Molecular characterization of lipoxygenase from barley grains and seedlings

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Contents

Abbreviations

ABA,	abscisic acid
AOC,	allene oxide cyclase
AOS,	allene oxide synthase
Cdi,	cathepsin D
COScoA,	acyl-CoA
COTG	acyl glycerol
DAF,	days after flowering
DAG,	days after germination
ETYA ,	eicosa-5,8,11,14-tetraynoic acid
FM,	full maturity
9(*S*)-HPOD,	9(*S*)-hydroperoxy-(10*E*, 12*Z*)-octadeca-10,12-dienoic acid
13(*S*)-HPOD,	13(*S*)-hydroperoxy-(9*Z*, 11*E*)-octadeca-9,11-dienoic acid
9(*S*)-HPOT,	9(*S*)-hydroperoxy-(10*E*, 12*Z* ,15*Z*)-octadeca-10,12,15-trienoic acid
13(*S*)-HPOT,	13(*S*)-hydroperoxy-(9*Z*, 11*E*, 15*Z*)-octadeca-9,11,15-trienoic acid
IAA,	indoleacetic acid
JIP,	jasmonate inducible protein
JA,	jasmonate or jasmonic acid
Lap,	leucine amino peptidase
LOX,	lipoxygenase
NP-40,	nonidet P-40
12-oxo-PDA	12-oxo-phytodienoic acid
PIN,	proteinase inhibitor protein
PKa,	protein kinase activity
RFLP,	restriction fragment length polymorphism
RIP,	ribosomal inactivating protein
Td,	threonine deaminase
VSP,	vegetative storage protein

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Errata

Conversion of the manuscript from a *Word Perfect Document* into a *Microsoft Word Document* prior to press, somehow resulted in a loss of most of the Greek symbols. To my regret, I did not notice the changes in time. Unfortunately, μM has been set as mM, while the original mM is unaffected. The same occurred for μl, μg and some other text symbols as listed here under.

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		11	T	50 μl of Tween 20
		15	T	50 μl of sample
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		3	T	linoleic acid (200 μl)
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		6	T	5 μg of prostaglandin B$_2$
		6	B	ChromSpher C$_{18}$ column (5 μM, ..)
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November 16, 1998, Jan van Mechelen.

General Introduction

Lipoxygenases in barley grain

The enzyme lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12,LOX) is widespread in nature (Galliard and Chan, 1980; Samuelsson et al, 1987; Siedow, 1991), as it is present in animal cells, plant cells including, fungi and yeast. The enzyme belongs to a class of dioxygenases that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids containing a (1*Z*,4*Z*)-pentadiene system to form fatty acid hydroperoxides.

In plants, the formed fatty acid hydroperoxides are subsequently converted into a series of products, including plant hormones such as jasmonic acid (JA). This hormone is involved in signal transduction pathways often associated with plant development and stress responses. Furthermore, some of the products of the lipoxygenase route have been implicated in the formation of aldehydes with off-flavour characteristics. Formation of such undesired flavour compounds occur during aging of beer (Doderer *et al.*, 1992; McElroy and Jacobsen, 1995). The presence of lipoxygenases in grains is therefore of particular interest to the brewing industry. Characterization of two cytosolic lipoxygenases from barley grains has been reported by Van Aarle (1991), Doderer *et al.* (1992) and Yang *et al.* (1993). Furthermore, expression of lipoxygenase genes during germination has been described by Holtman *et al.* (1996). Nevertheless, the exact number of LOX isoenzymes and genes present in barley remains to be established.

Current state of lipoxygenase research

Despite its wide distribution in the plant kingdom, the physiological role of lipoxygenase is only partly understood. The enzyme has been implicated to play a role in growth and in development (Hildebrand and Hymowitz, 1983), in fruit ripening (Ealing, 1994), in senescence (Vick and Zimmerman, 1983; Fobel *et al.*, 1987) and in response to stress by involvement in the synthesis of the plant hormones jasmonic acid and traumatin (Gardner, 1979). Furthermore, a role for lipoxygenase in the synthesis of abscisic acid has been proposed (Parry and Horgan, 1991a and 1991b; Creelman *et al.*, 1992). The presence of multiple lipoxygenase isoenzymes has been reported for many plant species. Several LOX isoenzymes have been characterized and their involvement in diverse physiological processes has been demonstrated.

In plants, lipoxygenase activity is observed in different tissues with high variation in temporal expression. Expression of lipoxygenase genes in plant tissues can follow an ordered genetically regulated program or can be induced as a result of environmental changes in particular stress-responses. Biochemical properties and the catalytic features of lipoxygenases have previously been reported in recent PhD theses from Utrecht University (Van Aarle, 1993; Hilbers, 1995; Nieuwenhuizen, 1997). The present thesis is focused on characterization of lipoxygenases from barley grain by cloning LOX encoding cDNAs and by studying the expression of its respective genes with molecular and biochemical methods.

Lipoxygenase reaction mechanism

Lipoxygenases contain a single non-heme iron per molecule of enzyme. A generally accepted model for the catalytic action of lipoxygenase implicates the oxidation of iron (De Groot *et al.*, 1975). The hydroperoxide formed at the start of the reaction oxidizes Fe(II)-lipoxygenase to the active Fe(III)-form (Fig. 1, step a). More hydroperoxide is formed by increasing amounts of Fe(III)-lipoxygenase. After binding of the substrate, a hydrogen of the methylene group between the two double bounds is split off resulting in a reduction of Fe(III) and the formation of a pentadiene-radical (step b). Under anaerobic conditions, the radical dissociates from the enzyme (step f), thereby possibly generating various secondary products. Under aerobic conditions molecular oxygen reacts with the radical to form a hydroperoxy radical (step c). Subsequently, the Fe(II) will be oxidized to Fe(III) (step d) and the formed hydroperoxide anion is protonated and dissociates from the Fe(III)-enzyme. The Fe(III)-enzyme will react for a next reaction cycle (step e).

The LOX-enzyme is measurable by monitoring the formation of conjugated double bounds at 234 nm (E=25 mM/cm). Under aerobic conditions, LOX activity can alternatively be measured following the consumption of molecular oxygen with a Clark-type oxygen electrode (Axelrod, 1974.; Grossman *et al.*, 1979).

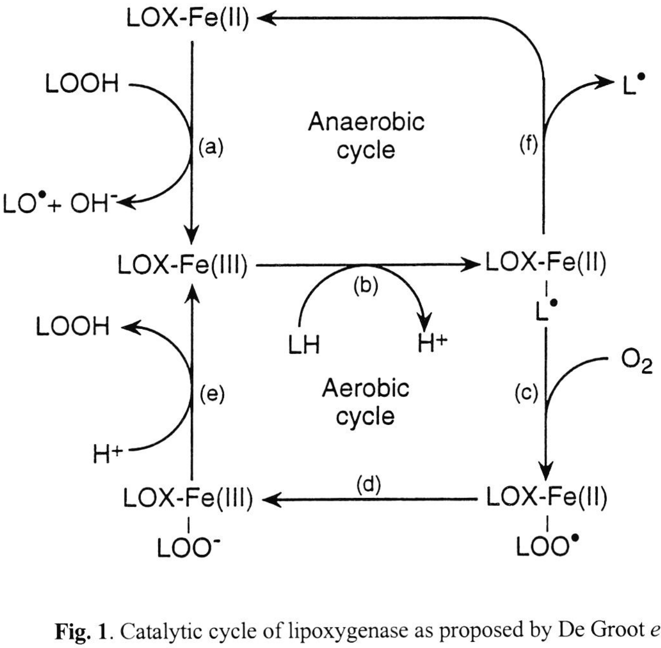

Fig. 1. Catalytic cycle of lipoxygenase as proposed by De Groot *et al.* (1975) and adapted from Hilbers (1995).

Lipoxygenase classification

Lipoxygenases catalyze the dioxygenation of polyunsaturated fatty acids with one or more 1*Z*,4*Z* pentadiene structures into optically active *Z,E* conjugated hydroperoxide fatty acids. In animals the main substrate for lipoxygenase is arachidonic acid that can become dioxygenated at the positions 5, 8, 11, 12 or 15 (Yamamoto, 1992). The enzymes are named after the position of arachidonic acid that they dioxygenate. Human 5-lipoxygenase synthesizes 5-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid, an intermediate in the synthesis of leukotrienes and lipoxins, which are known as messengers involved in transduction of inflammatory responses (Samuelsson, 1987).

Plant lipoxygenase isoenzymes can also be distinguished by product formation. The substrate of plant lipoxygenase is mainly linoleic acid. However, a general consensus for the classification of LOX-isoenzymes has not been established so far. Considering the fact that the product formed by a particular LOX can vary dependent on incubation condition (Feussner and Kühn, 1995), it will be difficult to classify LOX isoenzymes based upon the position that will be dioxygenated. It has been proposed to catagorize lipoxygenase in two major groups, namely those with a cytosolic designation and the ones with a transit peptide for chloroplast targeting (Shibata et al, 1994). Chloroplast LOXs have alternatively been named novel-lipoxygenases and have been catagorized under a LOX type 2 group of proteins (Peng *et al.*, 1994; Bell and Mullet, 1993; Vörös *et al.*, 1998).

The regiospecificity of the product depends on the orientation of the substrate binding site. For some of the LOX-enzymes it has been shown that the orientation site may alter under varying reaction conditions (Vick and Zimmerman, 1982; Vick, 1993). Furthermore, for some lipoxygenases the enzymatic characteristics have not been determined as yet.

Because consensus on the classification of plant lipoxygenases is lacking, the cDNAs isolated from barley grain and presented in this thesis are catagorized LoxA, LoxB and LoxC, respectively.

Structure

The initial studies on the structure of plant lipoxygenase were conducted on the enzymes from soybean cotyledons (Christoffer *et al.*, 1970 and 1972; Axelrod, 1974), comprising three isotypes. The isotypes differ in properties, like molecular mass (varying from 94 to 97 kDa), pH optimum and range (varying from 5.0 to 9.0) and isoelectric point (5.7 to 6.1). Because lipoxygenases from various sources contain many structural similarities, soybean lipoxygenase L-1 has served as a model for other lipoxygenases. X-ray structures of the Fe(II) form of soybean lipoxygenase L-1 have been published at 2.6 Å and 1.3 Å resolution (Boyington et al, 1993; Minor *et al.*, 1993 and 1996). The additional availability of primary structures deduced from three genes encoding soybean lipoxygenase isoenzymes (Shibata *et al.*, 1987 and 1988; Yenosfky *et al.*, 1988) and the production of point-mutated soybean LOX-1 in *E.coli* facilitated the characterization of its protein structure (Steczko *et al.*, 1992).

The soybean L-1 enzyme consists of 839 amino residues which are arranged in two domains. The smaller domain I comprises the 146 residues of the amino terminus. Domain II contains 23 a-helices and two antiparallel b-sheets. Of the a-helices, 17 are parallel or antiparallel and surround a long central helix of 43 amino acid residues. The catalytic domain has two cavities from the surface to the inside located iron-binding site. Cavity I is a 18 Å long and hydrophobic. Cavity II contains a hydrophobic stretch of 38 highly conserved amino residues among plant lipoxygenase, which includes 2 histidine residues essential for iron binding (Boyington *et al.*, 1994). These histidine residues (His 499 and His 504) together with His 690 and Ile 839 serve as ligands for iron binding (Fig. 2). Asn 694 is located on 3.16 Å distance of the iron molecule and is probably involved in protein binding (Minor *et al.*, 1996). Replacement of His 499, His 504 or His 690, respectively, fully abolished enzyme activity of recombinant protein (Steczko *et al.*, 1992).

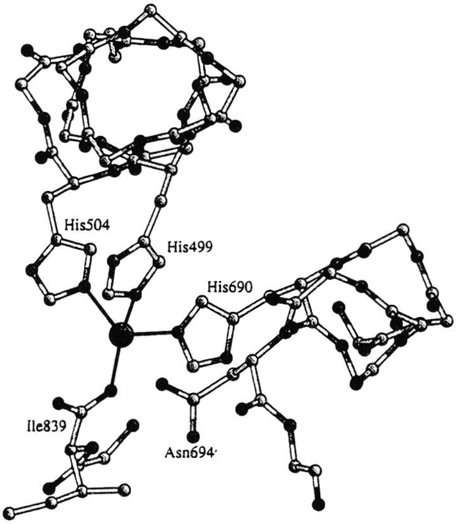

Fig. 2. The coordination of iron in the active site of soybean lipoxygenase L-1 (adapted from Boyington *et al.*, 1993).

