# Lipoxygenases in Plants – Their Role in Development and Stress Response

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Lipoxygenases catalyze the hydroperoxidation of polyunsaturated fatty acids and thus the first step in the synthesis of fatty acid metabolites in plants. Products of the LOX pathway have multiple functions as growth regulators, antimicrobial compounds, flavours and odours as well as signal molecules. Based on the effects of LOX products or on the correlation of increases in LOX protein and the onset of specific processes, a physiological function for LOXs has been proposed for growth and development and for the plant response to pathogen infection and wound stress.

## Introduction

Lipoxygenases (LOXs, linoleate:oxygen oxidoreductases, EC 1.13.11.12) are non-heme iron containing dioxygenases that catalyze the formation of hydroperoxy derivatives of polyunsaturated fatty acids. First described in plants as lipoxidase (Andre and Hou, 1932) and carotene oxidase, as an activity that degraded carotenoids (Sumner and Sumner, 1940), LOX has since been the subject of extensive studies. In particular in animal systems, the role of LOXs in inflammatory responses is well established. By converting arachidonic acid into hydroperoxyeicosatetraenoic acid, mammalian LOXs catalyze the first step in the synthesis of regulatory compounds such as leukotrienes and lipoxins, that play a role in inflammation, immunity, hypersensitivity and host defense reactions (Samuelson *et al.*, 1987).

In comparison, the role of plant LOXs is less clear. Despite detailed knowledge on structural and enzymatic features, the physiological functions for plant LOXs have yet to be elucidated. Based on a correlation between increases in LOX protein and transcripts and the onset of specific developmental processes, a role of LOXs in seed germination and senescence has been suggested. During the past years, evidence is accumulating for the involvement of LOXs in the plant response to environmental stimuli such as wounding and pathogen attack. Products of the LOX pathway, such as traumatin, jasmonic acid, oxylipins and volatile aldehydes, are supposed to play a role in signal transduction of the wound response, as antimicrobial substances in host-pathogen interactions, as regulators of growth and development and as aromatic compounds that affect food quality. However, analysis of the function of LOXs in plants is complicated by the presence of multiple isoforms and their heterogeneous tissue-specific and developmentally regulated expression patterns. This article attempts to highlight some aspects of the presumed role of plant LOXs.

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## Occurrence of LOXs in plants

LOXs have been detected in a large number of plant species (reviewed by Vick and Zimmerman, 1987; Siedow, 1991). Although LOX isoforms occur in most plant cells, the tissue-specific expression level of LOX within a plant can vary substantially depending on developmental and environmental conditions. Young and expanding tissues usually contain high levels of LOX enzyme, though increases in senescing tissue have been reported as well (Siedow, 1991). The first LOX enzymes characterized in detail were the isoforms which occur in high amounts in soybean seeds (Axelrod *et al.*, 1981). High levels of LOX are also present in soybean leaves, where the 94 kDa vegetative storage protein was identified as LOX (Tranbarger *et al.*, 1991), and in potato tubers (Pinsky *et al.*, 1971).

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LOX levels are regulated by various compounds such as jasmonic acid and its methyl ester which induce LOX gene expression in soybean (Bell and Mullet, 1991; Anderson *et al.*, 1989; Grimes *et al.*, 1992), *Arabidopsis thaliana* (Melan *et al.*, 1993), potato (Fidantsef *et al.*, 1993; Geerts *et al.*, 1994) and barley (Feussner *et al.*, 1995). Furthermore, nitrogen (Grimes *et al.*, 1993), wounding (Hildebrand *et al.*, 1989) and pathogens (Yamamoto and Tani, 1986; Ocampo *et al.*, 1986; Ohta *et al.*, 1991; Rojas *et al.*, 1993) modulate the level of LOX expression as do drought stress (Bell and Mullet, 1991), abscisic acid (Melan *et al.*, 1993) as well as auxin (Liu *et al.*, 1991).

Studies to determine the subcellular localization revealed the presence of LOX isoforms predominantly in the cytosol (Siedow, 1991), but also in chloroplasts (Douillard and Bergeron, 1981; Feussner *et al.*, 1995), mitochondria (Grossman *et al.*, 1972), vacuoles (Wardale and Lambert, 1980), nuclei (Feussner *et al.*, 1995) and lipid bodies (Feussner and Kindl, 1992) as well as in association with microsomal and plasma membranes (Droillard *et al.*, 1993; Macrí *et al.*, 1994; Nellen *et al.*, 1995). Because of this wide distribution of isoforms within the cell it is difficult to assign a general role for LOX. It has rather to be taken into consideration that different isoforms might exert distinct functions in plant growth and development.

## Structural features

Most of the information on the structure of LOXs is derived from studies on the isoforms found in soybean seeds, LOX-1, -2, -3a and -3b, which are soluble proteins consisting of a single polypeptide chain (Axelrod *et al.*, 1981). The subsequent characterization of LOXs from various other plants revealed a rather conserved size of the proteins with a molecular mass of about 95 kDa. Furthermore, comparison of the deduced amino acid sequences from cDNA and genomic clones from different plants shows high overall homology between LOXs (Siedow, 1991). While the N-terminal sequences seem to be specific for each isoform, other stretches of amino acids exhibit an especially high degree of similarity. These include the C-terminal amino acids as well as a region of 38 amino acids corresponding to residues 494–531 in soybean cotyledon LOX-1 (Steczko *et al.*, 1992). A special role was attributed to six conserved histidine residues in this region, five of which are also present in mammalian LOXs. Another histidine residue located 149 to 170 residues C-terminal of this region is also invariant in different LOXs. Since LOXs contain a single non-heme iron atom at their active site, which is necessary for the oxygenation reaction, it has been suggested that the conserved histidine residues might be involved in the binding of the iron atom. Replacement of the histidine at position 499, 504 or 690, respectively, of soybean LOX-1 resulted in enzymatically inactive proteins (Steczko *et al.*, 1992) that did not contain iron (Steczko and Axelrod, 1992). Crystallographic studies with soybean LOX-1 revealed that the histidines at these three positions, as well as the invariant C-terminal isoleucine are involved in iron ligand binding (Boyington *et al.*, 1993). In addition, the analysis of a soybean LOX-2 null mutant gene also showed that the exchange of the histidine residue equivalent to position 504 in LOX-1 with glutamine results in a non-functional gene product (Wang *et al.*, 1994).

