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Thermal injury and ozone stress affect soybean lipoxygenases expression

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The effects of thermal injury (cold and heat shock) and ozone treatment on lipoxygenases 1 (LOX-1) and 2 (LOX-2) of soybean seedlings have been investigated. Cold stress led to a decrease of the specific activities of both isoenzymes, attributable at least in part to a down-regulation of gene expression at the translational level. Both heat shock and ozone treatment enhanced lipoxygenases-specific activities, acting at the level of transcription of the genes. It is proposed that LOX-1 and LOX-2 are involved in the thermotolerance of soybeans and in the precocious aging induced by ozone.

Lipoxygenase-1; Lipoxygenase-2; Cold; Heat; Ozone; Soybean

1. INTRODUCTION

Lipoxygenases (E.C. 1.13.11.12) are non-heme iron-containing dioxygenases, which use molecular oxygen to produce hydroperoxides from unsaturated fatty acids containing one or more 1,4-*Z*,*Z*-pentadiene systems. Widespread in nature among animals and plants [1], lipoxygenases were isolated for the first time from soybeans (*Glycine max*), which contain 7 distinct isoenzymes [2]. Mature soybean seeds contain high levels of LOX-1 and LOX-2, which are encoded by two different genes [3,4] and have different kinetic features [5].

Many physiological roles have been proposed for plant lipoxygenases, including roles in growth and development, in the synthesis of regulatory molecules and in senescence (see [6] for a review). Moreover, lipoxygenases are involved in the plant's response to stress, i.e. pathogen attack [7], wounding [8], water deficit [9] and anoxia [10].

Soybeans are very sensitive to cold stress [11,12] and heat shock [13,14], which both affect protein synthesis and cell metabolism [15]. Thus, the possible modulation of lipoxygenase activity and expression by temperature shifts was investigated, in order to find out whether lipoxygenases take part in the homeostatic response [16] of soybeans to thermal injury.

Ozone treatment causes peroxidation of the lipids of plant membranes, leading to premature plant senescence [17]. Because lipoxygenases are involved in membrane deterioration during plant aging [18], we studied the possible effect of ozone stress on the expression of lipoxygenase genes, to ascertain whether or not ozone could play a role as regulator of these enzymes.

Evidence is presented for the involvement of lipoxygenases in the plant's response to temperature and ozone stresses.

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

2. MATERIALS AND METHODS

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

2.1. *Plant material and lipoxygenase assay*

Soybean (*Glycine max* (L.) Merrill) seedlings were germinated and homogenates were prepared as described [10]. Enzyme activity was measured polarographically, at 25°C, in a solution of 1.8 mM linoleic acid in air-saturated 0.1 M sodium borate buffer, pH 9.0 (LOX-1), or 0.1 M sodium phosphate buffer, pH 6.6 (LOX-2). Protein concentration was determined according to Bradford [19], using BSA as a standard.

2.2 *Determination of LOX content*

The amount of lipoxygenases 1 and 2 present in the seedling homogenates was estimated by enzyme-linked immunosorbent assay (ELISA), performed by coating immunoplates with 50 μg/well of each sample, as described [10]. Anti-LOX-1 monoclonal antibodies (mAbs) were produced at CIVO-TNO (Zeist, The Netherlands) using pure LOX- and a single hybridoma clone. They were purified according to [20]. Rabbit anti-LOX-2 polyclonal antibodies (pAbs) were produced and purified according to Vernooy-Gerritsen [21]. The anti-LOX-1 mAbs and the anti-LOX-2 pAbs were independently used as first antibody, diluted 500-fold. GAM-AP and GAR-AP (diluted 3000-fold) were used as second antibody and were reacted with the anti-LOX-1 mAbs and the anti-LOX-2 pAbs, respectively. Color develop-

ment of the alkaline phosphatase reaction was recorded at 405 nm. Calibration curves were drawn after reacting different amounts of LOX-1 and LOX-2, purified as described [22], with the anti-LOX-1 and anti-LOX-2 antibodies. The A_{405} values of the seedling homogenates were within the linearity range of the calibration curves. Controls were carried out by using non-immune sera from mouse (mAbs) and rabbit (pAbs). Controls also included wells coated with different amounts of BSA.