Lipoxygenase pathway in plants

The primary fatty acid hydroperoxide generated by plant lipoxygenase can subsequently be converted via several metabolic pathways into compounds, such as jasmonates, traumatin, C6 volatile aldehydes (hexanals) and C9 aldehydes (nonenals). The physiological functions of these products are only partly understood, so far jasmonate and traumatin are known to be plant hormones, while hexanals might play a role in cell signalling, a role for nonenal is even more obscure (Siedow, 1991). Nonenal has been identified to be an "off-flavour" in plant industrial products and is therefore an undesired product for many food industries.

Four pathways for degradation of fatty-acid hydroperoxides, generated by lipoxygenase, have been characterized in plants. One route is known as the hydroperoxide lyase catalysed route which comprises the conversion of hydroperoxides into aldehydes and ω-oxoacids by hydroperoxide lyase (Vick and Zimmerman, 1976). The second route is known as the allene oxide synthase or octadecanoid pathway and comprises the epoxydation of the hydroperoxides formed from the LOX reaction (Hamberg, 1987; Brash *et al.*, 1988). The two remaining routes have more recently been identified and make use of different substrates. In the peroxygenase route hydroperoxide is formed by lipoxygenase action. The hydroperoxide is used as co-substrate for the conversion of oleic acid into epoxy acid. The epoxy acid can be hydrated by an epoxyhydrolase into hydroxystearic acid derivatives. Furthermore, in this pathway a cytochrome P450 dependent reaction involves hydroxylation of stearic acid derivatives. The final product of this route is used for the biosynthesis of cutin, a polymer that covers plant cell walls (Blée and Schuber, 1993; Fig. 3).

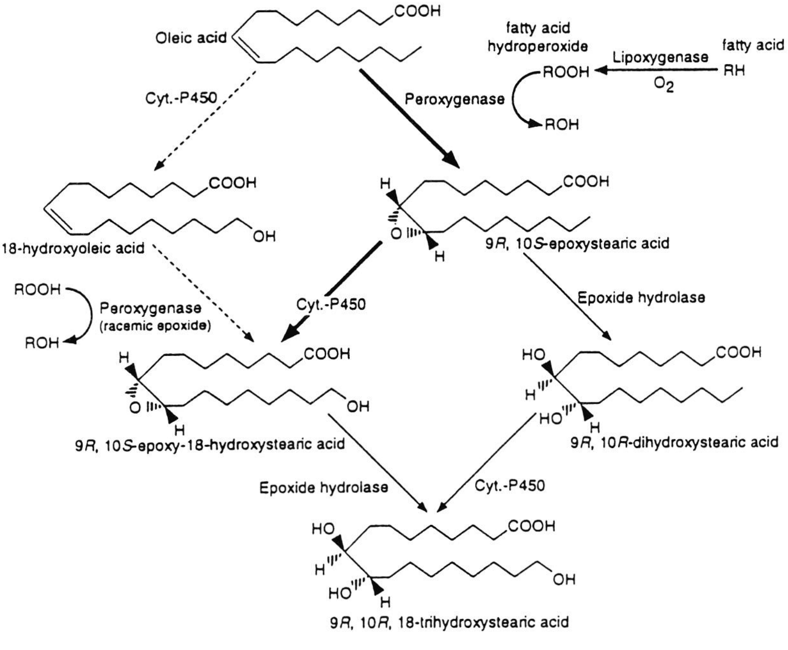

Fig. 3. The peroxygenase pathway in which lipoxygenase is involved in the conversion of fatty acid into hydroperoxide. The hydroperoxide serve as a cosubstrate for the conversion of oleic acid into epoxy acid (adapted from Blée and Schuber, 1993).

The reductase pathway makes use of storage acyl glycerols, which are directly oxygenated into 13-hydroperoxides (Fig. 4). These hydroperoxide intermediates can be used as substrates for β-oxidation, thereby generating energy for seedling growth after germination (Feussner *et al.*, 1997).

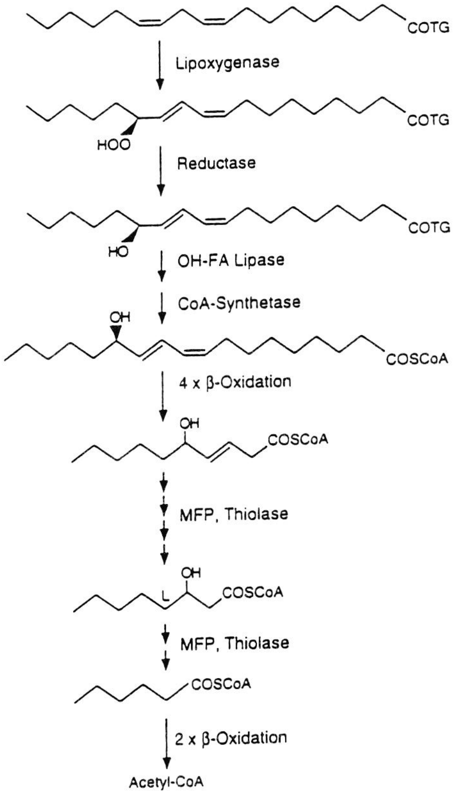

Fig. 4. The reductase pathway in which Acetyl-CoA is formed, providing energy for seedling growth (adapted from Feussner *et al.*, 1997).

Synthesis of volatile signalling molecules and traumatin via the hydroperoxide lyase pathway.

The action of hydroperoxide lyase involves the cleavage of 9-HPOD as well as that of cleavage of 13-HPOD. 9-HPOD is converted into 3*Z*-nonenal and 9-oxo-nonanoic acid. 3*Z*-nonenal is subsequently isomerized into 2*E*-nonenal (Philips *et al.*, 1979). 13-HPOD is converted into n-hexanal and 12-oxo-9*Z*-dodecenoic acid, which is subsequently converted into 12-oxo-10*E*-dodecenoic acid, known as traumatin or the traumatic acid (Zimmerman and Coudron, 1979).

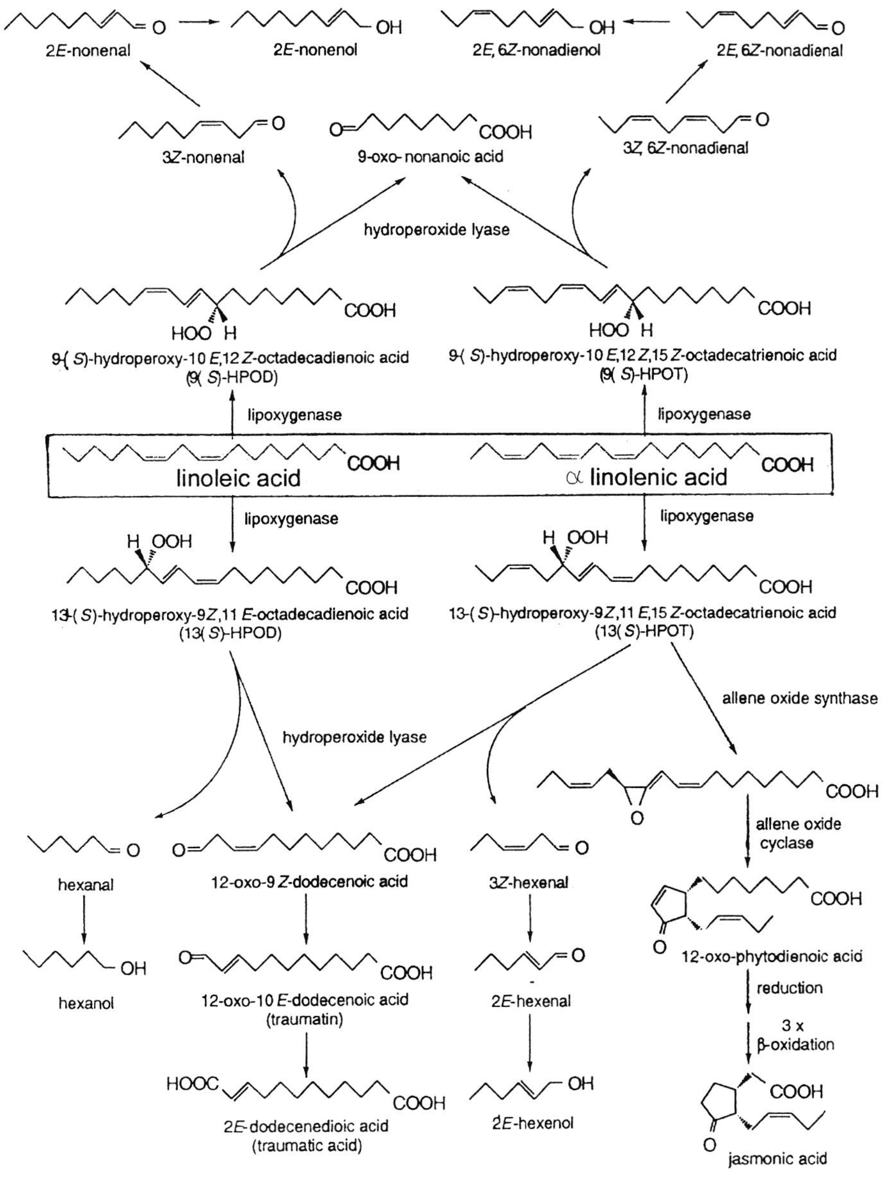

Fig. 5. Hydroperoxide lyase and the allene oxide synthase pathways (the latter is also known as the octadecanoid pathway), two routes in which plant lipoxygenases are involved. Substrates for lipoxygenase are printed in the block in the center (adapted from Hilbers, 1995).

Traumatin has been reported to mimic the physiological effects seen upon wounding of plant tissues. It causes abcission of cotyledon petioles in cotton explants. Auxin treatment can reduce traumatin induced abscission. Furthermore, traumatin increases upon wounding. Traumatin are volatile and can be rapidly diffused in the air after wounding.

The aldehydes generated by the hydroperoxide lyase route and their alcohol derivatives are often associated with characteristic plant odours (Gardner, 1985; Phillips and Galliard, 1987; Hatanaka, 1993). Some of the products from the hydroperoxide lyase pathway have also been implicated in signal mediation in host-pathogen interactions (Anderson *et al.*, 1989 and 1994; Slusarenko *et al.*, 1991; Farmer and Ryan, 1992; Croft *et al.*, 1993). 'Lipid-derived volatiles are directly formed upon pathogen attack by the action of lipoxygenases and hydroperoxide lyases.

Allene oxide synthase a key enzyme for jasmonate biosynthesis

Jasmonate is derived through 13(*S*)-HPOT by conversion into 12,13(*S*)-epoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid by allene oxide synthase as a first step. The unstable allene oxide is converted non-enzymatically into α-ketols and γ-ketols. Otherwise, the allene oxide can be converted in 12-oxo-phytodienoic acid, the precursor for jasmonate, by allene cyclase. Subsequently, three steps of β-oxidation will lead to the synthesis of jasmonate (Fig. 5).

Jasmonic acid methyl ester (MeJA) was first identified in the fungus *Lasiodiplodia theobromae* (Démole *et al.*, 1962). Later the interest became focused on methyl jasmonate from the fungus *Jasminum grandiforum* L. because of the usefulness of its flavour properties as a compound for the perfume industry. Lipoxygenase has drawn more attention because of its potential role in the synthesis of jasmonates (Siedow, 1991; Creelman and Mullet, 1997).

Jasmonates are potential inducers of expression of several genes, such as proteinase inhibitors in tomato (Farmer and Ryan, 1990), polyphenol oxidases in tomato (Constabel *et al.*, 1995), vegetative storage proteins in soybean (Anderson *et al.*, 1989; Grimes *et al.*, 1992), seed storage proteins and oil body membrane proteins (oleosins) in *Brassica napus* (Wilen *et al.*, 1991). Also the expression of lipoxygenase after jasmonate treatment has been reported for several plant species like: barley (Rouster *et al.*, 1997; Vörös et al, 1998); soybean (Grimes *et al.*, 1992); and *Arabidopsis* (Bell and Mullet, 1993; Melan *et al.*, 1993). The involvement of the octadecanoid pathway in induction of several stress and defense related genes have become well documented. But so far the implication of *de novo* synthesis of lipoxygenase upon MeJA treatment and its roles in systemic plant defense are poorly understood. Therefore, much of the current interest in plant-derived fatty acid signals is focused on the *in vivo* roles of lipoxygenase and jasmonic acid in regulating defense genes. However, many details are lacking and questions are: a) what are mediators for induction or maintenance of JA synthesis?; b) how does the feed-back mechanism operates?; c) are distinct lipoxygenases operating in such a cascade?