## The LOX pathway

LOXs catalyze the stereospecific dioxygenation of polyunsaturated fatty acids containing *cis,cis*-1,4-pentadiene structures leading to the formation of the respective hydroperoxy derivatives (Vick and Zimmerman, 1983). In plants, the most common substrates for LOXs are linoleic and linolenic acids, the major polyunsaturated fatty acids in plant membrane phospholipids (Lesham, 1987). Oxygen is introduced either at the 9- or 13-carbon of linoleic or linolenic acid, leading to the formation of 9- or 13-hydroperoxylinoleic or -linolenic acid, respectively. The ratio of 9- to 13-hydroperoxides differs for LOXs from various plant species (Vick and Zimmerman, 1987) and steric constraints within the LOX enzyme might be responsible for the stereospecific addition of oxygen (Siedow, 1991). Arachidonic acid, which has not been detected in higher plants, but which occurs in lipids of oomycete fungi including several important plant pathogens (Wassef, 1977), is also used as a substrate. While some plant LOXs, such as soybean LOX-1, oxygenate arachidonic acid predomi-

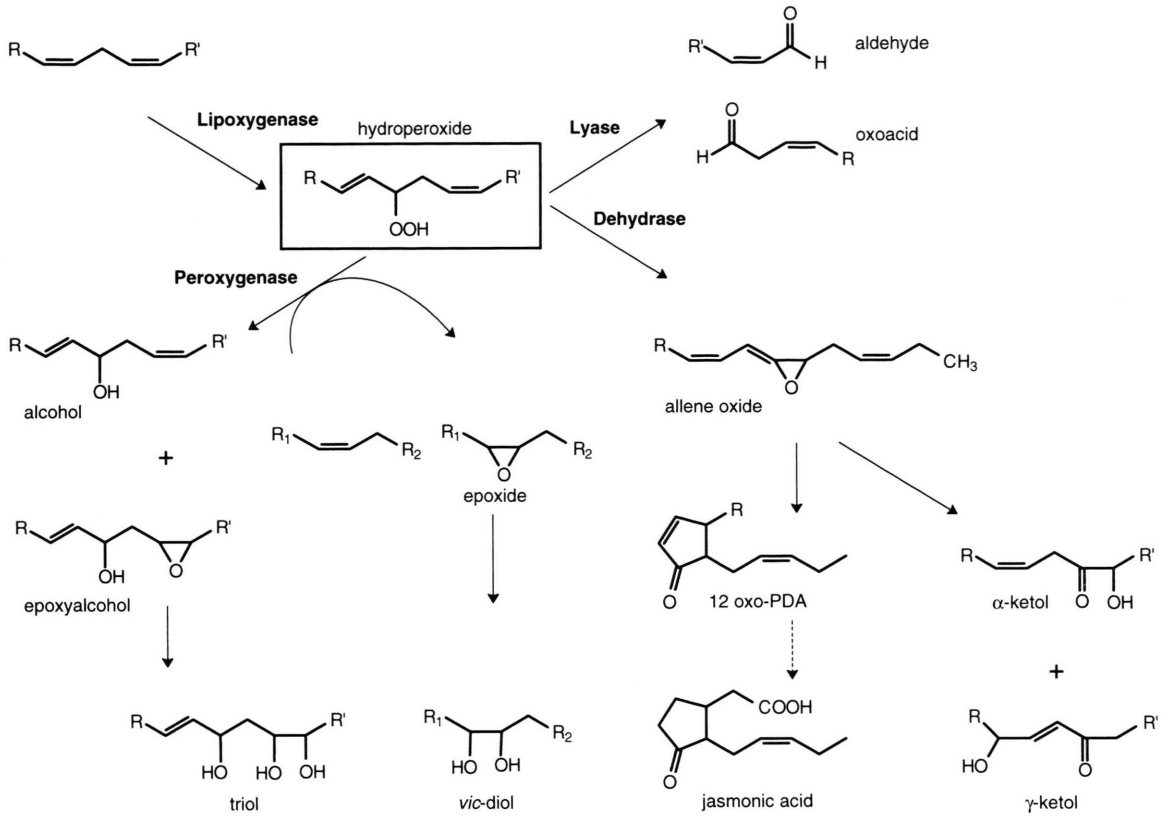


Fig. 1. The LOX pathway (by kind permission of E. Blée).

nantly at the 15-carbon (Axelrod *et al.*, 1981), LOX from potato tubers is one of the few plant enzymes capable of catalyzing the formation of 5-hydroperoxyeicosatetraenoic acid (Mulliez *et al.*, 1987) as well as its subsequent conversion to leukotriene $A_4$ (Shimizu *et al.*, 1984).

The mechanism of the LOX enzyme reaction was most extensively studied using soybean LOX-1 and a qualitatively similar behaviour was reported for LOX-2 and -3 (Gardner, 1991; Siedow, 1991). The single non-heme iron cofactor exists either as Fe(II) in the catalytically inactive or as Fe(III) in the active form of the enzyme (Schilstra *et al.*, 1994). The reaction starts with the binding of the unsaturated fatty acid to the active site of the Fe(III) form of the enzyme. The removal of a proton from the methylene group at the center of the 1,4-pentadiene structure, which appears to be the rate-limiting step for the reaction, leads to the formation of Fe(II) and a pentadienyl radical with a delocalized unpaired electron. In a second step, molecular oxygen reacts with the pentadienyl radical to produce a hydroperoxy radical. The subsequent reduction of this radical to the product of the reaction, the hydroperoxy derivative of the fatty acid, by Fe(II) restores the enzyme to its initial state (reviewed by Siedow, 1991).

