Modulation of soybean lipoxygenase expression and membrane oxidation by water deficit

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Abstract The modulation of the activity and expression of soybean lipoxygenases 1 (LOX-1) [Vliegenthart, J.F.G. and Veldink, G.A. (1982) in: Free Radicals in Biology (Pryor, W.A., Ed.) pp. 29–64, Academic Press, New York] and 2 (LOX-2) by water deficit (osmotic stress) has been investigated, by following gene expression at the transcriptional and translational levels. Osmotic stress enhanced the transcription of the genes of both isoenzymes, leading to increased levels of the corresponding mRNAs, protein contents and specific activities. Abscisic acid (ABA) did not mediate enhancement of LOX expression, but caused a decrease of LOX-2 activity and was ineffective on LOX-1. Water deficit also caused oxidative modification of soybean membrane pool lipids [Schmidt, W.E. and Ebel, J. (1987) Proc. Natl. Acad. Sci. USA 84, 4117–4121], attributable to the increase of conjugated hydroperoxides in the esterified fatty acids of the lipid bilayer.

Key words: Lipoxygenase-1; Lipoxygenase-2; Water deficit; Abscisic acid; Biomembrane; Soybean

1. Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase, E.C. 1.13.11.12) are non-heme iron-containing dioxygenases, which dioxygenate unsaturated fatty acids with at least one 1,4-*Z,Z*-pentadiene system to yield *Z,E*-conjugated hydroperoxides. These enzymes are ubiquitous in plants [1,2] and animals [3]. The first lipoxygenase was isolated from soybeans (*Glycine max*), which contain at least seven distinct isoenzymes [4], LOX-1 and -2 being the major ones [5,6]. Plant lipoxygenases are probably involved in a variety of physiological processes (see [2] for a review), including the plant's response to biological [7] and physical [8–10] stress factors.

Soybean seedlings osmoregulate when the supply of water is limited around the roots [11]. The mechanism of osmoregulation involves solute accumulation [11], growth inhibition [12], modulation of gene expression [13] and deterioration of cell membranes [14]. Here, the possible involvement of LOX-1 and -2 in the response of three-day-old, dark-grown soybean seedlings to osmotic stress has been investigated, as well as the effect of abscisic acid (ABA) on the expression of both isoenzymes. Finally, also the possible nature of the membrane deterioration due to osmotic stress has been investigated.

2. Materials and methods

Chemicals were of the purest analytical grade. Octadecyl-SPE columns were purchased from Baker. Goat anti-rabbit and goat anti-mouse IgGs conjugated with alkaline phosphatase (GAR-AP and GAM-AP, respectively) were from Bio-Rad. Yeast RNA, DIG oligonucleotide Tailing kit, nylon membranes and DIG Luminescent Detection kit were from Boehringer Mannheim. Hybond-mAP was purchased from Amersham.

2.1 Plant material and lipoxygenase assay

Soybean (*Glycine max* (L.) Merrill) seeds were soaked in tap water overnight at room temperature, then they were grown at 22°C, in the dark, for three days. Three-day-old (3d-old), dark-grown seedlings were subjected to water deficit and ABA treatment as described below, then they were homogenized and assayed for LOX-1 and -2 specific activities according to [8]. LOX-1 and -2 specific activities were expressed as μmol $O_2 \cdot min^{-1} \cdot mgP^{-1}$. Protein concentration of the homogenates was determined as reported [15], using BSA as a standard.

2.2 Enzyme-linked immunosorbent assay (ELISA)

The amounts of LOX-1 and -2 present in the homogenates of 3d-old, dark-grown seedlings were estimated by ELISA, performed by coating immunoplates with 50 μg of each sample per well [9]. Anti-LOX-1 monoclonal antibodies (mAbs) and anti-LOX-2 polyclonal antibodies (pAbs), produced and purified as described [9], were reacted with GAM-AP and GAR-AP (diluted 2000-fold), respectively. Color development of the alkaline phosphatase reaction was recorded at 405 nm. The A_{405} values of the samples were within the linearity range of the calibration curves [9]. Controls were carried out by using non-immune sera from mouse (for mAbs) or rabbit (for pAbs). Controls also included wells coated with different amounts of BSA.