The role of jasmonate has been correlated with lipoxygenases in several physiological processes associated with plant development, senescence, seed germination, tuberisation, fruit ripening, tendril coiling, flavonoid synthesis, wound responses, pathogen defense and osmotic stress responses (Creelman and Mullet, 1997). Since lipoxygenase is associated with many plant processes, it is difficult to postulate a model, which provides an integral function for this enzyme in plants.

Intracellular localization of lipoxygenases

The appearance of lipoxygenases varies per tissue, developmental stage and in response to environmental stimuli. A manner to distinguish lipoxygenases is based on the subcellular location of the enzyme. Therefore, two groups were classified, namely those found in the cytosol (soluble types) and those found associated with organelles like chloroplasts (Peng *et al.*, 1994; Bell *et al.*, 1995), vacuoles (Grimes *et al.*, 1992) or in pericarp tissue (Ealing, 1994).

Germinating seeds express high levels of cytosolic lipoxygenase (Hildebrand and Hymowitz, 1982; Shibata *et al.*, 1987; Feussner and Kindl, 1994). However, physiological functions for these soluble lipoxygenases have sparely been proposed. A role for these lipoxygenases can be assigned to the mobilization of storage lipids during germination (Feussner *et al.*, 1995a). They might be involved in regulating the rate of germination by its role in the *de novo* synthesis of JA. JA might lower the sensitivity for ABA during germination (Berger *et al.*, 1996). In dry grains ABA is controlling the quiescent state of the grain. Therefore, the grain can survive during periods of drought or low temperature. After hydration the grain starts to germinate. The level of ABA and the sensitivity for ABA in the grain is crucial for germination. However, contrarily many of the other physiological responses found upon jasmonate treatment are similar to the effects observed from ABA treatment, suggesting that their mode of action must be closely related (Sembdner and Parthier, 1993).

For the non-cytosolic LOX, a number of reports show that these LOXs are tightly bound to organelles. Chloroplast and vacuolar localization have been reported for various species like: barley (Peng *et al.*, 1994; Feussner *et al.*, 1995b), tomato (Ealing, 1994; Heitz *et al.*, 1997), *Arabidopsis* (Bell *et al.*, 1995) and potato (Royé et al, 1996). The organelle associated LOXs have been identified in leaf, floral, fruit and tuber tissues. The genes encoding these LOX types are responsive to jasmonate or Me-jasmonate and are associated with processes like stress, fruit ripening or senescense. The appearance of multiple lipoxygenase forms in one cell makes the understanding of specific functions for distinct isoenzymes difficult. Gaining insight into the physiological functions for distinct lipoxygenases has been facilitated by the use of mutant and transgenic plants overexpressing or down-regulating genes involved in the lipoxygenase path.

For example, two distinct lipoxygenase genes (*AtLox1* and *AtLox2)* have been identified in *Arabidopsis thaliana* (Melan *et al.*, 1993; Bell *et al.*, 1995). *AtLox1* is mostly expressed in roots and young seedlings, while *AtLox2* is mostly expressed in leaves and inflorescences. *AtLox1* lacks a transit sequence and is localized in the cytosol of root cells. *AtLox2* is localized in the chloroplasts of leaf cells and exhibits an N-terminal transit sequence for chloroplasts targeting. In plants transformed with an AtLox2 cDNA construct, cosuppression of the *AtLox2* gene occurred. The cosuppression coincided with a lack of JA induction in leaves after wounding, whereas in wild-type plants JA did accumulate after wounding. Also the expression of the JA-responsive *VSP* (vegetative storage protein) gene was reduced in those transgenic plants. Therefore, *AtLox2* seems to be involved in the wound inducible synthesis of jasmonate and thereby related to JA-responsive gene expression in leaves.

Other studies are focused on lipoxygenase and jasmonate in relation with altered expression of jasmonate inducible proteins (see for reviews: Farmer, 1994; Creelman and Mullet, 1997, Wasternack and Parthier, 1997).

The role of jasmonate in plants

Jasmonic acid and its derivatives, like its methyl ester, collectively referred as jasmonates (JA) are plant hormones derived from α-linolenic acid through the action of lipoxygenase. Much of the interest in studying lipoxygenase in plants is related to the finding that jasmonates are solely synthesized by the intermediate action of lipoxygenase via the octadecanoid pathway. Like mentioned before, some *LOX* genes are responsive to jasmonate (Bell and Mullet, 1993; Melan *et al.*, 1993; Rouster *et al.*, 1997; Vörös *et al.*, 1998). Beside *LOX*-genes, a broad range of genes involved stress responses can also be induced by jasmonate treatment. Jasmonates modulate the expression of genes involved in pathogen and insect resistance, like: systemin (Heitz *et al.*,1997), polyphenol oxidase (Constabel *et al.*, 1995), proteinase inhibitor proteins (Doares *et al.*, 1995; Farmer *et al.*, 1989; Kim *et al.*, 1992), thionins (Wasternack *et al.*, 1994), ribosome inactivating protein-60 (Reinbothe *et al.*, 1994a; Chaudhry *et al.*, 1994), proline-rich proteins (Creelman and Mullet, 1991) and the genes involved in phenyl-propanoid metabolism (Kreuzaler *et al.*, 1983).

Some of these gene products are also designated as jasmonate-induced-proteins (JIP's), a group of abundant polypeptides appearing after JA or MeJA treatment of plants (Reinbothe *et al.*, 1994b). Jasmonate induces a class of proteins named vegetative storage proteins, which also accumulate upon several stress conditions, like mechanical wounding, water deficit and removal of sink tissue (Tranbarger *et al.*, 1991; Franceschi and Grimes, 1991).

Jasmonate can also repress the expression of several proteins associated with chloroplasts and photosynthesis, such as small and large subunits of ribulose-1,5-biphosphate carboxylase/ oxygenase, the light harvesting chlorophyll a/b (cab) binding proteins and the 65- and 68- kDa proteins of photosystem II. The joined action of inhibition of synthesis of chloroplast proteins and the induction of proteolytic enzymes might cause characteristic senescence symptoms in plants (Reinbothe *et al.*, 1994b).

Jasmonate can induce the production of medically important secondary metabolites such as alkaloids in *Catharanthus* and *Cinchona* seedlings (Aerts *et al.*, 1994). In *Taxus* suspension cells, treatment with jasmonate or fungal elicitor induces genes involved in the taxane biosynthesis (Yukimune *et al.*, 1996), thereby providing an alternative system to produce the anti-cancer drug "*paclitaxel*", which is commonly extracted from young meristemes of the *Taxus brevifolia* tree in relatively limited yield.

Jasmonate in relation to senescence and photosynthesis repression

Degradation of membranes is a process that can occur in plant tissue as a result of changes in the environment (like pathogen attack) or through a developmental switch, often referred to "senescence". Senescence is the result of the coordinated action of genes and might therefore be assigned as a form of "programmed cell death" also named "apoptosis". Leaf senescense consists of a number of processes that involve chlorophyll loss, membrane deterioration, increased membrane permeability and protein degradation (Reinbothe *et al.*, 1994b). The process provides the plant with a system for disassembly of superfluous organs. It has been shown that ethylene (Gepstein and Thimann, 1982) as well as jasmonate (Hung and Kao, 1996; Siedow, 1991; Wasternack, 1994) are involved in the promotion of leaf senescence, although the role for ethylene is only partially known.

It has been shown that the level of ethylene production in detached maize leaves cultivated in the dark was very low in contrast to jasmonate levels (Kao, 1992). However, the jasmonate-dependent promotion of senescence in detached leaves could be lowered by inhibitors of ethylene activity, thus suggesting that ethylene sensitivity is involved in the regulation of jasmonate promoted senescense. Moreover, characteristic chlorophyll degradation found during leaf senescence could also be retarded by addition of lipoxygenase inhibitors, indicating that the octadecanoid pathway is involved in the senescence process.

Comparing the role of jasmonate for promoting senescence with the occurrence of increasing jasmonate levels during seedling germination, are somehow contradictory. An explanation might be found in the process of repression of chloroplastic protein synthesis. In germinating seedlings, it might be beneficial during early stages of cell division to repress the development of chloroplastic proteins, thus providing first protein sources for cell development. The jasmonate levels are declining rapidly after cell elongation, resulting in the onset of the photosynthetic apparatus. Repression of genes which are involved in chloroplastic metabolism occurs during leaf senescence. This gene repression provides the plant with the ability to reduce the utilization of carbon sources under unfavourable conditions. Furthermore, as a result of the combined action of phospholipase and LOX, formation of cytotoxic hydroperoxides might cause membrane deterioration. Such a process will finally lead to senescence. During senescence carbon and nitrogen sources become available, which can be used for the *de novo*-synthesis of JA responsive proteins, such as R̲ibosomal I̲nactivating P̲rotein (Chaudhry *et al.*, 1994).

For example, *in vitro* translation experiments, showed that RIP 60 could strongly reduce the synthesis of the rubisco small subunit (SSU) and the light harvesting chlorophyll a/b binding (LHCP) precursor proteins in a concentration dependent manner (Reinbothe *et al.*, 1994b). However, this reduction was only observed in polysomal extracts treated with jasmonate, desiccation or osmotic stress for a period longer than 24 h prior to isolation. Therefore, the polysomal complex seemed to be tagged, which makes the polysomal complex become susceptible for RIP 60 promoted cleavage. In addition, a process of translational control through phosphorylation of a helicase (factor elF-4A of the polysomal complex) has been found (Thach, 1992). Thus, these RIPs might only be active in combination with a specific state of the polysomes modulated through conditions like stress or senescence. Furthermore, repression of photosynthesis occurs in leaves during darkness, resulting in a night-day circadian rhythm. It was proposed that jasmonate could be released from the chloroplast into the cytosol during the night through an acidification of the internal chloroplast. As a result, repression of nuclear encoded jasmonate responsive genes associated with photosynthesis would occur (Creelman and Mullet, 1997).

Jasmonate in sink and storage tissues

Plants have the ability to store large amounts of proteins, starch and fructans. Processes involved in synthesis and transport of such molecules are frequently designated by the term "vegetative sink". Nutrients are moved from vegetative tissue to developing seeds and pods where they will be stored until usage. After germination the nitrogen and carbon sources will be mobilized for seedling growth. Transient storage of nutrients also occurs in chloroplasts and vacuoles of leaves. Later, nutrient sources can in return be mobilized from the chloroplast to sink tissue. In soybean leaves, 25, 29, and 94 kDa proteins have been isolated, which accumulate during vegetative growth in vacuoles of mesophyl cells and decrease during seed set (Staswick, 1990). These proteins are named Vegetative Storage Proteins (VSPs). The 94 kDa protein from soybean has been identified as a lipoxygenase (Tranbarger *et al.*, 1991). Expression of *VSP* genes, including the 94 kDa encoding LOX, increases dramatically in response to removal of reproductive sink tissue or by supplying exogenous nitrogen sources, like NH_4NO_3, NO_3^- and NH_4^+ (Saravitz and Siedow, 1995; Grimes *et al.*, 1993). Therefore, nitrogen availability seems to be a signal for regulating the expression of *VSP* genes. High levels of jasmonate are present in pods and seeds during development and during germination in meristematic and young growing tissue before elongation. Thus, lipoxygenase itself is presumed to be involved in regulating *VSP* gene expression due to its role in the synthesis of jasmonate (Saravitz and Siedow, 1995). Jasmonates also play an important role in the formation of tubers in potato (Pelacho and Mingo-Castel, 1991; Ravnikar *et al.*, 1993).