The hydroperoxy derivatives of linoleic and linolenic acids are further metabolized in plants by different enzymes including hydroperoxide lyase, dehydrase, and peroxygenase (Fig. 1; Gardner, 1991; Hamberg, 1993).

Volatile aldehydes and oxoacids are produced by the action of hydroperoxide lyase. For instance, with 13-hydroperoxylinolenic acid as a substrate, *cis*-3-hexenal and 12-oxo-*cis*-9-dodecenoic acid are formed whereas the 9-hydroperoxy derivative is converted to *cis*-3,*cis*-6-nonadienal and 9-oxo-nonanoic acid. These compounds as well as their me-

tabolites are components of the characteristic flavour and odour of fruits and leaves.

Other metabolites derived from lipid hydroperoxides are α- and γ-ketols, whose formation was attributed to the action of hydroperoxide dehydrase. Since allene oxides were identified as unstable intermediates in the synthesis of these ketols, the enzyme was also called allene oxide synthase (Hamberg and Gardner, 1992). Flaxseed allene oxide synthase was shown to contain cytochrome P-450 and to convert 13-hydroperoxylinoleic and -linolenic acids to the respective allene oxide, which yields ketol or cyclopentenone derivatives by either hydrolysis or intramolecular cyclization by enzyme action (Song and Brash, 1991; Hamberg and Gardner, 1992).

Zimmerman and Feng (1978) detected a cyclic fatty acid derivative of 13-hydroperoxylinolenic acid, 8-[2-(*cis*-2'-pentenyl)-3-oxo-*cis*-4-cyclopentenyl]-octanoic acid or 12-oxo-phytodienoic acid, whose synthesis also involves an epoxy intermediate and requires the presence of allene oxide synthase. The cyclization of the allene oxide 12,13-epoxy-linolenic acid is catalyzed by allene oxide cyclase (Hamberg and Gardner, 1992). 12-oxo-phytodienoic acid is a precursor for the synthesis of jasmonic acid. It is first reduced by 12-oxo-phytodienoic acid-reductase and the resulting 3-oxo-2-(2'-pentenyl)cyclopentaneoctanoic acid is subsequently shortened by three cycles of ß-oxidation to the 12-carbon product jasmonic acid, 3-oxo-2-(2'-pentenyl)cyclopentaneacetic acid (Vick and Zimmerman, 1983; 1984). Jasmonic acid has phytohormone-like activities and is involved in the regulation of developmental processes as well as in the plant's response to wounding and pathogens. The molecular actions of jasmonic acid were recently reviewed by Sembdner and Parthier (1993).

Further modifications of hydroperoxides of polyunsaturated fatty acids include the epoxidation by epoxygenase (Hamberg and Hamberg, 1990) or peroxygenase (Blée and Schuber, 1990) yielding epoxy fatty acids which can be converted to vicinal dihydroxy acids by the action of epoxide hydrolases (Blée and Schuber, 1992; Hamberg, 1993). Epoxy and hydroxy fatty acids have been shown to possess antimicrobial activity (Kato *et al.*, 1983; 1985).

## Possible functions of LOX in plant developmental processes

### *Senescence*

In analogy to mammalian systems where LOXs are involved in membrane degradation (Schewe *et al.*, 1986), a role of LOXs in plant senescence has been suggested. Loss of membrane integrity, an inherent feature of senescence, is thought to occur *via* lipid peroxidation, a process that is envisaged to be initiated or promoted by free radicals or enzymatically by LOXs (Thompson *et al.*, 1987). The activation of phospholipase A2 by $Ca^{2+}$ and calmodulin is considered to lead to the release of linoleic and linolenic acids from membrane phospholipids which are used as substrates by LOXs (Lesham, 1987). Since the LOX reaction itself produces reactive compounds, these might be responsible for further membrane deterioration. Also, LOX products may function as $Ca^{2+}$ ionophores and lead to further $Ca^{2+}$ influx after cycling to the membrane (Lesham, 1987). This cycling has been suggested to eventually lead to the membrane alterations observed during senescence.

LOX activity has been reported to increase in senescing plant tissue (reviewed by Thompson *et al.*, 1987). Moreover, a parallel increase in cytosolic as well as microsomal LOX activity and the production of superoxide radicals was observed in senescing bean cotyledons (Lynch and Thompson, 1984). Application of a LOX inhibitor, the activity of which is not due to scavenging of free radicals, resulted in a concomitant reduction of LOX activity and of superoxide anion formation. In addition, production of high levels of oxygen radicals was dependent on the presence of linoleic acid (Lynch and Thompson, 1984). These observations suggest that LOX is involved in the production of superoxide anions and may thus contribute to membrane degradation during senescence.

As reviewed by Siedow (1991), however, there is also considerable doubt concerning a function of LOXs in senescence. In particular, the occurrence of LOX activity predominantly in young and expanding tissues as well as studies on soybean LOX, which is not induced in senescing tissue, are generally cited as arguments against an involvement of LOX in this process. However, the role of distinct isoforms of LOX and in particular the function of membrane-associated LOXs (Rouet-

Mayer *et al.*, 1992; Droillard *et al.*, 1993; Nellen *et al.*, 1995), which may be more likely to play a role in senescence, still has to be elucidated. A LOX isoform which is active at the beginning of senescence in carnation petals is associated with the plasma membrane and might be an integral membrane protein (Rouet-Mayer *et al.*, 1992). Although soluble LOXs from tomato fruit have been shown to use fatty acids esterified in phospholipids after solubilization as substrates *in vitro* (Droillard *et al.*, 1993), it has not been shown that membrane-associated LOXs in plants can catalyze the hydroperoxidation of fatty acids esterified in membrane phospholipids *in vivo*.