2.3 Dot-hybridization analysis

Total RNA was isolated from soybean seedlings according to Logemann [16] and was used to further purify poly(A)$^+$RNA by mAP chromatography [17]. Poly(A)$^+$RNAs from different samples were blotted (3 μg/dot) onto positively charged nylon membranes, by means of a Bio-Dot apparatus (Bio-Rad). Two oligonucleotides were prepared with a Biosearch 8600 DNA Synthesizer and were separately used in dot-blot analyses: 5′-CTGCTGAGCCATCAGGGTTAAC-3′, specific for the LOX-1 mRNA [5], and 5′-CTCCTCTGTTCAGGATTCCCG-A-3′, specific for the LOX-2 mRNA [6]. The probes were labeled at their 3′-ends with terminal transferase in the presence of DIG-dUTP [18]. (Pre)hybridization of the filters and chemiluminescent detection of the samples were performed as reported [9]. The amounts of LOX-1 and LOX-2 mRNAs were quantified by laser densitometry [19], comparing the peak areas of the samples with those of the calibration curves [9]. Controls were made by blotting different amounts of yeast RNA.

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Abbreviations: LOX-1, lipoxygenase-1; LOX-2, lipoxygenase-2, ABA, abscisic acid; mAP, messenger affinity paper; BSA, bovine serum albumin, DIG-dUTP, digoxigenin-labeled deoxyuridine-triphosphate

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2.4. Membrane isolation and spectrophotometric analysis of lipid extracts

Three-day-old, dark-grown soybean seedlings were homogenized to isolate a whole membrane fraction as described [20]. The free fatty acids were separated from the esterified fatty acids on octadecyl-SPE columns according to Kühn [21], then the absorbances of the fractions were recorded at 205 nm and 235 nm on a Hewlett Packard 8450A double-beam diode array spectrophotometer. The oxidative modification of the membrane lipid fraction was expressed by means of the oxidative index, i.e. the A_{235}/A_{205} ratio [21]. The amount of conjugated hydroperoxides present in the samples was calculated using the extinction coefficient at 235 nm of 25,000 $M^{-1} \cdot cm^{-1}$ [22].

2.5. Water deficit and ABA treatment

Three-day-old, dark-grown soybean seedlings were subjected to water deficit (osmotic stress) by immersing roots in 200 mM, 400 mM, 600 mM or 800 mM mannitol solutions for 5 h in the dark, as described [23]. After treatment, seedlings were homogenized and the homogenates were kept at −80°C until used for protein determination, LOX activity assay and immunochemical analysis. ABA treatments were performed by immersing 3d-old, dark-grown seedling roots in 50 μM, 100 μM, 200 μM, 300 μM or 400 μM ABA solutions for 5 h in the dark.

Each data point reported in this paper is the mean of three independent experiments. Standard deviation values not indicated in the figures were <8%.

3. Results

3.1. Effect of water deficit and ABA on lipoxygenase activity

Dark-grown, 3d-old soybean seedlings subjected to water deficit showed changes in LOX-1 and -2 specific activities (Table 1). Osmotic stress induced an increase of both specific activities, proportional to the concentration of mannitol used. Due to solubility limits, mannitol concentrations higher than 800 mM could not be used, therefore this concentration value was chosen to further investigate the responsiveness of lipoxygenases to water deficit. The time course of the 800 mM mannitol-induced increase of LOX-1 and -2 specific activities showed a plateau at 170% and 155% of the control, respectively, after 4 h of treatment (Fig. 1). Such increase of LOX-1 and -2 activities could not be reversed by a subsequent incubation of the seedlings under well watered conditions.