Signals in stress response

Mechanical wounding and damage of leaves by pathogen attack induce rapid transcriptional activation of defense genes. In response to wounding, local and systemic genes will be activated in order to protect the plant. Distinct signals involved in defense reponse are: a) oligosaccharide fragments of the plant or the pathogen (Ryan and Farmer, 1991); b) α-linolenic acid liberated from cell- or chloroplastic- membranes (Creelman and Mullet, 1997); c) jasmonates (Creelman and Mullet, 1997; Wasternack and Parthier, 1997); d) polyamines (Wang and Buta, 1994); e) ABA (Dammann *et al.*, 1997); f) IAA (Thornburg and Li, 1990); g) Ethylene (O'Donnell *et al.*, (1996); h) salicylic acid (Shulaev *et al.*, 1995); i) electric signals (Rhodos *et al.*, 1996; Peña-Cortés *et al.*, 1995); j) wind (Mauch *et al.*, 1997); k) systemin (Constabel *et al.*, 1995). Several reports are focused on fitting these molecules together in the signalling plant defense cascade. However, a significant part of the research on the transduction network for regulating the effects of stress-response has concentrated on the implications of ABA and JA in separated and/or combined pathways. Thus, lipoxygenase might be involved in a variety of stress responses. Its role in stress response due to the synthesis of JA is evaluated.

Lipoxygenase is involved in wound response

A crucial signal molecule in this defense cascade is called systemin. Systemin is an 18-amino acid polypeptide that is transported through the phloem and is an inducer of proteinase inhibitor protein synthesis in a sytemic fashion (Narvaez-Vasquez *et al.*, 1995). Systemin is cleaved from a 200 amino acid precursor called prosystemin (McGurl and Ryan, 1992). Methyl jasmonate has been implicated with the action of systemin in the distribution of systemic stress signals, since wounding, application with MeJA or systemin induce expression of proteinase inhibitor protein (*pin*) genes and distinct protease (*swrp)* genes in tomato leaves (Constabel *et al.*, 1995). Proteolysis after pathogen attack is proposed to be an important physiological process in plant defense. Members of this group of wound induced defense genes are carboxypeptidases, leucine aminopeptidases and cysteine proteinases (Schaller and Ryan, 1996). The role for proteinases is likely to up-regulate protein synthesis, thereby providing a pool of free amino acids required for the *de novo*-synthesis plant defense proteins.

Furthermore, transgenic tomato plants, expressing an antisense prosystemin cDNA, have reduced levels of proteinase inhibitor proteins after wounding (McGurl *et al.*, 1992). Moreover, transgenic tomato plants overexpressing a prosystemin cDNA, have a constant level of two proteinase inhibitor proteins (Constabel *et al.*, 1995).

However, the action of systemin was shown to be ABA dependent, since ABA-deficient mutants do not induce *pin2* expression after application with systemin (Peña-Cortés *et al.*, 1995 and 1996).

It has also been shown that expression of several wound and ABA-inducible defense genes can be suppressed by aspirin, an inhibitor of the jasmonate synthesis in the octadecanoid pathway (Peña-Cortés *et al.*, 1993). Aspirin pretreated tomato plants were completely blocked in the expression of *pin2*, cathepsin D (*Cdi*) and Threonine deaminase (*Td*) genes after wounding in local as well as in systemic cells, whereas control plants showed induction of those genes. Detached leaves incubated with aspirin and additionally supplied with 12-oxo-PDA or jasmonate were able to overcome the aspirin-mediated inhibition, whereas 13(*S*)-HPOT had no effect, thus showing that aspirin blocks the allene oxide synthase step (AOS) or the allene cyclase step (AOC) in the octadecanoid pathway.

Similar observations were obtained from studies on a tomato mutant (JL5), deficient in wound-inducible gene expression. This mutant showed reduced resistance against herbivore attack (Howe *et al.*, 1996). Crossing the mutant line with a tomato line overexpressing the prosystemin gene could not restore defense gene expression. Also the application of elicitors or systemin itself did not induce defense gene expression. Some of the exogenously applied precursors for JA synthesis from the octadecanoid pathway could induce defense gene expression in the tomato mutant. Exogenously applied 13(*S*)-HPOT, the first metabolic product after the lipoxygenase step could not induce *pin2* expression, whereas the more down-stream precursor 12-oxo-PDA or MeJA itself could, indicating that LOX is involved in systemin mediated *pin2* expression. Observations provide information that systemin induced defense gene expression can be mediated through ABA and the octadecanoid pathway. Furthermore, it has been shown that intracellular levels of free α-linolenic acid increase to a 15-fold excess over that required for jasmonate synthesis within 1 h after wounding of tomato leaves (Conconi *et al.*, 1996). The increase of free α-linolenic acid correlated in time with the accumulation of JA. The content of total free fatty acids remained relatively unchanged, indicating that an extensive loss of lipids did not occur. The relatively highest lipid decrease was assigned to a release of lipids from the chloroplasts.

Some models, explaining systemic stress signal mediation by the interaction of systemin, ABA and JA have been proposed (Creelman and Mullet, 1997; Wasternack and Parthier, 1997; Dammann *et al.*, 1997; Peña-Cortés *et al.*, 1995). Alternatively, mediation of stress signals conducted by electric signals have been shown in tomato plants (Rhodos *et al.*, 1996).

Based upon the data presented, a model for systemic signal mediation by systemin, ABA, and the octadecanoid pathway is presented in Fig. 6. The model also includes involvement of electric signals and the action of a protein kinase in the ABA-JA mediated signal transduction chain.

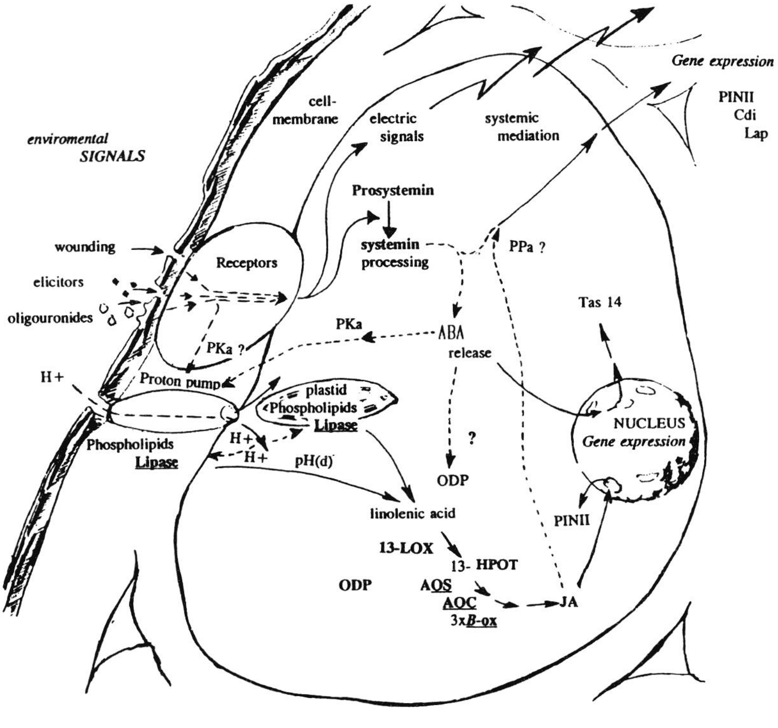

Fig. 6. Model for signal mediation of local and systemic response in tomato plants leading to induction of stress responsive genes by involvement of the octadecanoid pathway (ODP).
ABA, abscisic acid; LOX, lipoxygenase; JA, jasmonate; AOS, allene oxide synthase; AOC, allene oxide cyclase; 13-(*S*)HPOT, 13(*S*)-hydroperoxy-(9*Z*, 11*E*, 15*Z*)-octadeca-9,11,15-trienoic acid; β-ox, β-oxidation; PPa, protein phosphatase activity; PKa, protein kinase activity; PINII, proteinase inhibitor protein; Cdi, cathepsin D (Cdi); Td, Threonine deaminase; Lap, leucine amino peptidase; Tas 14, an ABA responsive gene.

Several studies showed the involvement of protein kinase activity in stress-signal mediation. For example, in tobacco it has been shown that a MAP-kinase is involved in wound induced accumulation of jasmonate (Seo *et al.*, 1995). Furthermore, addition of yeast extract-elicitor to *Phaseolus* suspension cells led to a rapid increase of jasmonate levels (Blechert *et al.*, 1995). Addition of staurosporine (an inhibitor of protein kinase activity) could inhibit jasmonate formation after elicitation. The involvement of proton-pump of which the activity is dependent on a phosphorylation step was suggested. In the presence of staurosporine, rapid alkalization in the medium of *Phaseolus* suspension cells was abolished. Also the *de novo* synthesis of jasmonate was repressed. Raising the pH by addition of base had no effect. The authors suggested that the action of the proton pump resulted in a cytosolic acidification, which led to an enzyme-catalyzed liberation of α-linolenic acid from the plasma membrane, so providing substrate for jasmonate biosynthesis. Similar observations were obtained from tomato suspension cells (Felix and Boller, 1995). Wounding, systemin or chitosan treatment led to a rapid alkalinization of the medium, which process could be blocked by adding protein kinase inhibitors to the medium. The systemin promoted alkalinization did also correlate with induction of several genes involved in ethylene biosynthesis. Use of an inactive systemin analogue did not cause alkalinization of the medium, neither *pin2* expression nor induction of genes involved in the ethylene biosynthesis pathway. Recently, cDNAs encoding for a receptor-like protein kinase, responsive to ABA and stress have been identified in wheat (Holappa and Walker-Simmons, 1995) and in *Arabidopsis thaliana* (Hong *et al.*, 1997). Furthermore, jasmonate or ABA induced *pin2* gene-expression in tomato leaves can be blocked by cycloheximide, suggesting that a late involvement of *de novo* protein synthesis is necessary for *pin2* gene expression (Peña-Cortés *et al.*, 1995).

A simplified model explaining systemic stress-response in tomato leaves, involves the processing of systemin, which is transported through the phloem to distant leaves. Subsequently, a systemin dependent mobilization of ABA occurs. ABA operates as an intracellular mediator for activation of phospholipases (in a protein kinase dependent fashion). α-Linolenic acid is mobilized from membranes to be used as substrate for the octadecanoid pathway resulting in an increase of jasmonate, leading to *pin2* expression (Fig. 6).

Lipoxygenase independent wound response

In potato plants it has been shown that two distinct proteinase inhibitor genes are present and activated through separated signal transduction pathways (Dammann *et al.*, 1997). Wounding of roots results in expression of a root specific *pin2* (*pin2*-R) gene mediated through a local route, while the systemic mediated response resulted in the expression of a leaf specific *pin2* (*pin2*-L). To determine whether lipoxygenase is contributing in one of these routes, first potato plants were subjected to jasmonate. High levels of *pin2* mRNAs and some other defense genes (e.g.: leucine peptidase (*Lap*), threonine deaminase (*Td*) and cathepsin D inhibitor (*Cdi*)) were detected in leaves. In contrast, in roots only the *pin*2-R gene was locally induced. In a dose-response experiment, it was shown that local gene expression accumulated by much lower JA concentrations suggesting differences of JA-perception between root and leaf cells.

In potato plants three distinct LOX-classes (LOX1, LOX2 and LOX3) have been identified (Sanchez-Serrano, 1986; Royo *et al.*, 1996) of which LOX2 and LOX3 have shown highest affinities for α-linolenic acid. LOX2 is expressed in leaves, while LOX3 occurs in leaves and roots. After wounding, the levels of LOX2 and LOX3 transcripts rise in leaves. The accumulation of LOX3 mRNA rises to a maximum within 30 min after wounding, whereas LOX2 rises to a maximum after 24 h. The difference in spatial expression suggests different roles for these lipoxygenase isoforms in the synthesis of jasmonate.

Subjecting potato roots to ABA resulted in comparable systemic expression of *pin2*-L and defense genes in leaves, suggesting involvement of ABA in JA-mediated stress responses (Dammann *et al.*, 1997).