*Seed germination*

Regulation of LOX gene expression and enzyme activity in germinating seedlings has been reported for several plants including watermelon (Vick and Zimmerman, 1976), rice (Ohta *et al.*, 1986), lupine (Beneytout *et al.*, 1988), soybean (Altschuler *et al.*, 1989), cucumber (Matsui *et al.*, 1992, Feussner and Kindl, 1992), French bean (Eiben and Slusarenko, 1994), and *A. thaliana* (Melan *et al.*, 1994). The occurrence of distinct isoforms of LOX is induced during germination and in some cases, *de novo* synthesis of LOX has been demonstrated (Ohta *et al.*, 1986; Park and Polacco, 1989). In contrast, the well characterized LOX isoforms from soybean seeds, whose levels decrease upon germination (Park and Polacco, 1989; Kato *et al.*, 1992), do not appear to be important for germination. Soybean isolines deficient in either of the three LOX genes encoding the major seed isoforms do not show significant differences in seed germination compared to a soybean line with functional LOX genes (Pfeiffer *et al.*, 1992).

The actual function of LOX in the process of germination remains unknown, although several hypotheses have been put forward. Thus, LOXs might be involved in the disruption of cellular membranes, thereby enabling transport of storage compounds to the embryo (Hildebrand, 1989). The presence of a LOX isoform as the major protein of lipid bodies in cucumber and soybean seedlings suggests that LOXs might contribute to or initiate the mobilization of lipids during germination (Feussner and Kindl, 1992). Interestingly, enhancement of enzyme activity and alteration of its regiospecificity takes place after binding of the cucumber LOX isoform to the lipid body membrane (Feussner and Kühn, 1995).

In addition, the detection of LOX protein predominantly in the epidermis of *A. thaliana* cotyledons led to another suggestion of a role for LOX in seedling development. Thus, LOX might either be involved in protecting the seedlings from pathogens by producing antimicrobial substances or, alternatively, growth or expansion of the epidermis might be regulated by products of the LOX pathway (Melan *et al.*, 1994).

*Tuber formation in potato*

A developmental process whose different facets have been extensively studied is the formation of tubers in potato. The identification of a product of the LOX pathway, tuberonic acid, as a stimulus for tuberization implied a role of LOXs in this process. Tuberization in potato is dependent on developmental as well as environmental factors (Ewing, 1987). Tubers are formed at the end of stolons, diageotropic shoots with reduced leaf growth. After cessation of stolon growth, cell enlargement precedes increases in cell division, leading to radial expansion which is accompanied by starch deposition and accumulation of a characteristic set of tuber proteins.

The photoperiod is considered to be one of the major factors determining tuber formation (Ewing, 1987). In general, plants exposed to short days will form tubers. Phytochrome appears to play a role in tuber formation, as red light pulses during the dark period reduce tuberization, an effect that can be reversed to a certain extent by far red light (Batutis and Ewing, 1982). However, the critical photoperiod is influenced by different additional factors, such as age of the mother tuber, genotype and environmental conditions. Cold temperatures, low nitrogen levels, increased leaf area and high light intensities favour tuberization (Ewing, 1987).

Grafting experiments (Gregory, 1956; Chapman, 1958) led to the hypothesis that tuber induction is triggered by a stimulus that is synthesized in the leaves of induced potato plants. The upper part of a plant kept under inducing conditions, i.e. short days, is able to cause tuberization in a non-induced plant after grafting. The stimulus even passes through stem segments from different species in-

tergrafted between induced potato scions and non-induced potato stocks. Moreover, tobacco segments that have been kept under short days to induce flowering are able to promote tuber formation when grafted to non-induced potato plants (Ewing, 1987). These observations suggested that a compound is being produced under inducing conditions in leaves of potato plants which is transmissible and able to induce tuber formation.

Based on a bioassay with single-node stem segments, tuber-inducing activities were detected in potato leaves and old tubers (Koda and Okazawa, 1988). The substance which was found to be present at higher levels in leaves of plants exposed to short days (Koda *et al.*, 1988) was isolated and identified as 3-oxo-2-(5'-ß-D-glucopyranosyloxy-2'-*cis*-pentenyl)-cyclopentane-1-acetic acid (Yoshihara *et al.*, 1989). This compound as well as its aglycone, tuberonic acid, were able to induce an appreciable degree of tuberization in *in vitro* tuberization systems. The structural similarity of tuberonic and jasmonic acid (tuberonic acid being 12-hydroxyjasmonic acid) led to the observation that jasmonic acid and its methyl ester are also potent inducers of tuber formation in potato (Koda *et al.*, 1991; Pelacho and Mingo-Castel, 1991).

Furthermore, a positive correlation between tuber formation and the presence of hydroxylated jasmonic acids was established. In *Solanum demissum*, a potato species that strictly requires short days for tuberization, hydroxylated jasmonic acids occur only in leaflets of plants kept under short days (Helder *et al.*, 1993). The content of jasmonic acid itself was not significantly different under short and long days, suggesting that the hydroxylation rather than the synthesis of jasmonic acid is under day length control. Indeed, spraying of jasmonic acid alone is not sufficient to induce tuberization in short day-requiring potato species when they are exposed to non-inducing conditions (Jackson and Willmitzer, 1994).

It remains to be established whether LOX, as an important enzyme in the biosynthetic pathway of jasmonic acid, plays a direct role in the control of tuber formation in plants. Application of the LOX inhibitor salicylhydroxamic acid to the roots of short day-requiring plants did not lead to a reduction of tuber formation (Helder *et al.*, 1993). However, a disadvantage of studies using LOX inhibitors is that their concentrations at the intracellular sites of jasmonic acid biosynthesis might be too low (Helder *et al.*, 1993). Moreover, many LOX inhibitors act generally as radical scavengers and their specificity for distinct isoforms remains to be established. Therefore, a more detailed analysis is necessary to assess the role of LOX in tuberization.