2.3. *Labeling of oligonucleotides*

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 [3]; 5'-CTCCTCTGTTCAGGATTCCCGA-3', specific for the LOX-2 mRNA [4]. The probes were labeled at their 3'-end with terminal transferase in the presence of DIG-dUTP, according to Schmits [23].

2.4. *Isolation of poly(A)⁺RNA and dot-hybridization analysis*

Total RNA was isolated from soybean seedlings and checked for purity and intactness according to Logemann [24]. Poly(A)⁺RNA was purified from total RNA by mAP chromatography [25] and was blotted (3 μg/dot) onto positively charged nylon membranes by means of a Bio-Dot apparatus (Bio-Rad). Filters were pre-hybridized and hybridized at 45°C as described [26], using 50 ng/ml DIG-labeled probe in the hybridization buffer. After washing, the filters were subjected to chemiluminescent detection, using the DIG Luminescent Detection Kit according to the manufacturer's instructions. The amounts of LOX-1 and LOX-2 mRNAs were quantified by means of laser densitometry [27], using an Ultroscan XL Enhanced Laser Densitometer (LKB). A calibration curve was drawn by plotting the peak areas corresponding to different amounts of poly(A)⁺RNA isolated from untreated 2-day-old soybean seedlings (control) vs. the poly(A)⁺RNA concentration. The peak areas of the unknown samples were within the linearity range of the calibration curve. Controls were made by blotting different amounts of yeast RNA.

2.5. *Thermal injury and ozone treatment*

Two-day-old soybean seedlings, grown in a greenhouse at 22°C in the dark, were incubated at different temperatures (in the range 5–50°C) for 5 h in the dark, as reported [28], then they were collected and frozen at −80°C until use. Ozone treatments were performed essentially as reported [17], generating ozone by means of an electrical discharge ozonizer (ROTAX). Jars containing dark-grown, 2-day-old seedlings were flushed with a stream of air and ozone, yielding an ozone flux of 5 ml/min, as determined according to Zwick [29]. During the treatment of the seedlings the plant cabinet was kept at 22°C in the dark. Controls were made by subjecting soybeans to the same treatment with the ozonizer switched off. At fixed times, seedlings were harvested and frozen at −80°C until use.

Each data point reported in this paper is the mean of three independent experiments (S.D. <5%).

3. RESULTS

3.1. *Lipoxygenase activity upon thermal injury and ozone stress*

Dark-grown, 2-day-old soybean seedlings, subjected to temperature shifts from 5°C to 50°C, showed changes in LOX-1 and LOX-2 specific activities (Fig. 1). Incubations at 5°C and at 45°C were the most effective in decreasing and increasing, respectively, the activity of both isoenzymes. Therefore, these temperatures were chosen to further investigate the effect of thermal stress on lipoxygenases. Cold induced a fast decrease of LOX activities which leveled off at 45% of the control after

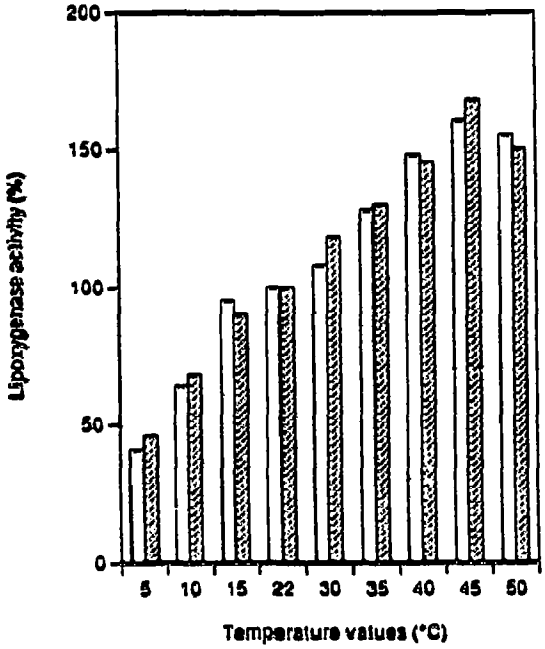

Fig. 1. LOX-1 (empty bars) and LOX-2 (hatched bars) specific activities after thermal injury of the seedlings. Activities are expressed as a percentage of the control (22°C) values, i.e. 1.50 (LOX-1) and 5.10 (LOX-2) μmol $O_2 \cdot min^{-1} \cdot mg\ P^{-1}$.