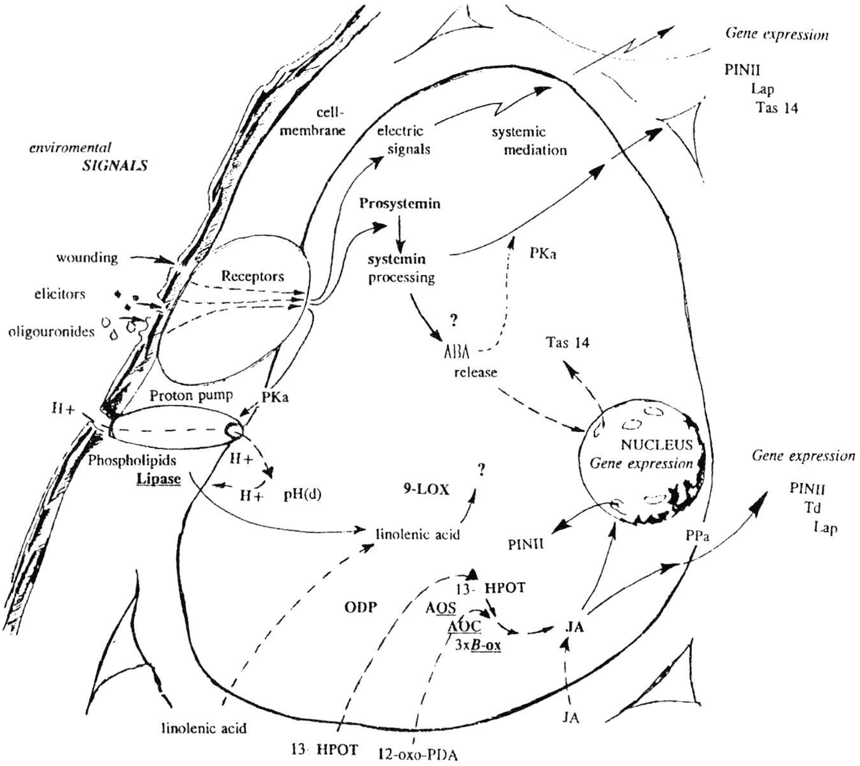

Fig. 7. Model for signal mediation of local and systemic response in potato plants for the activation of stress responsive genes in a LOX independent manner (proposed by Dammann *et al.*, (1997)). ABA, abscisic acid; LOX, lipoxygenase; JA, jasmonate; AOS, allene oxide synthase; AOC, allene oxide cyclase; 13-(*S*)HPOT, 13(*S*)-hydroperoxy-(9*Z*, 11*E*, 15*Z*)-octadeca-9,11,15-trienoic acid; β-ox, β-oxidation; PPa, protein phosphatase activity; PKa, protein kinase activity; PINII, proteinase inhibitor protein; Cdi, cathepsin D (Cdi); Td, Threonine deaminase; Lap, leucine amino peptidase; Tas 14, an ABA responsive gene.

To obtain more information of the involvement of the octadecanoid pathway in signal mediation, different substrates from the JA biosynthesis pathway were tested for their ability to induce stress-response. Application of α-linolenic acid did not induce systemic response. Also application of 13-(*S*)HPOT could not induce *pin2*-L expression, whereas jasmonate treatment did. These results indicate that products of the octadecanoid pathway could potentially induce expression of the *pin2*-L gene. The absence of 13-(*S*)HPOT consuming enzyme activity in root cells might be causing the lack of *pin2*-L expression. Supplying roots with JA in the presence of okadaic acid (a membrane permeable inhibitor of protein phosphatases), reduced the systemic expression of defense genes in leaves, whereas the root specific *pin2* (R) expression was not affected. An okadaic sensitive phosphatase seems to be necessary for the systemic JA-mediated activation of defense genes in leaves. Subsequently, it was shown that inhibitors of JA-biosynthesis as well as okadaic acid could not abolish systemic ABA induced *pin2*-L expression. The protein kinase inhibitor staurosporine could block ABA mediated gene expression, suggesting that also a protein kinase is required in this ABA dependent route.

In conclusion Dammann *et al.* (1997) showed for potato plants that JA is a potential inducer for systemic *pin2* gene expression, although JA is not required for induction of systemic *pin*2 gene expression. Thus, systemic *pin*2 gene expression is not depending on the action of the octadecanoid pathway (Fig. 7).

Lipoxygenase is induced by pathogens

Jasmonic acid induces plant defense genes and might thereby protect the crop. Application of MeJA to plants can cause local and systemic protection against pathogens. Numerous studies have been performed by comparing defense gene responses after pathogen inoculation of cultivars possessing susceptible and hypersensitive (resistent) phenotypes (Kogel *et al.*, 1995; Hammond-Kosack *et al.*, 1994; Freialdenhoven *et al.*, 1994; Rouster *et al.*, 1997; Molina *et al.*, 1997). The *in vivo* mobilization of jasmonate after infestation by various fungi or pests is often correlated with an induction of lipoxygenase gene expression resulting in increased resistance against pathogens. Differences in lipoxygenase expression caused by inoculation with virulent or avirulent pathogen races have been demonstrated (Ohta *et al.*, 1991; Croft *et al.*, 1993; Melan *et al.*, 1993). In beans lipoxygenase activity was increasing during hypersensitive response after inoculation with an avirulent race of the plant pathogenic bacterium *Pseudomonas syringae* pv. phaseolicola. In contrast, no lipoxygenase induction was observed in the susceptible response after inoculation with virulent bacteria. In *Arabidopsis* inoculation with an avirulent race of the pathogen *Pseudomonas syringae* resulted in a much faster and higher expression of LOX compared with inoculation with a virulent bacterial race (Melan *et al.*, 1993). Thus, lipoxygenase activity might be determined by signals providing pathogen resistance. However, no role for jasmonate nor lipoxygenase could be demonstrated in barley during compatible or incompatible interaction with the powdery mildew fungus "*Erysihe gramines F.* sp. hordei" (Hause *et al.*, 1997). A strong jasmonate inducible gene (*JIP23*) was used to determine pathogen directed jasmonate mobilization in infected barley leaves. Where exogenously applied MeJA induced the *JIP-23* gene on the susceptible as well as on the resistant barley line, no *JIP-23* gene expression was observed after pathogen inoculation. Instead accumulation of chitanase class II protein occurred in a relatively short period in comparison with the susceptible line.

Addition of fungal elicitors to barley suspension cells led to an increase of endogenous jasmonate levels within 2 h. These jasmonate levels induce the expression of phytoalexins, such as flavonoids, terpenoids and alkaloids (Gundlach *et al.*, 1992). Phytoalexins are known antimicrobial compounds. Because jasmonates are mediators of phytoalexin synthesis as well as other defense compounds, their role in pathogen defense is significant. In another study of pathogen related gene expression, tobacco cell suspensions were treated with elicitor from *Phytophthora parasitica* var. nicotianae (Rickauer *et al.*, 1997). The elicitor induced a rapid and transient increase in jasmonate levels, which could be abolished when cells were preincubated with ETYA (a lipoxygenase inhibitor). Pretreatment with ETYA also inhibited the induction of proteinase inhibitor protein by fungal elicitor. Addition of MeJA, or α-linolenic acid caused *pin* expression, whereas linoleic acid had no effect. The expression of other defense related genes encoding glucanases, chitinases, hydroxyproline rich proteins or glycine rich glycoproteins were induced in a basically similar manner by fungal elicitor or MeJA, although ETYA-pretreatment could only partially abolish the expression of these defense genes. Another defense related gene, encoding the sesquiterpene cyclase (5-*epi*-aristolochene synthase) could only be induced by the elicitor and not by MeJA. Results indicate that also during hypersensitivity response, distinct pathways are involved in mediation leading to defense gene expression.

In wheat it was shown that glycopeptide elicitors from germ tubes of the rust fungus *Puccinia graminis* induce the expression of a 100-kDa lipoxygenase in leaf, while chitosan, chitin oligosaccharides and MeJA induce the expression of two distinct lipoxygenases in leaf with molecular masses of 92 and 103 kDa, respectively (Bohland *et al.*, 1997). Taken together, these data show the involvement of the lipoxygenase pathway in plant defense systems, although also LOX-independent routes for defense response occur.

During plant pathogen interaction, secondary metabolism of the plant can alter pathogen growth or protein synthesis. For example products of the lipoxygenase pathway in soybean seeds have been implicated with an increased resistance to mycotoxin contamination (aflatoxins) produced by *Aspergillus nidulans* (Burow *et al.*, 1997). *Aspergilli* invade soybean through cracks generated by insect damage or drought stress. *In vitro* experiments showed that 13-HPOD reduced the production of aflatoxin by *Aspergillus*, while 9-HPOD prolonged the synthesis of aflatoxins. The authors suggested that induction of 13-LOX might therefore be beneficial in resistance against pathogen attack.

Lipoxygenase is present during development and germination

Multiple lipoxygenase isoforms have been identified in a large number of plant species during seed development and germination. Most studies describe the temporal and spatial expression of the LOX-isoforms (reviewed by Siedow, 1991). However, mutants (Hildebrand and Hymowitz, 1983; Kitamura *et al.*, 1983; Davies and Nielsen, 1986; Wang *et al.*, 1996; Suzuki and Matsukura, 1997) or transgenic plants (Bell *et al.*, 1995) lacking distinct isoforms are not significantly altered in their development and germination capacities. Based upon these observations it is difficult to designate essential functions for lipoxygenase during plant development and germination. Initial studies were performed on legume seedlings, such as soybean and pea, because of the high lipoxygenase levels found during germination. So far most of these data are restricted to the appearance of lipoxygenase, the biochemical properties and the temporal and spatial expression patterns. Increasing lipoxygenase activity during early stages of germination have been reported for species like; soybean (Park and Polacco., 1989), rice (Ohta *et al.*, 1986), cucumber (Matsui *et al.*, 1988), *Arabidopsis* (Bell and Mullet, 1993) and barley (Holtman *et al.*, 1996). The function of high levels of lipoxygenase expression during germination remains unclear. Lipoxygenase might be operating in mobilization of esterified storage lipids for the generation of energy during germination (Feussner *et al.*, 1995a). In soybean seedlings, six distinct lipoxygenase isoforms have been detected by immunogold labelling with the a cross-reacting LOX antibody. Lipoxygenases occurred in the cytoplasm and associated with protein bodies or in the vacuoles, but not associated with lipid bodies, mitochondria or plasma membranes (Wang *et al.*, 1996). These results suggested that lipoxygenases from soybean seedlings are not directly involved in reserve lipid mobilization.

Alternatively, lipoxygenase has been implicated with jasmonate levels occurring in seedlings. Jasmonate might stimulate germination of dormant apple embryos through increasing alkaline lipase activity, thereby providing the mobilization of lipid reserves. The free lipids can be used in seedling growth (Ranjan and Lewak, 1992). It is interesting that expression of LOX-genes after seed imbibition coincides with increasing levels of JA, while otherwise JA can block germination. In soybean seedlings JA levels are highest in youngest tissues and lowest in zones after elongation. JA levels decline during seedling growth (Creelman and Mullet, 1995). JA has been implicated with inhibition of IAA-induced cell elongation in oat coleoptiles (Ueda *et al.*, 1994; Miyamoto *et al.*, 1997). Addition of sucrose could reverse the JA-modulated repression of cell wall elongation.

In barley high levels of lipoxygenase activity are measured over the first 24 h after seed imbibition, followed by a rapid decrease of activity. Then the activity gradually increases until day seven (Holtman *et al.*, 1996). Such a pattern has also been reported for lipoxygenases of other species, like soybean (Hildebrand and Hymowitz, 1983). The lipoxygenase content is correlated with the stage of germination. Thus, the level of expression can vary in time depending on factors that influence the germination velocity, such as germination capacity, moisture content, light, nutrients and temperature. Two isoenzymes (LOX1 and LOX2) have been characterized in barley during germination (Doderer *et al.*, 1992). LOX1 is present in both quiescent and germinating barley grains, whereas LOX2 is considerably less detectable in quiescent grain. The major difference between the isoenzymes is the product specificity with linoleic acid as a substrate. In barley LOX1 produces 9-HPOD, whereas LOX2 forms 13-HPOD. The high levels of activity measured during the first 24 h of imbibition are mainly caused by the action of LOX1, which is mobilized from the quiescent grain. The low activity detected at 24 h of imbibition is caused by the period needed for the *de novo* synthesis of LOX1 and LOX2. Comparable levels of LOX 1 and LOX 2, detected from day 2 until day 7 after germination, resulted in a almost equal distribution of 9-HPOD and 13-HPOD products.

Aim of this thesis

Lipoxygenases are well conserved during evolution, suggesting that their existence might provide for structural functions in the maintenance of plant. So far the physiological functions of plant lipoxygenases have only been partially understood. Research has been focused on the occurrence of lipoxygenase in grains and seeds, although the importance of lipoxygenase expression during development and germination has not yet been established. On the other hand, one of the presumed unfavorable aspects of lipoxygenase in economically important food-crops is its possible involvement in the formation of undesired substances, because of unpleasant taste characteristics.