## Possible function of LOX in environmental stress responses

### *Wounding*

Plants respond to wounding by a variety of defense mechanisms including the fortification of cell walls, induction of defense-related genes, synthesis of antimicrobial compounds as well as the initiation of processes leading to wound healing. Products of the LOX pathway have been identified as compounds involved in the plant response to wound stress. *Trans*-2-dodecenedioic acid, which is synthesized from 13-hydroperoxylinolenic acid (Zimmerman and Coudron, 1979; Vick and Zimmerman, 1987), was first isolated from mesocarp of wounded bean plants as the wound hormone traumatic acid and shown to enhance cell proliferation at the wound site (English *et al.*, 1939). It was also able to induce wound periderm formation in potato tuber disks. Furthermore, abscission of cotyledonary petioles of cotton explants induced by traumatic acid was accompanied by proliferation of cells in the protective layer after separation, leading to callus formation (Strong and Kruitwagen, 1967). Later, the active compound was postulated to be 12-oxo-*trans*-10-dodecenoic acid which is supposed to autooxidize to *trans*-2-dodecenedioic acid during the isolation procedure (Zimmerman and Coudron, 1979). The increase of this ω-keto fatty acid, traumatin, after wounding in some plant species and its effect on cell proliferation at wound sites suggest that traumatin acts as a signal for wound healing.

Another product of the LOX pathway involved in the plant response to wounding is jasmonic acid, as well as its derivatives, the jasmonates. Like traumatin, jasmonic acid is also synthesized from 13-hydroperoxylinolenic acid (Vick and Zimmerman, 1983) and plays a role as a plant growth regulator with various physiological activities (reviewed by Sembdner and Parthier, 1993). A function of jas-

monates in the wound induction of plant genes was first demonstrated for the expression of the proteinase inhibitors I and II of potato and tomato (Farmer and Ryan, 1990; Farmer *et al.*, 1992). Accumulation of these proteins is systemically induced by insect chewing, mechanical wounding and oligouronide treatment (Ryan, 1987). In search of a common inducing signal, Farmer and Ryan (1990) reported that methyl jasmonate induces the synthesis of proteinase inhibitors in unwounded plants. A model for the events of the signal transduction leading from perception of the wound signal to activation of wound-induced genes was proposed (Farmer and Ryan, 1992). According to this model, perception of specific stimuli like wounding or oligouronides leads to the activation of a membrane-bound phospholipase which releases linolenic acid from the plasma membrane. Hydroperoxidation of linolenic acid by LOX yields the precursor for the synthesis of jasmonic acid, which induces the expression of a number of genes whose products are assumed to be involved in defense reactions.

This octadecanoid acid pathway appears indeed to be functional in plants. Using the proteinase inhibitors I and II of tomato as a model system, the effect of precursors of jasmonic acid on their accumulation was studied (Farmer and Ryan, 1992). Thus, linolenic acid, 13-hydroperoxylinolenic acid and 12-oxo-phytodienoic acid can induce the accumulation of both proteinase inhibitors in tomato leaves whereas unsaturated fatty acids, which either do not represent substrates for LOX or do not act as precursors in the synthesis of jasmonic acid, fail to do so (Farmer and Ryan, 1992). Furthermore, inhibitor studies support the model of octadecanoid acid signalling in plants (Doares *et al.* 1995). Peña-Cortes *et al.* (1993) applied different LOX as well as hydroperoxide dehydrase inhibitors to detached tomato leaves and observed a loss of induction of proteinase inhibitor genes. This block was overcome by addition of the respective products of the enzyme reaction. These results emphasize the importance of the LOX pathway in the induction of proteinase inhibitor genes by wounding.

The rapid increase in jasmonate levels after wounding of plant tissue, which can also be prevented by LOX inhibitors (Peña-Cortes *et al.*, 1993), is at least partly due to *de novo* synthesis of jasmonic acid (reviewed by Farmer, 1994). Although LOX activity and mRNA accumulation are also induced by wounding in various plants (Hildebrand *et al.*, 1989; Bell and Mullet, 1991), this increase occurs rather late and thus cannot account for the rapid *de novo* synthesis of jasmonic acid. Therefore, LOX isozymes that are constitutively expressed in leaves are more likely to be responsible for the rapid synthesis of jasmonic acid after wounding. Inhibition of LOX gene expression in leaves by expression of antisense constructs would be a convenient tool to study the effects on jasmonic acid accumulation and expression of wound-inducible genes.

*Pathogen attack*

The plant defense response to pathogen attack comprises the activation of defense genes, synthesis of antimicrobial compounds, such as phytoalexins, and, in many cases, hypersensitive cell death which correlates with and might be causally linked to the development of resistance (Atkinson, 1993). Recognition of the pathogen by a plant is the prerequisite to start the sequence of events leading to resistance. Model systems including cell suspension cultures have assisted the identification of elicitor molecules derived from either the pathogen or the plant, that are recognized by plant cells and induce the activation of defense genes and phytoalexin accumulation (Ebel and Scheel, 1995). It is assumed that elicitors bind to specific receptors at the plasma membrane and initiate one or more intracellular signal transduction pathways. Early events include changes in ion fluxes across the plant plasma membrane, leading to an alkalinization of the growth medium and an increase in the intracellular $Ca^{2+}$ concentration, as well as the occurrence of an oxidative burst and changes in the phosphorylation pattern of proteins (Ebel and Scheel, 1995; Nürnberger *et al.*, 1994; Mehdy, 1994; Legendre *et al.*, 1993; Suzuki and Shinshi, 1995). The production of intracellular signal molecules is supposed to ultimately lead to the activation of defense gene transcription.