5 h (Fig. 2A). Heat induced a fast decrease of LOX activities which reached a maximum of 165% of the control value after 5 h of incubation (Fig. 2C). The incubation of the seedlings for 5 h at 22°C after treatment at 5°C reversed the cold-induced inhibition of LOX activities (Fig. 2B). However, the heat effect could not be reversed by a subsequent incubation of the heat-stressed seedlings at 22°C.

Lipoxygenase activities doubled in seedlings exposed to ozone for 3 h (Fig. 2D). The increase was proportional to the duration of the treatment and was irreversible. Further incubation of the ozone-stressed soybeans under a normal atmosphere for 6 h did not affect the lipoxygenase specific activities.

3.2. *Lipoxygenase content in temperature- and ozone-stressed seedlings*

The amount of LOX-1 and LOX-2 present in the homogenates was determined by ELISA, the calibration of which is shown in Fig. 3A. The anti-LOX-1 mAbs did not cross-react with LOX-2 in the ELISA test, while the anti-LOX-2 pAbs cross-reacted 10-fold less with LOX-1 than with LOX-2. Therefore, these antibodies were used to specifically detect LOX-1 and LOX-2 in the samples. The results obtained by reacting the homogenates with the anti-LOX-2 pAbs were the same as the ones obtained with the anti-LOX-1 mAbs. It is shown in Fig. 3B that the LOX-1 content of seedlings incubated for different lengths of time at 5°C decreased, levelling off at 75% of the control after 5 h. A further incubation of