In cereal grains, lipoxygenases have been characterized extensively at the biochemical level. But hardly any information was available on their primary structures.

The aim of this thesis was to obtain information of the number of lipoxygenase isoenzymes from barley grain and to study the physiological functions of distinct isoenzymes by a combination of molecular and biochemical analysis.

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Chapter 2

Primary structure of a lipoxygenase from barley grain as deduced from its cDNA sequence*

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Abstract

A full length cDNA sequence for a barley grain lipoxygenase was obtained. It includes a 5' untranslated region of 69 nucleotides, an open reading frame of 2586 nucleotides encoding a protein of 862 amino acid residues and a 3' untranslated region of 142 nucleotides. The molecular mass of the encoded polypeptide was calculated to be 96.392 Da. Its amino acid sequence shows a high homology with that of other plant lipoxygenases identified to date.

Lipoxygenases (Linoleate: oxygen oxidoreductase, E.C. 1.13.11.12) are a group of enzymes that catalyse the oxygenation of fatty acids containing a cis,cis-1,4-pentadiene system to form conjugated hydroperoxydienes. The physiological role of plant lipoxygenase is still poorly understood. The enzyme is involved in the biosynthesis of regulatory molecules like jasmonic acid and traumatic acid and may play a role in growth and development, in plant senescence and in responses to various types of stress like wounding and pathogen attack (1).

Barley grains contain at least two distinct lipoxygenase isoenzymes, lipoxygenase 1 and 2, which have been purified from germinating grains and characterized (2). Lipoxygenase 1 is present in both quiescent and germinating barley grain, while lipoxygenase 2 appears during germination. The isoenzymes differ in the broadness of their pH activity range, in their isoelectric point and in the product formed upon incubation with linoleic acid. Lipoxygenase 1 solely forms 9-hydroperoxides, whereas 13-hydroperoxides are the major products formed by lipoxygenase 2.

To date many plant lipoxygenases have been cloned and sequenced, but most of the data are limited to dicotyledons (3-12). For monocotyledons complete coding sequences of two rice lipoxygenases have been published, one encoding a grain lipoxygenase (13), the other a vegetative enzyme (14). Their homology with dicotyledon lipoxygenases is relatively low. As a first step in studying the physiological role of lipoxygenase in barley grains we have obtained a full length cDNA sequence (LoxA) coding for a barley grain lipoxygenase.

A lambda ZAP expression library of 2 x 10^6 plaques, from poly $(A)^+$ mRNA isolated from developing barley grains (8 - 20 days after pollination, cv. Triumph) (15) was screened. In developing barley grains both lipoxygenase 1 and 2 are present (data not shown). Immunological screening of 67,000 recombinant phages with anti-barley lipoxygenase antibodies, cross-reacting with both lipoxygenase 1 and 2 (16), revealed three positive clones, containing an identical insert based on restriction analysis as well as partial sequence analysis (results not shown). One of these (pLoxA1; Fig. 1, position: 802-2818) was sequenced and showed an open reading frame encoding a polypeptide of 518 amino acid residues with a homology of 74,4 % to rice lipoxygenase L-2 (13). According to the expected size of the polypeptide it corresponds to approximately 70 % of a full size barley lipoxygenase.

In order to clone a full size barley LoxA cDNA the following strategy was used. First a reverse primer, which included a XhoI restriction site located on the 5' part of the insert of pLoxA1 (fig. 1, primer 1, reverse position: 1295-1264, 5'-CCCTCGAGGTTCTTCTCTATGTGCTCCGCC-3'), was synthesized and used for cDNA synthesis on poly $(A)^+$ mRNA extracted from barley grains (cv. Triumph) germinated for 48 hours at 25 °C according to the protocol for ZAP-cDNA synthesis (Stratagene). The cDNA products were cloned into a lambda ZAP vector. A 489 bp 5' XhoI fragment of the pLoxA1 insert (Fig. 1, position: 802-1292) was used to screen 5.10^6 pfu. Six clones were obtained, of which the longest clone (pLoxA2) contained an insert of 1.12 Kb, which combined with pLoxA1 resulted in an almost full length coding sequence (Fig. 1, position: 169-1292).

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                                                      [ <- primer 4
   1 AGTGAAAAACAGTGTGCTGGTGCCATTGGTTGGAGCAGTGAAAGCGAGGAGAGGAGGCCA 60

              ]
  61 AGAACAAGATGCTGCTGGGAGGGCTGATCGACACCCTCACGGGGGCGAACAAGAGCGCCC 120
   1         M  L  L  G  G  L  I  D  T  L  T  G  A  N  K  S  A  R 18

 121 GGCTCAAGGGCACGGTGGTGCTCATGCGCAAGAACGTGCTGGACCTCAACGACTTCGGCG 180
  19   L  K  G  T  V  V  L  M  R  K  N  V  L  D  L  N  D  F  G  A 38
                   [   <- primer 2                          '
 181 CCACCATCATCGACGGCATCGGCGAGTTCCTCGGCAAGGGCGTCACCTGCCAGCTTATCA 240
  39   T  I  I  D  G  I  G  E  F  L  G  K  G  V  T  C  Q  L  I  S 58
       ]         SalI
 241 GCTCCACCGCCGTCGACCAAGACAACGGCGGTCGCGGGAAGGTGGGCGCGGAGGCGGAGC 300
  59   S  T  A  V  D  Q  D  N  G  G  R  G  K  V  G  A  E  A  E  L 78

 301 TGGAGCAGTGGGTGACGAGCCTGCCGTCGCTGACGACGGGGGAGTCCAAGTTCGGCCTCA 360
  79   E  Q  W  V  T  S  L  P  S  L  T  T  G  E  S  K  F  G  L  T 98

 361 CCTTCGACTGGGAGGTGGAGAAGCTCGGGGTGCCGGGCGCCATCGTCGTCAACAACTACC 420
  99   F  D  W  E  V  E  K  L  G  V  P  G  A  I  V  V  N  N  Y  H 118

 421 ACAGCTCCGAGTTCCTGCTTAAAACCATCACCCTCCACGACGTCCCCGGCCGCAGCGGCA 480
 119   S  S  E  F  L  L  K  T  I  T  L  H  D  V  P  G  R  S  G  N 138
              [     primer 3 ->     ]
 481 ACCTCACCTTCGTCGCCAACTCATGGATCTACCCCGCCGCCAACTACCGATACAGCCGCG 540
 139   L  T  F  V  A  N  S  W  I  Y  P  A  A  N  Y  R  Y  S  R  V 158
                      *[-> intron in pLoxiP1]
 541 TCTTCTTCGCCAACGACACGTACCTGCCGAGCCAGATGCCGGCGGCGCTGAAGCCGTACC 600
 159   F  F  A  N  D  T  Y  L  P  S  Q  M  P  A  A  L  K  P  Y  R 178

 601 GCGACGACGAGCTCCGGAACCTGCGTGGCGACGACCAGCAGGGCCCGTACCAGGAGCACG 660
 179   D  D  E  L  R  N  L  R  G  D  D  Q  Q  G  P  Y  Q  E  H  D 198

 661 ACCGCATCTACCGCTACGACGTCTACAACGACCTCGGCGAGGGCCGCCCCATCCTCGGCG 720
 199   R  I  Y  R  Y  D  V  Y  N  D  L  G  E  G  R  P  I  L  G  G 218

 721 GCAACTCCGACCACCCTTACCCGCGCCGCGGCCGCACGGAGCGCAAGCCCAACGCCAGCG 780
 219   N  S  D  H  P  Y  P  R  R  G  R  T  E  R  K  P  N  A  S  D 238
                  BglII
 781 ACCCGAGCCTGGAGAGCCGGCTGTCGCTGCTGGAGCAGATCTACGTGCCGCGGGACGAGA 840
 239   P  S  L  E  S  R  L  S  L  L  E  Q  I  Y  V  P  R  D  E  K 258

 841 AGTTCGGCCACCTCAAGACGTCCGACTTCCTGGGCTACTCCATCAAGGCCATCACGCAGG 900
 259   F  G  H  L  K  T  S  D  F  L  G  Y  S  I  K  A  I  T  Q  G 278
                    prot. pep. seq. 1.
 901 GCATCCTGCCGGCCGTGCGCACCTACGTGGACACCACCCCCGGCGAGTTCGACTCCTTCC 960
 279   I  L  P  A  V  R  T  Y  V  D  T  T  P  G  E  F  D  S  F  Q 298

 961 AGGACATCATCAACCTCTATGAGGGCGGCATCAAGCTGCCCAAGGTGGCCGCCCTGGAGG 1020
 299   D  I  I  N  L  Y  E  G  G  I  K  L  P  K  V  A  A  L  E  E 318

1021 AGCTCCGTAAGCAGTTCCCGCTCCAGCTCATCAAGGACCTCCTCCCCGTCGGCGGCGACT 1080
 319   L  R  K  Q  F  P  L  Q  L  I  K  D  L  L  P  V  G  G  D  S 338
```

Fig. 1. The LoxA cDNA sequence and the deduced amino acid sequence. Nucleotide 1-242 and 489-557 originate from the genomic iPCR clone (p*LoxiP*1), nucleotide 169-1292 originate from cDNA clone pLoxA2 and nucleotide 802-2818 originate from pLoxA1. Translation start site is on position 69 and translation terminates on position 2657. Putative polyadenylation signal sequences are underlined. Primer sequences are printed in bold.

```
1081 CCCTGCTTAAGCTCCCCGTGCCCCACATCATCCAGGAGAACAAGCAGGCGTGGAGGACCG 1140
 339  L  L  K  L  P  V  P  H  I  I  Q  E  N  K  Q  A  W  R  T  D  358

1141 ACGAGGAGTTCGCACGGGAGGTGCTCGCCGGCGTCAACCCGGTCATGATCACGCGTCTCA 1200
 359  E  E  F  A  R  E  V  L  A  G  V  N  P  V  M  I  T  R  L  T  378

1201 CGGAGTTCCCGCCAAAAAGTAGTCTGGACCCTAGCAAGTTTGGTGACCACACCAGCACCA 1260
 379  E  F  P  P  K  S  S  L  D  P  S  K  F  G  D  H  T  S  T  I  398
 [  <- primer 1                XhoI  ]
1261 TCACGGCGGAGCACATAGAGAAGAACCTCGAGGGCCTCACGGTGCAGCAGGCGCTGGAAA 1320
 399  T  A  E  H  I  E  K  N  L  E  G  L  T  V  Q  Q  A  L  E  S  418

1321 GCAACAGGCTGTACATCCTTGATCACCATGACCGGTTCATGCCGTTCCTGATCGACGTCA 1380
 419  N  R  L  Y  I  L  D  H  H  D  R  F  M  P  F  L  I  D  V  N  438

1381 ACAACCTGCCCGGCAACTTCATCTACGCCACGAGGACCCTCTTCTTCCTGCGCGGCGACG 1440
 439  N  L  P  G  N  F  I  Y  A  T  R  T  L  F  F  L  R  G  D  G  458

1441 GCAGGCTCACGCCGCTCGCCATCGAGCTGAGCGAGCCCATCATCCAGGGCGGCCTTACCA 1500
 459  R  L  T  P  L  A  I  E  L  S  E  P  I  I  Q  G  G  L  T  T  478

1501 CGGCCAAGAGCAAGGTTTACACGCCGGTGCCCAGCGGCTCCGTCGAAGGCTGGGTGTGGG 1560
 479  A  K  S  K  V  Y  T  P  V  P  S  G  S  V  E  G  W  V  W  E  498

1561 AGCTCGCCAAGGCCTACGTCGCCGTCAATGACTCCGGGTGGCACCAGCTCGTCAGCCACT 1620
 499  L  A  K  A  Y  V  A  V  N  D  S  G  W  H  Q  L  V  S  H  W  518

1621 GGCTGAACACTCACGCGGTGATGGAGCCGTTCGTGATCTCGACGAACCGGCACCTTAGCG 1680
 519  L  N  T  H  A  V  M  E  P  F  V  I  S  T  N  R  H  L  S  V  538