During interactions of pathogens with their hosts, alterations in LOX activity have been observed indicating that the enzyme may be involved in the plant response to infection. In particular, since LOX activity increases specifically during incom-

patible interactions in several plant-pathogen systems, it has been argued that LOXs play a role in the development of resistance *via* the hypersensitive cell death. Thus, Yamamoto and Tani (1986) reported the *de novo* synthesis of two LOX isozymes during the incompatible interaction of oats and the crown rust fungus, *Puccinia coronata* f.sp. *avenae*, and suggested a causal relationship between the presence of these LOX isozymes and resistance. Similarly, Ocampo *et al.* (1986) showed that LOX activity increased during the hypersensitive response of wheat leaves infected with avirulent races of rust fungi and suggested a direct involvement of LOX in the sequence of events leading to rapid cell death. Injection of a specific elicitor preparation from *Cladosporium fulvum* increased LOX activity in tomato leaves of a resistant, but not a susceptible cultivar (Peever and Higgins, 1989). Moreover, the occurrence of a specific LOX isozyme, which mainly produces 13-hydroperoxylinolenic acid, after inoculation of rice plants with an avirulent strain of the fungus *Magnaporthe grisea* coincided with the growth inhibition of infection hyphae (Ohta *et al.*, 1991) and provides correlative evidence for the role of LOX in the resistance response. Isolation and characterization of a cDNA clone, presumably encoding this specific rice LOX, revealed an isoform which might be located in the chloroplasts (Peng *et al.*, 1994). In addition, in cotyledons of coffee (*Coffea arabica)*, a significant increase in LOX activity was detected during the incompatible interaction with the coffee leaf rust fungus, *Hemileia vastatrix* (Rojas *et al.*, 1993).

Increases in LOX activity in plants resistant to bacteria have been reported as well, for instance upon infiltration of cucumber cotyledons with *Pseudomonas syringae* pv. *pisi*, which results in an incompatible interaction (Keppler and Novacky, 1987). The activity of LOX isozymes in French bean, *Phaseolus vulgaris,* also increases specifically after inoculation of leaves with avirulent strains of *Pseudomonas. syringae* pv. *phaseolicola* (Croft *et al.*, 1990). LOX mRNA, protein and activity were induced in tomato leaves undergoing a hypersensitive response after infection with *Pseudomonas syringae* pv. *syringae* (Koch *et al.*, 1992). In *A. thaliana*, the LOX1 gene is more rapidly induced after infiltration of leaves with *P. syringae* strains carrying an avirulence gene as compared to inoculation with virulent bacteria (Melan *et al.*, 1993).

These correlative data on increases in LOX activity during incompatible interactions raise the question as to whether LOXs might have a function in the development of the hypersensitive response and thus might be involved in conferring resistance. Indeed, LOXs have been envisaged to play a role at various levels in the plant response to pathogens. They might initiate or contribute to membrane damage during the hypersensitive response due to their ability to peroxidize lipids and to generate reactive oxygen species. Also, the production of several antimicrobial substances proceeds *via* the LOX pathway. Moreover, LOXs have recently been implicated in the production of signal molecules that are involved in the activation of defense responses. In addition, in potato, LOX is supposed to function as a receptor and metabolizing enzyme for the elicitor molecule arachidonic acid. In the following paragraphs, these individual steps will be discussed.

### Contribution to membrane damage during the hypersensitive response

Rapid cell death at infection sites, the hypersensitive response, is considered to be one of the mechanisms of resistance of plants against pathogens. Early physiological changes during the hypersensitive death include irreversible membrane damage which is supposed to be due to alterations of membrane lipids. The peroxidation of polyunsaturated fatty acids of membranes has been suggested to contribute to membrane alterations during the hypersensitive response (Keppler and Novacky, 1986). The hydroperoxy derivatives of the fatty acids can undergo autocatalytic degradation, producing radicals and thus initiating a chain reaction of lipid peroxidation. Subsequently, membranes are damaged and electrolyte leakage and rapid collapse of tissue occurs (Keppler and Novacky, 1987).

Lipid peroxidation can be initiated by active oxygen species or is suggested to occur enzymatically by LOX (Keppler and Novacky, 1987). The rapid and transient generation of reactive oxygen species, the oxidative burst, is characteristic of the plant's early response to pathogens or elicitors and is assumed to be involved in mediating the hypersensitive cell death (Mehdy, 1994). Thus, hydrogen peroxide, which is involved in oxidative crosslink-

ing of cell wall components leading to fortification of cell walls, was also shown to act as a local trigger for the programmed cell death of infected cells as well as a diffusible signal for the induction of defense genes in neighbouring cells (Levine *et al.*, 1994).

The generation of active oxygen species in plant-pathogen interactions leading to resistance has been observed in many systems. In potato, membrane fractions from tubers undergoing a hypersensitive response induced by incompatible races of *Phytophthora infestans* produce superoxide anions (Doke, 1983). Upon inoculation of cultured tomato cells with a race-specific elicitor of *C. fulvum*, active oxygen species are generated within 2 min (Vera-Estrella *et al.*, 1992). In addition, in soybean cell suspensions, non-specific fungal elicitors were shown to induce the generation of active oxygen species (Apostol *et al.*, 1989). Interactions of pathogenic bacteria with plants are likewise able to induce oxidative burst (Ádám *et al.*, 1989; Keppler *et al.*, 1989; Baker *et al.*, 1993) as are viruses (Doke and Ohashi, 1988).

The crucial role of superoxide radicals was demonstrated by studies showing that application of superoxide dismutase to cucumber cotyledons infiltrated with avirulent strains of *P. syringae* reduced lipid peroxidation and thus membrane alterations (Keppler and Novacky, 1987). Moreover, cellular antioxidants decreased electrolyte leakage and delayed the development of the hypersensitive response of tobacco inoculated with *P. syringae* (Ádám *et al.*, 1989).