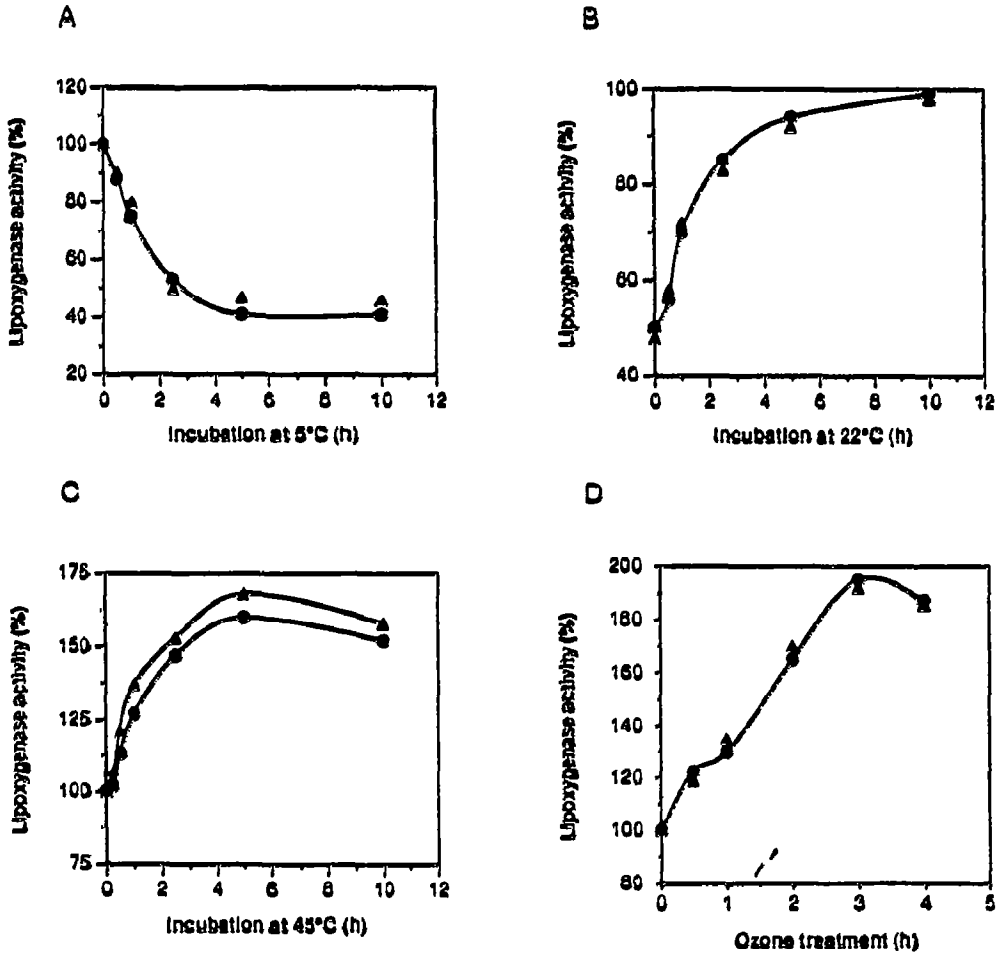

Fig. 2. LOX-1 (●) and LOX-2 (▲) specific activities were measured upon incubation of the seedlings: (A) at 5°C; (B) at 22°C, following incubation for 5 h at 5°C; (C) at 45°C and (D) under ozone. The untreated control had the same LOX-1 and LOX-2 activities as the control in Fig. 1.

the stressed seedlings at 22°C fully reversed the cold effect in 5 h (Fig. 3B). Soybeans subjected to heat shock showed an increase in the LOX-1 content, which reached a maximum of 150% of the control after 5 h (Fig. 3C). In analogy, the ozone treatment led to an increase in the LOX-1 content, which leveled off at 190% of the control after 3 h (Fig. 3D).

3.3. *Dot-hybridization analysis*

The steady-state levels of mRNAs coding for LOX-1 and LOX-2 were quantified by dot-hybridization, which yielded a linear relationship between the amount of poly(A)$^+$RNA and the peak areas recorded (Fig. 4A). The results obtained by hybridizing with the LOX-2 probe were identical to the ones obtained by hybridizing with the probe specific for the LOX-1 mRNA. It is shown in Fig. 4B that the incubation at 5°C did not change the level of LOX-1 mRNA compared to the control. On the other hand, the heat shock caused an increase in the LOX-1 mRNA up to 150% of the untreated control (Fig. 4C). A similar trend was observed in the ozone-stressed samples, whose LOX-1 mRNA level increased to a maximum of 180% of the control after 3 h of treatment (Fig. 4D).

4. DISCUSSION

Soybean LOX-1 and LOX-2 specific activities changed when the seedlings were subjected to thermal shifts, the trend being a decrease at low temperatures and an increase at high temperatures (Fig. 1). Both cold- and heat-induced changes of LOX activities were fast (Fig. 2A and C), but heat had an irreversible effect, at variance with cold (Fig. 2B). The LOX content of the samples followed the same trend as the activity. In particular, cold-stressed soybeans displayed a 75% decrease in the amount of LOX, a decrease which was fully reversible (Fig. 3B) and less pronounced than the decrease of activity in the same samples (45% of the control). Moreover, the cold injury did not change the LOX mRNA levels (Fig. 4B). Therefore, cold regulates LOX activity in part by acting at the translational level of gene expression. Cold-induced modifications of the enzyme conformation [15] and changes of the level of