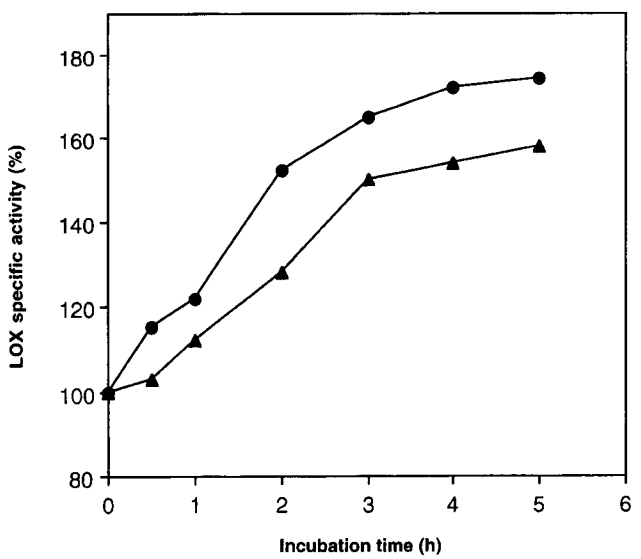

Fig. 1. Time courses of the water deficit-induced increases in LOX-1 (circles) and -2 (triangles) specific activities. LOX-1 and -2 specific activities are expressed as percentage of the control values, i.e. 2.60 (LOX-1) and 6.50 (LOX-2) μmol $O_2 \cdot min^{-1} \cdot mgP^{-1}$.

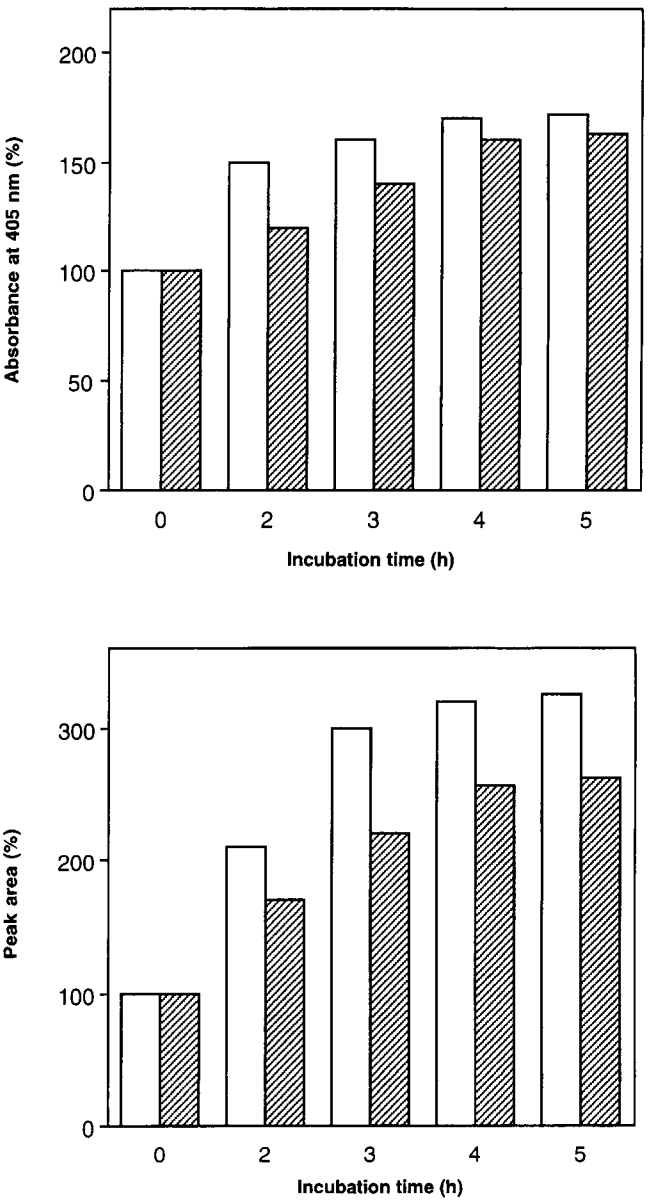

Fig. 2. (Upper panel) LOX-1 (empty bars) and LOX-2 (hatched bars) content in soybeans incubated in 800 mM mannitol. The amounts of LOX are expressed as percentage of the A_{405} control values, i.e. 0.15 (LOX-1) and 0.21 (LOX-2). (Lower panel) LOX-1 (empty bars) and LOX-2 (hatched bars) mRNA levels of the same samples as in the upper panel. The amounts of LOX mRNAs are expressed as percentage of the peak areas of the control samples, i.e. 1.60 AU · mm (LOX-1) and 2.12 AU · mm (LOX-2).

