Preferential induction of a 9-lipoxygenase by salt in salt-tolerant cells of *Citrus sinensis* L. Osbeck

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**Abstract.** Recent findings in our laboratory suggested that in citrus cells the salt induction of phospholipid hydroperoxide glutathione peroxidase, an enzyme active in cellular antioxidant defense, is mediated by the accumulation of hydroperoxides. Production of hydroperoxides occurs as a result of non-enzymatic autoxidation or via the action of lipoxygenases (LOXs). In an attempt to resolve the role of LOX activity in the accumulation of peroxides we analyzed the expression of this protein under stress conditions and in cells of *Citrus sinensis* L. differing in sensitivity to salt. Lipoxygenase expression was induced very rapidly only in the salt-tolerant cells and in a transient manner. The induction was specific to salt stress and did not occur with other osmotic-stress-inducing agents, such as polyethylene glycol or mannitol, or under hot or cold conditions, or in the presence of abscisic acid. The induction was eliminated by the antioxidants dithiothreitol and kaempferol, thus once more establishing a correlation between salt and oxidative stresses. Analyses of both in vitro and in vivo products of LOX revealed a specific 9-LOX activity, and a very fast reduction of the hydroperoxides to the corresponding hydroxy derivatives. This suggests that one of the metabolites further downstream in the reductase pathway may play a key role in triggering defense responses against salt stress.

**Key words:** Antioxidant – *Citrus* (salt tolerance) – Lipoxygenase – Oxidative stress – Oxylin – Salt tolerance

**Introduction**

Lipoxygenases (LOXs; EC 1.13.11.12) refer to a class of non-heme iron-containing dioxygenases that catalyze the hydroperoxidation of long-chain alkyl chains containing (1Z,4Z)-pentadiene structures. Lipoxygenases are ubiquitous among eukaryotic organisms and have been demonstrated to exist in many tissues of numerous higher plants and animals (Rosahl 1996). Several isozymes differing in their cellular localization and substrate specificity have been described (Feussner and Wasternack 1998). Lipoxygenases are mostly soluble enzymes and sequences of many cDNA clones have shown high homology between isozymes within a plant (i.e. soybean) (Bunker et al. 1995) and between clones isolated from different plants (Siedow 1991). In plants, the substrates of LOXs are mainly linoleic acid (18:2) and linolenic acid (18:3). These polyenoic fatty acids can be converted by LOXs into hydroperoxy fatty acids with the hydroperoxy group positioned either at C-9 or C-13 of the 18-carbon chain (Feussner and Wasternack 1998). Lipoxygenases can also dioxygenate fatty acids esterified in glycerolipids such as phospholipids and apparently glycolipids, but free fatty acids are the preferred substrates for most LOXs, at least in vitro (Rosahl 1996). As described in Fig. 1, the LOX-derived hydroperoxy polyenoic fatty acids (Hydro Peroxy OctadecaDienoic, HPOD; derived from 18:2 or Hydro Peroxy OctadecaTrienoic, HPOT; derived from 18:3) can be further converted in different reactions of the LOX pathway (Blee 1998): (i) by a peroxidase or reductase leading to hydroxy polyenoic fatty acids (HOD or HOT), (ii) by a LOX leading to keto polyenoic fatty acids, (iii) by a divinyl ether synthase leading to vinyl ether-containing polyenoic fatty acids, (iv) by an allene oxido synthase leading to jasmionic acid and methyl jasmonate, and (v) by a hydroperoxide lyase leading to...
Active oxygen species bring about peroxidation of membrane lipids that lead to membrane damage (Scandalios 1993). Since lipid peroxidation is a symptom most easily ascribed to oxidative damage and an oxidative stress component is involved in many stresses, it is often used as an indicator of the stress (Bohnert and Sheveleva 1998). Oxidative stress has been implicated in salt stress (Hernandez et al. 1993, 1995; Gossett et al. 1994, 1996; Gueta-Dahan et al. 1997), in water deficit (Leprince et al. 1990; Gogorcena et al. 1995; Olsson 1995), in cold stress (Bohnert and Sheveleva 1998), in freezing tolerance (McKersie et al. 1993; Zhang and Kirkham 1996; Bruggermann et al. 1999), in senescence (Olsson 1995; Thompson et al. 1998), and in biotic stresses caused by fungal (Kondo et al. 1993) and microbial invasion (Popham and Novacky 1991).

It was shown that salt stress caused a high level of lipid peroxidation in citrus cells (Gueta-Dahan et al. 1997) and in the cultivated tomato (Lycopersicon esculentum), while the level of peroxidation in the wild relative, salt-tolerant, tomato (L. pennellii) was only marginal (Shalata and Tal 1998). It should be noted that, in these studies, lipid peroxidation was estimated by malondialdehyde. This compound is a breakdown product of lipids as a result of peroxidation initiated with stress-induced accumulation of active oxygen species. It is a rather indirect method to evaluate the stress-induced damage caused to membrane lipids, and does not monitor the actual substrates and the pathways involved. Lipid hydroperoxides can be produced both via enzymatic and non-enzymatic processes. The non-enzymatic pathway is initiated with excess active oxygen species, interacting with free iron or copper ions to form lipid radicals (Fenton reaction). The enzymatic reaction can be catalyzed by LOXs. Techniques have been developed to enable a detailed characterization of the various lipid hydroperoxide products, the identification of their source and the determination whether enzymatic or non-enzymatic pathway produced them (Feussner et al. 1997a; Kohlmann et al. 1999; Weichert et al. 1999).

In citrus cells, we have previously shown that a phospholipid hydroperoxide glutathione peroxidase (PHGPX), an enzyme that reduces lipid hydroperoxides, and its corresponding gene csa, are over-expressed under salt and other stresses (Faltin et al. 1998; Avsian-Kretchmer et al. 1999). Based on the facts that (i) under salt stress, the induction of both csa transcript and the protein PHGPX was observed earlier in the salt-sensitive cells than in the salt-tolerant ones, and (ii) treatment with organic hydroperoxide resulted in a similar induction of the gene in both cell lines, it was suggested that stress-induced accumulation of lipid peroxides directly induces the expression of csa. As mentioned above, two pathways can form lipid peroxides. In order to assess the relative contribution of each of these pathways to the induction of csa, we initiated the present study on LOXs at the protein level under similar stress conditions used to study csa expression (Avsian-Kretchmer et al. 1999).