1681 TGACGCACCCGGTGCACAAGCTGCTGAGCCCGCACTACCGCGACACCATGACCATCAACG 1740
 539  T  H  P  V  H  K  L  L  S  P  H  Y  R  D  T  M  T  I  N  A  558

1741 CGCTGGCGCGGCAGACGCTCATCAACGCCGGCGGCATCTTCGAGATGACGGTGTTCCCGG 1800
 559  L  A  R  Q  T  L  I  N  A  G  G  I  F  E  M  T  V  F  P  G  578

1801 GCAAGTTCGCGTTGGGGATGTCGGCCGTGGTGTACAAGGACTGGAAGTTCACCGAGCAGG 1860
 579  K  F  A  L  G  M  S  A  V  V  Y  K  D  W  K  F  T  E  Q  G  598

1861 GACTGCCGGACGATCTCATCAAGAGGGGCATGGCGGTGGAGGACCCGTCGAGCCCGTACA 1920
 599  L  P  D  D  L  I  K  R  G  M  A  V  E  D  P  S  S  P  Y  K  618

1921 AGGTGCGGTTGCTGGTGTCGGACTACCCGTACGCGGCGGACGGGCTGGCGATCTGGCACG 1980
 619  V  R  L  L  V  S  D  Y  P  Y  A  A  D  G  L  A  I  W  H  A  638

1981 CCATTGAGCAGTACGTGAGCGAGTACCTGGCCATCTACTACCCGAACGACGGCGTGCTGC 2040
 639  I  E  Q  Y  V  S  E  Y  L  A  I  Y  Y  P  N  D  G  V  L  Q  658

2041 AGGGCGATACGGAGGTGCAGGCGTGGTGGAAGGAGACGCGCGAGGTCGGGCACGGCGACC 2100
 659  G  D  T  E  V  Q  A  W  W  K  E  T  R  E  V  G  H  G  D  L  678

2101 TCAAGGACGCCCCATGGTGGCCCAAGATGCAAAGTGTGCCGGAGCTGGCCAAGGCGTGCA 2160
 679  K  D  A  P  W  W  P  K  M  Q  S  V  P  E  L  A  K  A  C  T  698

2161 CCACCATCATCTGGATCGGGTCGGCGCTGCATGCGGCAGTCAACTTCGGGCAGTACCCCT 2220
 699  T  I  I  W  I  G  S  A  L  H  A  A  V  N  F  G  Q  Y  P  Y  718

2221 ACGCGGGGTTCCTCCCGAACCGGCCGACGGTGAGCCGGCGCCGCATGCCGGAGCCCGGCA 2280
 719  A  G  F  L  P  N  R  P  T  V  S  R  R  R  M  P  E  P  G  T  738


2281 CGGAGGAGTACGCGGAGCTGGAGCGCGACCCGGAGCGGGCCTTCATCCACACCATCACGA 2340
 739  E  E  Y  A  E  L  E  R  D  P  E  R  A  F  I  H  T  I  T  S  758

2341 GCCAGATCCAGACCATCATCGGCGTGTCGCTGCTGGAGGTGCTGTCGAAGCACTCCTCCG 2400
 759  Q  I  Q  T  I  I  G  V  S  L  L  E  V  L  S  K  H  S  S  D  778

2401 ACGAGCTGTACCTCGGGCAGCGGGACACGCCGGAGTGGACCTCGGACCCAAAGGCCCTGG 2460
 779  E  L  Y  L  G  Q  R  D  T  P  E  W  T  S  D  P  K  A  L  E  798
```

Fig.1. (Continued).

```
2401 ACGAGCTGTACCTCGGGCAGCGGGACACGCCGGAGTGGACCTCGGACCCAAAGGCCCTGG 2460
779   E  L  Y  L  G  Q  R  D  T  P  E  W  T  S  D  P  K  A  L  E  798

2461 AGGTGTTCAAGCGGTTCAGCGACCGGCTGGTGGAGATCGAGAGCAAGGTGGTGGGCATGA 2520
799   V  F  K  R  F  S  D  R  L  V  E  I  E  S  K  V  V  G  M  N  818
                      prot. pep. seq. 2.
2521 ACCATGACCCGGAGCTCAAGAACCGCAACGGCCCGGCTAAGTTTCCCTACATGCTGCTCT 2580
819   H  D  P  E  L  K  N  R  N  G  P  A  K  F  P  Y  M  L  L  Y  838

2581 ACCCCAACACCTCCGACCACAAGGGCGCCGCTGCCGGGCTTACCGCCAAGGGCATCCCCA 2640
839   P  N  T  S  D  H  K  G  A  A  A  G  L  T  A  K  G  I  P  N  858

2641 ACAGCATCTCCATCTAATCTAAGCCATCGGCAACCATGGATGAATAAAGGGCGTTCGCCA 2700
859   S  I  S  I  *                                              862

2701 CGTACGAAACTTGTCGAGAGATTGGTGTAGTGTGTGTCTGTGACAGTACTATGTCAGCAG 2760

2761 TTGCTCTTTAAGCCGAATAAATAAAGCAGATTTGCTTCCAAAAAAAAAAAAAAAAAAA 2818
```

Fig.1.(Continued).

Using inverse PCR cloning (17) a PCR fragment of approximately 500 bp was obtained, which provided the missing 5' coding part of the LoxA cDNA. Genomic BglII digested DNA fragments (cv. Triumph) of 2.5 - 3.0 kb, enriched for sequences upstream of the BglII site at position 817, were circularized by ligation and subsequently linearized by digestion with SalI. PCR was performed with the following primers: primer 2 (Fig. 1, reverse position: 242-219, 5'-GCTGATAAGCTGGCAGGTGACGCC- 3') and primer 3 (Fig. 1, sense position: 489-512, 5'TTCGTCGCCAACTCATGGATCTAC 3'). PCR products were cloned into KS bluescript plasmids (Stratagene)(18).

Out of 2000 transformants one clone (p*LoxiP*1) was selected by probe hybridisation (using the insert of pLoxA2, Fig. 1, position: 169-1292). The insert of p*LoxiP*1 partially overlapped with perfect homology to the insert of pLoxA2 (Fig. 1, position: 169-242 and 489-557), whereas homology with pLoxA2 downstream nucleotide position 557 ended due to the appearance of an intron (sequence not shown). The inverse PCR clone contained the sequence of the 5' region of the LoxA cDNA (Fig. 1, position: 1-168), resulting in a full-length coding sequence.

The transcription start was mapped 68 nucleotides upstream of the first in frame ATG codon in the coding region by primer extension analysis. Poly $(A)^+$ RNA, extracted from barley embryos (cv. Triumph) germinated for 48 hours at 18 °C, was primed with primer 4 (fig. 1, reverse position: 69 - 50, 5'-TCTTGTTCTTGGCCTCCTCT-3') and analyzed by gel electrophoresis (Fig. 2). The corresponding sequence was obtained with primer 4 and a 5 Kb *LoxA* genomic clone comprising upstream 5' sequences and 500 bp of the coding sequence, isolated from a *H. vulgare* cv. Himalaya genomic library. The 5' leader sequence of this clone and the p*LoxiP*1 were identical

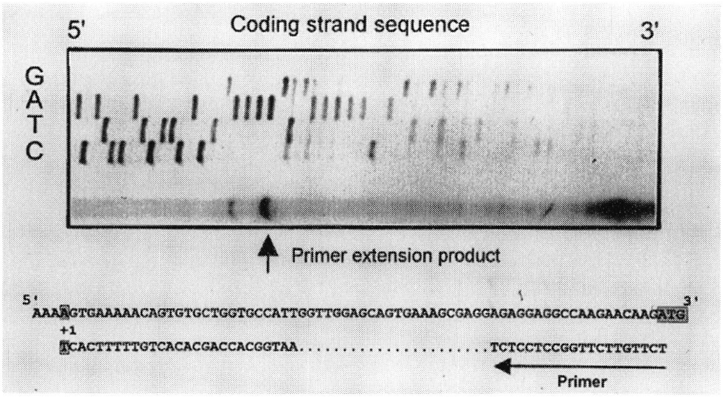

Fig. 2. LoxA transcription start site. Primer extension on poly (A)$^+$RNA (0.2 mg) was performed with ^{32}P-labelled primer 4 and (M-MLV) reverse transcriptase. The purified labelled DNA was separated on a 6% sequencing gel togethe with the sequence generated with primer 4 and the *LoxA* genomic clone.