Three-day-old, dark-grown soybean seedlings exposed to different concentrations of ABA (for 5 h, in the dark) showed an irreversible decrease in LOX-2 specific activity, which levelled off at 80% of the control value upon treatment with 200 μM ABA (Table 2). Unlike LOX-2, LOX-1 was unaffected by ABA, whatever the concentration used (Table 2). The time-course of the 200 μM ABA-induced decrease of LOX-2 specific activity showed a plateau at 80% of the control value after 5 h of incubation (not shown).

3.2. Lipoxygenase content in mannitol- and ABA-treated seedlings

Incubation of 3d-old, dark-grown soybean seedlings in

800 mM mannitol induced an increase in the amount of both LOX-1 and -2, proportional to the duration of the treatment (Fig. 2, upper panel). After 4 h of osmotic stress, the content of both isoenzymes levelled off at about 165% of the control. Unlike water deficit, ABA did not induce any change in LOX content, whatever the duration of the treatment up to 5 h (data not shown).

3.3. Dot-hybridization analysis

Poly(A)$^+$RNAs extracted from seedlings subjected to osmotic stress were hybridized with probes specific for LOX-1 or -2 mRNAs. In Fig. 2 (lower panel) it is shown that the steady state levels of both mRNAs increased upon treatment with 800 mM mannitol, longer incubations yielding higher levels of the specific mRNAs. LOX-1 mRNA reached a maximum of 320% of the control after 4 h of incubation, whereas LOX-2 mRNA amounted at 260% of the control value after the same treatment. The incubation of 3d-old, dark-grown soybean seedlings in 200 μM ABA did not influence the steady state level of either mRNA, whatever the duration of the treatment up to 5 h (data not shown).

3.4. Oxidative modification of biomembranes of mannitol-treated seedlings

A membrane pool was isolated from 3d-old, dark-grown soybean seedlings subjected to osmotic stress, then the free fatty acid fraction was separated from the esterified fatty acid fraction and both were analysed. In Fig. 3 it is shown that the oxidative index of the esterified lipids increased with prolonging the incubation in 800 mM mannitol, whereas the free fatty acids did not show any change of the A_{235}/A_{205} ratio under the same conditions.

4. Discussion

Soybean seedlings respond to water deficit with a fast increase in specific activity of their major lipoxygenases, LOX-1 and -2 (Table 1, Fig. 1). Such increase was paralleled by the increase of LOX content and mRNA (Fig. 2), indicating that osmotic stress modulates the expression of lipoxygenase genes at the transcriptional level. The changes in the steady state levels of LOX mRNAs were larger than the changes in LOX activity and content, a finding which is in line with the known accumulation of mRNAs upon water deficit in stressed seedlings [24]. These results extend preliminary experiments on two new classes of lipoxygenase genes isolated from soybeans in different developmental stages [23]. Abscisic acid is generally reported to mediate the plant response to water deficit [25], although such mediation does not always occur [26]. Here, it is shown that osmotic stress enhances LOX-1 and -2 expression without the intermediacy of ABA. Under our experimental conditions, ABA treatment led to some decrease of LOX-2 specific activity, being ineffective towars LOX-1 (Table 2). This finding seems noteworthy in the light of the role of type-2 lipoxygenases in the biosynthesis of abscisic acid [27], which might suggest a feedback inhibition of the enzyme activity by the phytohormone. The effects of ABA on the LOX-mediated metabolism of linoleic acid in maize embryos [28] corroborate this hypothesis. Moreover, the different modulation of lipoxygenases 1 and 2 by ABA extends the concept of a differ-

