieties and germination conditions.

A comparison between LOX-1 and LOX-2 revealed that the only major difference in temporal expression between the two isoenzymes was observed in the quiescent grain. In the dry grain a high level of LOX-1 was observed, as was evident from hydroperoxide measurements, which showed that lipoxygenase-mediated oxidation of linoleic acid resulted primarily in the production of the LOX-1 product, namely 9-hydroperoxide. In addition, the LOX-1 protein was clearly demonstrable in

western blots. LOX-2 protein was also detectable in western blots but, according to the ratio of hydroperoxides formed from linoleic acid, hardly contributed to the total lipoxygenase activity. The high ratio of 9- to 13-hydroperoxides formed by lipoxygenase from quiescent grains is in agreement with literature values (Yabuuchi, 1976; Baxter, 1982; van Aarle et al., 1991; Yang et al., 1993). LOX-2 was described to be either absent (Yabuuchi, 1976; Yang et al., 1993) or present at a low level (Baxter, 1982; van Aarle et al., 1991), possibly depending on the barley cultivar used (van Aarle et al., 1991).

From 2 d after germination onward, LOX-1 and LOX-2 behaved similarly. This was evident from the almost constant ratio of hydroperoxides formed by lipoxygenase from 2 to 9 d of germination, which is in agreement with data from Yabuuchi (1976) and Yang et al. (1993) and is further supported by the detection of comparable protein patterns for the two isoenzymes. Also in the presence of light, no differences in the expression patterns of LOX-1 and LOX-2 protein in the leaf were observed (data not shown). However, under these circumstances in 4-d-old leaves, total lipoxygenase activity was 2-fold lower than in etiolated leaves (W.L. Holtman, unpublished data). This is in accordance with results described for soybean seedlings, in which a phytochrome-mediated decrease of LOX-1 and LOX-2 activity was found that was due to transcriptional regulation of gene expression (Maccarrone et al., 1991).

Not only were the temporal expression patterns of LOX-1 and LOX-2 during germination similar, but their distributions in the germinating embryo tissues were also similar, with the highest levels being found in leaves and roots. This result differs from the results of Yang et al. (1993), who observed, based on the ratio of lipoxygenase-derived hydroperoxides formed, that in roots of 5-d-old germinated barley, only LOX-2 was present.

Little is known about the regulation of the activity levels of lipoxygenase isoenzymes. During early germination the level of immunoreactive LOX-1 in the extracts decreased and subsequently increased again. This could possibly be due to degradation of lipoxygenase protein. During the later stages of germination the levels of loxA mRNA corresponded to those of LOX-1 protein, suggesting a pretranslational regulation. A similar pretranslational regulation was observed for LOX-2.

A third lipoxygenase isoenzyme may be present in germinating barley, as suggested by the expression of a third lipoxygenase mRNA. The protein it encodes probably is present at low levels if at all, since it was not observed during purification of lipoxygenase isoenzymes from germinating barley (Doderer et al., 1992). This would be in agreement with the low mRNA level for this isoenzyme. It may be present at higher levels, for example during grain development, in the mature plant or under stress conditions (J.R. von Mechelen, M. Smits, A. Graner, A.C. Douma, N.J.A. Sedee, R.C. Schuurink, F. Heidekamp, and B.E. Valk, unpublished data).

The physiological role of LOX-1 and LOX-2 is unclear at present. The enzymes give rise to different subsets of lipoxygenase products. The major difference in their expression is in the quiescent grain, whereas over the course of germination the two enzymes behave similarly. In soybean, the best-studied plant with respect to lipoxygenase, two classes of lipoxygenase isoenzymes have been described (Kato et al., 1992). Isoforms of the first class, namely L-1, L-2, and L-3, are already present in the quiescent grain and gradually disappear during germination. They have been inferred to play a role in the development of germination capability (Hildebrand et al., 1991) or in plant defense (Kato et al., 1993). LOX-1 from barley is also present in the quiescent grain but also appears to be expressed during germination. Therefore, in the quiescent grain, LOX-1 may function in plant defense or may be involved in the development of germination capability, whereas in the germinating grain, LOX-1 may have a different function. In cotyledons of soybean three isoenzymes of a second class, L-4, L-5, and L-6, appeared after germination, and these may play a role in growth and development (Kato et al., 1992) or, as was indicated for L-4, they may function as storage proteins (Kato et al., 1993). LOX-2 from barley resembles those isoenzymes in this respect, because it shows a similar expression pattern. Further indications regarding the physiological roles of LOX-1 and LOX-2 may be obtained from a study of their location in the tissue and at the subcellular level. The Mab generated as part of this study will form an excellent tool in this respect.

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