The initiation of lipid peroxidation has also been proposed to be mediated enzymatically by LOX due to the concomitant increase in LOX activity and membrane lipid peroxidation during the early stages of the hypersensitive response (Croft *et al.*, 1990). In addition, the LOX-mediated hydroperoxidation of polyunsaturated fatty acids produces active oxygen species like singlet oxygen (Kanofsky and Axelrod, 1986) or superoxide radicals (Lynch and Thompson, 1984). Thus, LOX has been envisaged to contribute to enzyme-independent lipid peroxidation by its ability to produce active oxygen species (Croft *et al.*, 1990). Alternatively, LOX might act as a scavenger of potentially toxic free fatty acids (Keppler and Novacky, 1987). Although it is not clear whether LOX is indeed involved in the initiation of lipid peroxidation or is induced merely as a response to radical-induced membrane alterations, LOX appears to be able to further contribute to membrane damage.

## Synthesis of antimicrobial substances

Another aspect of the role of LOX in plant defense against pathogens lies in the antimicrobial nature of many products of the LOX pathway. Both the direct action of these substances against pathogens as well as their possible role in fatty acid signalling between plants and microorganisms has been suggested as a possible mechanism in conferring resistance.

The primary products of the LOX reaction, hydroperoxy derivatives of polyunsaturated fatty acids, show antifungal activity. Thus, 9-hydroperoxylinolenic acid efficiently affects growth of the rice blast fungus, *M. grisea*, by inhibiting spore germination, germ tube growth and formation of appressoria (Ohta *et al.*, 1990). Ricker and Bostock (1994) determined the antifungal activity of derivatives of linoleic and arachidonic acids and found that cystospore germination of *Phytophthora capsici* was significantly inhibited by both 9- and 13-hydroperoxylinoleic acids as well as by the dihydroxy derivatives of arachidonic acid, 5,6- and 5,12-dihydroxyeicosatetraenoic acids. In addition, germination of *P. infestans* cystospores was inhibited by low concentrations of 15-hydroperoxyeicosatetraenoic acid (Ricker and Bostock, 1994). Mono- and trihydroxy derivatives of linolenic acid which arise by lipid hydroperoxide-decomposing activities are active against pathogens at even lower concentrations than the hydroperoxy fatty acids (Ohta *et al.*, 1990).

Other metabolites derived from the LOX pathway are unsaturated aldehydes and their oxidized and reduced derivatives. Antibacterial activity at low concentrations has been demonstrated for *trans*-2-hexenal and at higher concentrations for *cis*-3-hexenol (Croft *et al.*, 1993). These compounds arise from 13-hydroperoxylinolenic acid and are produced as volatiles in significantly higher amounts during the incompatible interaction of bean and *P. syringae* pv. *phaseolicola* as compared to the compatible one. The occurrence of *trans*-2-hexenal and *cis*-3-hexenol which are produced before the onset of the accumulation of isoflavonoid phytoalexins correlates with the

decrease in bacterial growth during the hypersensitive response (Croft *et al.*, 1993).

Resistance of soybean against *Aspergillus flavus* also requires a functional LOX pathway. Application of LOX substrates to soybean cotyledons results in efficient inhibition of spore germination which can be partially reversed by LOX inhibitors (Doehlert *et al.*, 1993). The active compound was identified as hexenal which acts as an antifungal substance (Doehlert *et al.*, 1993 and ref. therein). Its activity towards other bacteria, fungi and arthropods has also been described (reviewed by Farmer, 1994). In addition, hexenal and hexanol are components of a mixture of volatiles produced by corn seedlings after insect chewing that have been suggested to serve the secondary function of attracting natural enemies of herbivores (Turlings *et al.*, 1990).

Jasmonic acid itself has also been identified as an antifungal compound in rice (Neto *et al.*, 1991). *In vitro*, both the discharge of zoospores from sporangia of *P. infestans* and the growth of mycelia in liquid medium were inhibited in a dose-dependent manner by jasmonic acid and methyl jasmonate (Cohen *et al.*, 1993). The protection of barley plants against *Erysiphe graminis* f.sp. *hordei* by spraying of jasmonic acid is thought to be due to direct action against the fungus (Schweizer *et al.*, 1993). However, the growth of mycelia of other phytopathogenic fungi is not inhibited by jasmonic acid (Schweizer *et al.*, 1993) and no significant inhibition of cystospore germination of *P. capsici* is observed in the presence of methyl jasmonate (Ricker and Bostock, 1994). Schweizer *et al.* (1993) therefore suggested that jasmonic acid does not act as a general toxin but rather exhibits its antifungal activity by inhibiting specific fungal differentiation processes.

## Synthesis of signal molecules

The observation that endogenous levels of jasmonic acid increase in cell cultures after elicitation within 30 minutes (Gundlach *et al.*, 1992) raised the question as to whether the lipid-based signal transduction pathway proposed by Farmer and Ryan (1992) might also be functionally significant for the events leading to defense gene activation during plant-pathogen interactions. Cell cultures from both mono- and dicotyledonous plants respond to elicitation by a yeast cell wall preparation with a transient increase in the levels of jasmonic acid as well as its precursor, 12-oxo-phytodienoic acid (Müller *et al.*, 1993). Furthermore, the level of linolenic acid is increased, possibly due to its release from lipids after elicitation (Müller *et al.*, 1993). Jasmonates were shown to induce the accumulation of secondary metabolites and of mRNA encoding phenylalanine ammonia lyase (Gundlach *et al.*, 1992). In parsley cells, 12-oxo-phytodienoic acid induced the synthesis of the flavonoid apiin and the accumulation of transcripts encoding enzymes of the secondary phenolic metabolism like phenylalanine ammonia lyase, 4-coumarate:CoA ligase and chalcone synthase (Dittrich *et al.*, 1992) as well as phytoalexin synthesis (D. Scheel, pers. communication). Thus, it has been postulated that jasmonic acid might function as an intracellular signal molecule in elicitation.