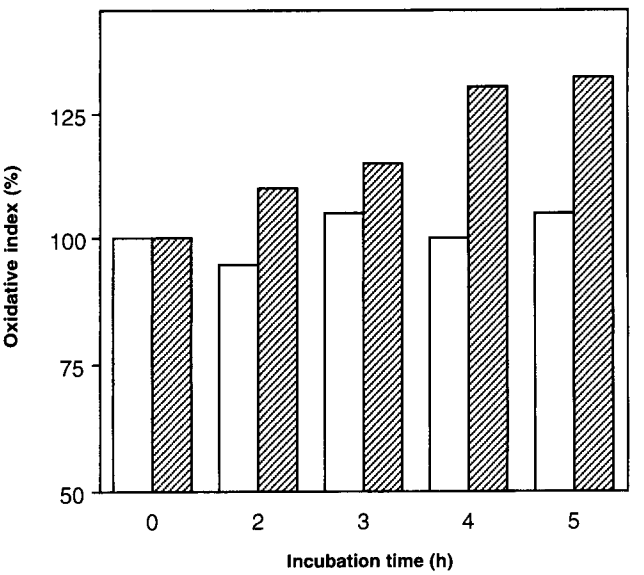

Fig. 3. Oxidative modification of membrane pool lipids isolated from 3d-old, dark-grown soybean seedlings subjected to water deficit. The A_{235}/A_{205} ratios of the free fatty acids (empty bars) and the esterified fatty acids (hatched bars) present in the lipid fraction are expressed as percentage of the control values, i.e. 0.15 and 0.46, respectively.

Table 1
Effect of osmotic stress on lipoxygenase 1 (LOX-1) and 2 (LOX-2) specific activities of 3d-old, dark-grown soybean seedlings

Mannitol concentration (mM)	LOX-1 specific activity	LOX-2 specific activity
0	2.60 ± 0.21 (100)	6.50 ± 0.52 (100)
200	2.86 ± 0.23 (110)	6.83 ± 0.55 (105)
400	2.99 ± 0.24 (115)	7.15 ± 0.57 (110)
600	3.38 ± 0.27 (130)	8.13 ± 0.65 (125)
800	4.55 ± 0.36 (175)	10.40 ± 0.83 (160)

Osmotic stress was induced by immersing seedling roots in mannitol solutions for 5 h in the dark. LOX specific activities, expressed as μmol $O_2 \cdot min^{-1}$ mgP^{-1}, are also expressed as percentage of the control, arbitrarily set to 100 (values in parentheses).

Table 2
Effect of Abscisic acid (ABA) on lipoxygenase 1 (LOX-1) and 2 (LOX-2) specific activities of 3d-old, dark-grown soybean seedlings

ABA concentration (μM)	LOX-1 specific activity	LOX-2 specific activity
0	2.60 ± 0.21 (100)	6.50 ± 0.52 (100)
50	2.34 ± 0.19 (90)	6.18 ± 0.49 (95)
100	2.73 ± 0.22 (105)	5.52 ± 0.44 (85)
200	2.60 ± 0.21 (100)	5.20 ± 0.42 (80)
300	2.47 ± 0.20 (95)	5.20 ± 0.42 (80)
400	2.60 ± 0.21 (100)	5.20 ± 0.42 (80)

Seedling roots were immersed in ABA solutions for 5 h in the dark. LOX-1 and -2 specific activities are expressed as in Table 1

ential regulation of plant isoenzymes by this phytohormone [29].

Water deficit has also been reported to impair cell membrane functioning [14]. Here, it is shown that osmotic stress increases the oxidative index of biomembranes, by increasing the hydroperoxide content of the lipid ester fraction (Fig. 3). This finding is interesting, because changes in membrane hydroperoxides lead to changes in membrane fluidity and permeability, ultimately giving rise to dysfunctioning of the lipid bilayer. Moreover, the observed enhancement of both LOX activity and membrane oxidative state in response to water deficit corroborates the hypothesis of a role of LOX in plant membrane deterioration [30].

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