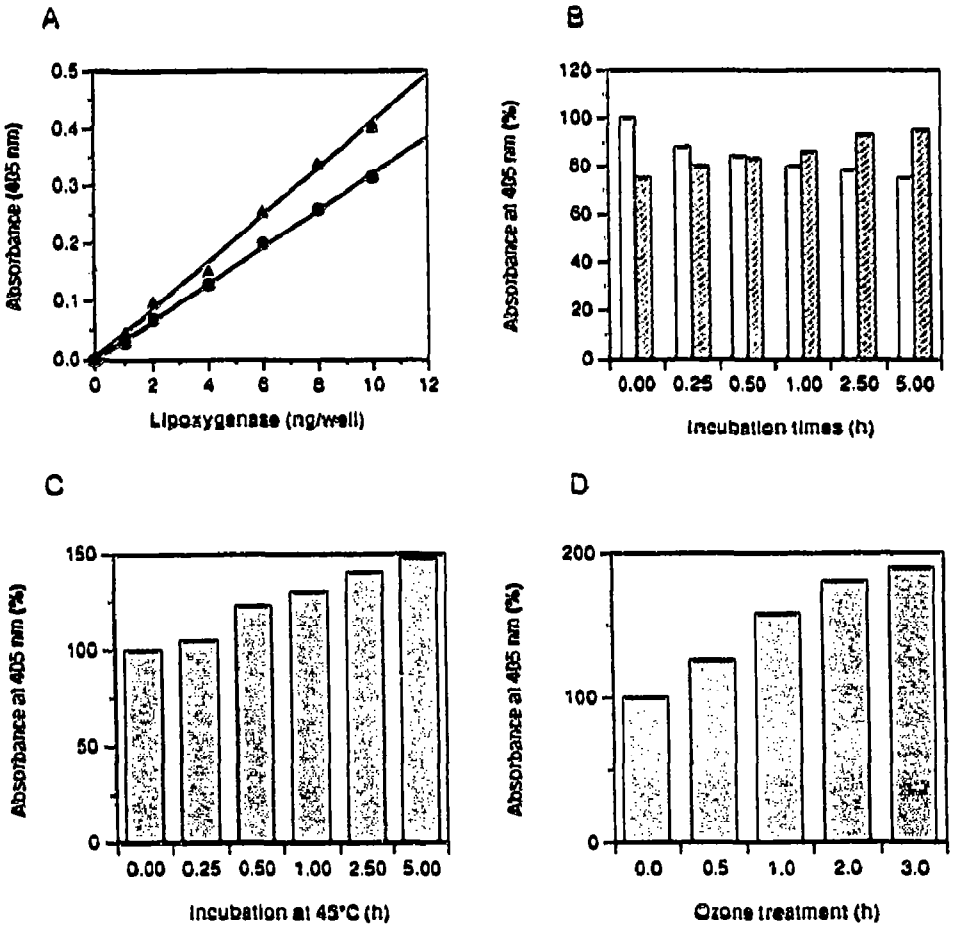

Fig. 3. (A) Reactivity of pure LOX-1 (●) with anti-LOX-1 mAbs and pure LOX-2 (▲) with anti-LOX-2 pAbs in the ELISA test. LOX-1 content of seedlings incubated: (B) at 5°C (hatched bars) and at 22°C following incubation of 5 h at 5°C (empty bars); (C) 45°C and (D) under ozone. The amounts of LOX-1 are expressed as a percentage of the untreated control (A_{405} = 0.150).

active enzyme present in the cells [12] might contribute to the cold effect as well. Heat shock induced an increase in the LOX content (Fig. 3C), thus acting on the synthesis and/or degradation of the enzymes. The analysis of the steady-state levels of the LOX mRNAs showed that heat shock yielded an increase in the LOX mRNAs (Fig. 4C), an increase which well paralleled the increases of LOX activity and content. Therefore, heat regulates LOX genes expression at the level of transcription.

Cold stress of seedlings alters the properties of plasma membranes [30] and acts as a regulator of enzymes involved in the synthesis of membrane constituents [12]. Lipoxygenases are involved in maintaining membrane integrity, both in animals and plants [31], therefore the down-regulation of LOX could play a role during cold acclimatization, conferring to soybean a better resistance [32]. Heat shock causes dramatic changes in plants, including soybeans [13,14]. The increase of LOX activity and expression upon heat shock of the seedlings suggest that LOX might take part in the heat shock system of soybeans [13]. Unlike heat shock proteins involved only in the homeostatic response to heat shock [16], LOX induction was not easily reversible. This feature can be explained by recalling that heat stress induces accelerated aging of plants [33] and that LOX activity increases in senescing plant tissues [8], thus mediating the process of membrane deterioration [18]. Therefore, the increase of LOX expression upon heat shock could also be involved in the heat-promoted senenescence of the seedlings, which renders LOX regulation more complex.