Although a specific LOX isoform has been purified from tobacco cell cultures that is induced by glycopeptide elicitors from *Phytophthora parasitica* var. *nicotianae* (Fournier *et al.*, 1993), the rather late increase in LOX activity as well as the predominant production of 9-hydroperoxylinolenic acid argues against a role of this induced LOX in the synthesis of elicitor-induced jasmonic acid. Therefore, analysis of the activation of preexisting LOXs, which are more likely responsible for the synthesis of jasmonic acid after elicitation, might elucidate the role of LOXs in the process of elicitor-induced gene expression. Interestingly, a plasma membrane-associated LOX in soybean appears to be stimulated by low levels of hydrogen peroxide (Kulkarni *et al.*, 1990; Macrí *et al.*, 1994). This observation suggests that the elicitor-induced oxidative burst might directly induce the synthesis of a signal molecule by activating the LOX enzyme, thus linking components of the different signal transduction pathways that activate subsets of defense genes (Farmer and Ryan, 1992; Nürnberger *et al.*, 1994; Levine *et al.*, 1994).

In plants, a functional significance of jasmonates in the signal transduction leading to resistance against pathogens has not been demonstrated. Chemically induced or genetically based resistance of barley plants against powdery mildew (*E. graminis* f.sp. *hordei*) is not correlated with higher levels of jasmonates (Kogel *et al.*, 1995). In potato, jasmonates do not induce a hypersensitive response

in aged tuber disks (Choi *et al.*, 1994). In this system, the accumulation of different isoprenoid phytoalexins in response to jasmonate or the fungal elicitor arachidonic acid correlates with the induction of different genes encoding a key enzyme of phytoalexin biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, suggesting the presence of distinct arachidonic acid- and jasmonate-response pathways (Choi *et al.*, 1994). In this context, it has been speculated that jasmonates might exert an alternative function in signalling by conditioning plant tissue for optimal response to elicitors or pathogens (Choi *et al.*, 1994).

### Metabolism of fatty acid elicitors

The discovery of the LOX substrates arachidonic acid and eicosapentaenoic acid as elicitors of the hypersensitive response during the interaction of *P. infestans* and potato (Bostock *et al.*, 1981) led to the postulation of another, direct involvement of LOX in the signal exchange during pathogenesis. The lipids of *P. infestans* and other oomycetes contain eicosapolyenoic acids like arachidonic acid (Wassef, 1977; Bryan *et al.*, 1985) which are released from sporangia after inoculation of potato leaves (Ricker and Bostock, 1992). Arachidonic acid acts as an elicitor of sesquiterpenoid phytoalexin accumulation in potato tubers (Bostock *et al.*, 1981) and fruits of pepper (Bloch *et al.*, 1984) as well as of isoflavonoid phytoalexin accumulation in leaves of French bean (Longland *et al.*, 1987). A possible involvement of LOX in mediating this response was postulated based on the finding that LOX inhibitors such as salicylhydroxamic acid and eicosatetraynoic acid prevent the accumulation of sesquiterpenes elicited by arachidonic acid (Preisig and Kuc, 1987). Moreover, the hypersensitive response of cell death and tissue browning in potato callus cultures cannot be elicited by arachidonic acid in mutant calli of potato which do not exhibit LOX activity (Vaughn and Lulai, 1992). LOX activity itself increases twofold within 30 min in potato tubers treated with arachidonic acid (Bostock *et al.*, 1992). Metabolites of arachidonic acid are rapidly produced by LOX after application of the elicitor (Ricker and Bostock, 1994). However, there are conflicting data on the elicitation capabilities of these hydroperoxyeicosatetraenoic acids. No elicitor activity of hydroperoxyeicosatetraenoic acids was found by Ricker and Bostock (1994). Furthermore, in the presence of fungal glucan preparations, which enhance the elicitor activity of arachidonic acid (Maniara *et al.*, 1984; Preisig and Kuc, 1985; Bostock *et al.*, 1986), a reduction of the amount of hydroperoxy derivatives of arachidonic acid was observed (Ricker and Bostock, 1994), suggesting that the metabolism of arachidonic acid by LOX is not necessary for elicitor activity. In contrast, 5-hydroperoxyeicosatetraenoic acid was reported to induce phytoalexin accumulation at lower concentrations than arachidonic acid itself (Castoria *et al.*, 1992). Thus, it is not clear whether LOXs are indeed necessary for elicitor activity of arachidonic acid. Alternatively, in analogy to animal systems, it has been speculated that arachidonic acid might also act by activating protein kinase C (Naor *et al.*, 1988), leading to the activation of other kinases and eventually to phosphorylation of transcription factors (Després *et al.*, 1995). Evidently, further analyses are needed to elucidate the role of LOX in arachidonic acid-mediated elicitation of phytoalexin accumulation in potato.

## Summary and Outlook

LOXs catalyze the formation of hydroperoxy derivatives of polyunsaturated fatty acids which are further metabolized to compounds with different biological activities in plant growth and development and in defense reactions. Based on the action of these products of the LOX pathway as well as on the correlation of increases in LOX protein and transcript levels and the onset of specific processes, functions for LOXs in developmentally regulated processes as well as in the plant response to wounding and pathogen attack have been proposed. However, since there are mostly correlative data available linking LOXs to these processes, it has not been possible to assign specific functions for LOXs so far. Inhibitor studies suffer the drawback of multiple effects in addition to not specifically inhibiting distinct isoforms. Therefore, genetic approaches with mutant plant lines lacking specific LOX isoforms offer the possibility to study the effect of "loss of function" mutations. In addition, the molecular cloning of LOX cDNAs and genes allows the ectopic expression of sense and antisense constructs in transgenic

plants and should lead to the expression or reduction of specific isoforms. First experiments indeed show a reduction in the accumulation of jasmonate and jasmonate-inducible transcripts in cosuppressed *Arabidopsis* plants lacking detectable LOX2 protein (Creelman *et al.*, 1995). Thus, this functional approach might lead to the elucidation of the physiological role of LOXs in plants.

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