LOX activities were irreversibly doubled by 3 h of ozone treatment (Fig. 2D), which increased LOX content (Fig. 3D) and mRNA level (Fig. 4D) to the same extent as activity. Thus, ozone modulates LOX expression acting on the transcription of the LOX genes. Ozone causes several damages to plants [34], mainly oxidizing membrane lipids [35], and induces premature plant senescence through membrane deterioration [17]. Lipoxygenases are involved in the control of membrane stability [6,8,18] and could mediate the ozone effect, contributing to its toxicity by producing free radicals and alkyl hydroperoxides [36].

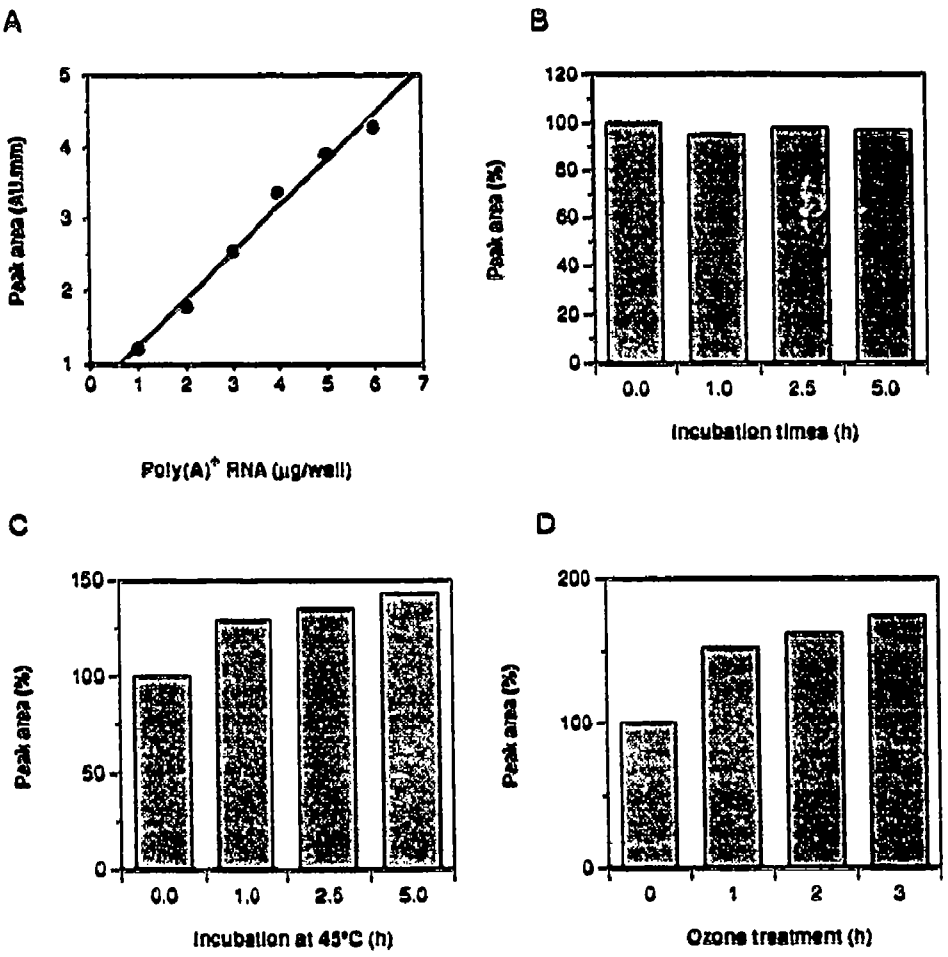

Fig. 4. (A) Reactivity of control poly(A)⁺RNA with the probe specific for the LOX-1 mRNA. Inset. Dots corresponding to 1, 2, 3, 4, 5 and 6 µg poly(A)⁺RNA (A–F, respectively) and to 30 µg yeast RNA (G). LOX-1 mRNA levels of seedlings incubated: (B) at 5°C; (C) at 45°C and (D) under ozone. The relative amounts of LOX-1 mRNA are expressed as a percentage of the untreated control (peak area = 2.4 AU·mm).

The observation that cold stress, heat shock and ozone can ultimately act on cell membranes corroborates the hypothesis of a major role for lipoxygenases in the control of membrane integrity.

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