```
                                                                  ref.
barley LoxA: ML--L-G--G----LIDTLT-GA-N----KSARLKGTVVLMRKNVLD 32
rice    L-2: ML----G--G----IIGGLT-G--N----KNARLKGSLVLMRKNALD 30 (13)
soybean  L1: MFS-A----G--------------H----K---IKGTVVLMPKNELE 21  (3)
soybean  L2: MFS-VPGVSG-----I--LNRGGGH----K---IKGTVVLMRKNVLD 32  (4)
soybean  L3: ML----G--G----L---LHRG--H----K---IKGTVVLMRKNVLD 25  (5)
pea      L2: MFPNVT---G----L---LNKG--H----K--R--GTVVLMRKNVLD 27  (6)
pea      L3: MFS---GVTG-----I--LNRG--H----K---IKGTVVLMRKNVLD 28  (7)
lentil  Lox: MASLL-F--G----------RG-Q-----K---LKGTVILMQKNVLD 25  (8)
potato  Lox: M---I----GQ---ITSGLF-G-GH--DSK--KVKGTVVMMNKNVLD 32  (9)
Arab   Lox1: MF-------GELRDL---LT-GGGNETTTK--KVKGTVVLMKKNVLD 34 (10)
Frenchb Lox: MF-------G-----I--LNRG--H----K---IKGTVVLMTKNVFD 24 (11)
tomlox  A  : ML---GQLVGG---LI-----G-GHH-DSK--KVKGTVVMMKKNALD 32 (12)
tomlox  B  : M-SL--G--G----I---VDAILGKDDRPK---VKGRVILMKKNVLD 32 (12)
             o        o                   o    +o++++o+oo+++
```

Fig. 3. Comparison of the deduced N-terminal sequences of plant lipoxygenases. The alignment reveals the low similarity of N-terminal ends. The regions with high similarity start at barley amino acid residue K at position 20 and are indicated by [+]. Residues identical in all sequences are indicated by [o]. Gaps (-) are introduced to obtain maximal homology in the alignment. The number at the end of each line corresponds to the position of the last shown residue. Abbreviations: Nlox, novel lipoxygenase from rice; L1, lipoxygenase 1; L2, lipoxygenase 2; L3, lipoxygenase 3; arab Lox, a lipoxygenase from Arabidopsis; Frenchb Lox, a lipoxygenase from French bean; tomlox, a lipoxygenase from tomato.

The open reading frame of barley LoxA cDNA has a length of 2586 nucleotides, encoding a polypeptide of 862 amino acid residues (Fig. 1). The deduced amino acid sequence aligns with the amino acid sequence of two peptide fragments of barley lipoxygenase 1 (2) at amino acid residue positions 274-294 and 832-845 (Fig. 1).

The molecular mass of this barley lipoxygenase, calculated to be 96.392 Da by GCG-Wisconsin computer prediction (19), is in agreement with the molecular mass of 90 kDa found for barley lipoxygenase isoenzymes using SDS-PAGE (2).

The coding region of LoxA cDNA showed a high homology to that of other plant lipoxygenases (Table 1). Especially striking is the high percentage of deduced amino acid similarity (86.4 %) and identity (74.9 %) between barley LoxA and rice lipoxygenase L-2. The N-terminus of the deduced protein sequence is the least conserved part as compared to other plant lipoxygenases (Fig. 3). Also, here barley is most similar to rice lipoxygenase L-2, indicating that these monocotyledon grain lipoxygenases are evolutionary closely related.

	amino acid		DNA	
Barley LA with:	similarity	identity	identity	Ref.
rice L-2	86.4	74.9	81.7	(13)
rice NLox	60.9	41.2	n.c.	(14)
soybean L1	72.8	55.2	57.9	(3)
soybean L2	73.0	56.2	58.5	(4)
soybean L3	75.0	56.8	58.5	(5)
pea L2	73.7	56.3	58.0	(6)
pea L3	73.5	55.4	59.5	(7)
lentil Lox	73.4	55.7	58.0	(8)
potato Lox	74.6	58.1	58.5	(9)
Arab Lox1	75.7	57.2	59.5	(10)
Frechb Lox	74.2	56.7	58.6	(11)
tomlox A	75.2	58.9	58.9	(12)
tomlox B	73.8	55.9	57.1	(12)
human L5	50.2	26.8	n.c.	(20)

Table 1. Percentage similarity and identity of the deduced amino acid (and DNA) sequences of barley LoxA with heterologous lipoxygenases compared by a BESTFIT Wisconsin GCG program. [n.c.]: significant calculation of percentage identity is not possible for the complete sequence.

In contrast the novel lipoxygenase isolated from rice leaves (NLox) has a low similarity. The N-terminal sequence of this rice lipoxygenase is not comparable with other lipoxygenase sequences due to the presence of a putative N-terminal transit peptide (14).

The C-terminal part of the protein contains the amino acid residues conserved among plant lipoxygenases, in particular the 5 histidines appearing within a stretch of 38 amino acids (1) at position 512-549. Two of the three histidines important for enzymatic activity (21) are present in this region. Furthermore the barley LoxA deduced sequence contains a stretch of 13 amino acids at position 704-716, which is highly conserved among plant lipoxygenases (ASALHAAVNFGQY, [1]) with the exception of a conservative substitution of alanine by glycine in barley. This region includes His_{708}, the third histidine residue required for iron binding and catalytic activity (21). Finally, the 9 C-terminal amino acid residues deduced from LoxA are identical to those of most other known plant lipoxygenases (RGIPNSISI, [1] except for the conservative substitution of arginine by lysine in the barley sequence.

In the 142 bp 3' untranslated region of the LoxA cDNA sequence two consensus sequences for polyadenylation were found at positions 2683-2688 and 2776-2785.

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Chapter 3

Molecular characterization of two lipoxygenases from barley*

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Chapter 3

Abstract

Two full length lipoxygenase cDNA sequences (LoxB and LoxC) from barley (*Hordeum distichum* cv. L. Triumph) are described. The cDNAs share high homology with the barley LoxA cDNA. Southern blotting experiments indicate single copy numbers of the three lipoxygenase genes. RFLP mapping revealed the presence of single lipoxygenase loci. LoxA and LoxB map on chromosome 4 and LoxC on chromosome 7. Two isoenzymes, LOX1 and LOX2 have been purified previously from germinating barley and characterized. LOX 1 is encoded by LoxA, while LOX 2 is encoded by LoxC. The product related to the third cDNA (loxB) has not been identified so far, suggesting a low protein abundance for the corresponding isoform in barley. Transcripts corresponding with these LOX genes are predominantly observed in grain and in seedling, whereas transcripts corresponding to LoxB and LoxC are also observed in mature vegetative tissue. No lipoxygenase mRNA could be detected in aleurone layer of germinating grain. No significant differences in lipoxygenase mRNA levels were observed in developing grains grown under dormant or non-dormant conditions, suggesting that LOX is not directly involved in induction of grain dormancy.

Introduction

Lipoxygenase (LOX, linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing dioxygenase that catalyses the oxidation of polyunsaturated fatty acids containing a 1,4-pentadiene system to form a Z,E-conjugated hydroperoxide fatty acid. Despite its wide distribution in the plant kingdom, the physiological role of LOX in cereal grains has only partly been elucidated. In plants the enzyme has been implicated in growth and development, senescence and responses to stress, partly through involvement in the synthesis of jasmonic acid (5) and traumatic acid (42).

The occurrence of LOX enzymes in cereal grain kernels has been well documented (14). Barley contains at least two distinct isoenzymes, LOX1 and LOX2, which have been purified and characterized from grain kernels (1, 10, 50). The major difference between the isoenzymes is the product they form when linoleic acid is used as a substrate. LOX1 produces the 9-hydroperoxide-octadecadienoic acid (9-HPOD) of linoleic acid, whereas LOX2 forms the 13-HPOD. Also for other cereals the occurrence of two or more isoforms of grain and seedling LOX has been described (14, 22).

LOX2 is present in early stages of grain development, whereas LOX1 accumulates in the later stages of grain development (36). LOX1 is present in both quiescent and germinating barley grains, while LOX2 is not detected before 24 h after germination (19). Because of the subcellular localisation, these isoforms they can be classified as cytosolic LOXs (also catagorized as LOX1-type proteins) in comparison with the organelle associated LOXs (LOX2--type proteins), which are predominantly found to be associated with chloroplasts in barley leaf (12, 47). A genomic fragment containing the barley LOX1 promoter element was previously isolated (33). Transient expression studies showed that the *Lox1* gene (LoxA) is also expressed in barley leaf in response to methyl-jasmonate (Me-JA) or wounding but not in response to ABA, drought or mildew infection.

The function of high levels of lipoxygenase expression during development and germination remains unclear. LOX activity has also been implied with the mobilization of storage lipids in cucumber seedlings (11). Storage acyl glycerols can be converted into 13-hydroxyperoxide derivatives which are subsequently reduced to 13-hydroxy derivatives, substrates for *B*-oxidation thereby providing an energy source for seedling growth. Similar observations in barley have recently been proposed where Lox 2 (LoxC) direct utilises storage lipids as a substrate for hydroperoxidation (20). However, the involvement of LOX in the biosynthesis of JA from linolenic acid via the octadecanoid pathway is probably one of its most important roles (5, 49).

High levels of JA are found during the first 24 h after germination in coleoptiles from soybean, whereas much lower levels are found in the hypocotyl after elongation (4). The JA levels decline rapidly during further germination. In wheat grains, LOX activity correlated with increased levels of JA upon imbibition (7).

Many of the physiological responses found upon JA treatment are similar to the effects observed from ABA treatment, suggesting that their mode of action must be closely related. JA treatment reduces seed germination velocity by inhibition of cell elongation (25). Application with growth retardant tetcyclacis (an inhibitor of ABA degradation) does abolish expression of JA induced proteins in barley seedlings (49). In tomato and potato plants ABA has been involved in signal mediation leading to the induction of JA responsive proteins (28, 29, 6). Although numerous of reports show that JA and ABA are both involved in a similar kind of modulation of gene expression, little is known about the functions and underlying mechanisms.

LOX expression can vary in response to environmental changes, which might lead to differences in endogenous levels of JA or JA-perception. Also existence of grain dormancy has shown to be directed by environmental conditions (37, 38). Several studies have shown the involvement of ABA in grain dormancy (27, 32, 39). Studies of a possible involvement of LOX or JA in the induction of grain dormancy have so far not been reported.

In the current paper the sequences of two cDNAs encoding LOX1-type proteins from barley are described. Three barley *LOX1*-type cDNAs have been characterised and the chromosomal location of the corresponding genes has been determined. The cDNAs have been linked with the isoforms appearing in barley grain. Northern analysis indicates differences in levels of temporal and spatial expression in the barley grain. The possible involvement of LOX in the occurrence of grain dormancy was evaluated, using two barley varieties containing differences in susceptibility for induction of dormancy. LOX expression was examined in developing grains from both varieties, grown under climate conditions known to be stimulating or omitting the occurrence of dormancy .

Materials and methods

Plant material.

Barley plants (*Hordeum distichum* L. Triumph) were grown in a phytotron under the following conditions: alternately 16 h light (300 mmol.m-^{2}.s^{-1}) at 15 °C and 8 h darkness at 12 °C. The relative humidity was kept at 80 %. Temperature was increased to 18-20 °C when grains reached yellow ripe stage. Spikes were tagged at their flowering stage and collected at different stages of development from 5 days after flowering to full maturity (FM). Maturity was obtained approximately 75 days after flowering. Collected grain kernels were immediately frozen in liquid nitrogen prior to storage at -80 °C, until analysis. Kernels from dormant and non-dormant developing grain (*Hordeum distichum,* L. Triumph and L. Kristina) were obtained from experiments previously described (38). Grain kernels were cultivated between three layers of moist filter paper in a plastic green house (33 x 32 x 20 cm) at 25°C in the dark. Germinated kernels were daily collected following the schedules named in the results section. Scutellum, leaf, root and aleurone were isolated prior to freezing in liquid nitrogen.

Isolation of lipoxygenase cDNA sequences

Initially a lambda ZAP expression library of 2 x 10^{6} primary plaques (41) from poly(A)$^{+}$ mRNA isolated from developing barley (*Hordeum distichum* L.cv. Triumph) grains (8-20 days after pollination) was screened. The probe consisted of the 1.5 kb 3' part of the LoxA cDNA clone (46). Screening resulted in the isolation of clones encoding partial sequences for two new types of LOX (LoxB and LoxC). The lacking sequences were cloned by use of Access reverse transcriptase (rt)-PCR (Promega) and rapid amplification of cDNA ends (RACE)-PCR (Gibco-BRL) systems. Prior to PCR, first strand cDNA was synthesized from RNA isolated from two days germinated barley seedlings. For amplification of upstream coding sequences sense-primers were developed based on comparison of conserved regions of the cDNA sequences of rice L2 (26) and barley Lox A. Antisense primers were derived from partial sequences of Lox B and Lox C to enable gene specific amplification. Primer sequences used for LoxB isolation are marked in figure 1 and for LoxC are marked in figure 2. The cDNA fragments used for cloning of the 5' parts were obtained by respectively two and three rounds of RACE-PCR. PCR fragments were cloned in the *Sma*I site of pUC 18 and sequenced according to standard protocols (35). The nucleotide sequence data and protein structure predictions were analysed using the Wisconsin GCG program (9).

Oligo sequences used for rt-PCR cloning of Lox B: GACCGC(G/A)TGTACCG(C/T)TATGAN (sense oligo position 725-745); and ACAGCAGACATCTCAAGCGCGTAT (antisense oligo position 1923-1900). Oligo sequences used for RACE-PCR cloning of LoxB: round 1, CCCAAAGCGCTCATCGCGGGGG (antisense position 943-922); round 2, CTCCTTAATCTTGTACTTAGGAATTC (antisense position 901-879).

Oligo sequences used for rt-PCR cloning of Lox C: GT(G/C)CTCATGCGCAAGAACN (sense oligo position 136-154); and CTACCTTGATGATCTCCGG (antisense oligo position 1123-1102). Oligo sequences used for rt-PCR cloning of LoxC: round 1, GAGGAGGAAGTCGCCGCCCTT (antisense position 1092-1072); round 2, GACGAAGACGATGGTACCGCG (antisense position 495-475); round 3, ATGAACGGCAGGTTCGTCGGCAGG (antisense position 335-312).

Southern and Northern hybridisation

Chromosomal mapping and single copynumber detection in Southern analysis was done with probes containing 163, 251 and 283 nucleotides of the 3' untranslated part of LoxA, LoxB and LoxC. Same probes were used for northern analysis unless stated otherwise. Southern analysis was done with DNA isolated from seedlings germinated for 3 days according to the protocol of Dellaporta (8). Genomic DNA (10 mg) was digested with *Bgl*I or *Bam*HI (20 units/digestion) overnight at 37 °C. Five pg of linearized plasmid was loaded on genomic gels for copy number estimation. Gene-Screen plus membranes were used for Southern blotting and hybridisation according to the protocol of the manufacturer (Dupont).

Northern blots were also derived on Gene-Screen plus membranes following the manufacturers procedure. RNA was isolated according to the protocol of Slater (43). Of each sample 5 mg of total RNA was denaturated by glyoxal and dimethylsulfoxide and separated on an agarose gel (35). After Northern analysis, blots were stripped and reprobed with a 28S ribosomal probe to control equality of RNA loading.

Chromosomal mapping

Genetic mapping was performed in a progeny comprising 110 F_2 derived F_3 families of an interspecific cross between *H. vulgare* (cv. Vada) and *H. spontaneum* (line 1B-87). In order to minimize errors due to segregation of heterozygous F_2 individuals equal amounts of leaf material from 10 F_3 individuals were pooled prior to DNA extraction. Wheat barley addition lines carrying disomic additions of individual barley chromosomes of the cultivar Betzes (except for chromosome 5, which is not available in an addition line) in a wheat background (cv. Chinese Spring) were used for the chromosomal assignment of RFLP bands (21). Linkage analysis was carried out using "Mapmaker" computer software (23). The following parameters were preset: two point analysis LOD 3.5, three point analysis LOD 3.0 and a maximum recombination value of 0.3. Recombination values were converted into map distances (centiMorgans, cM) by choosing the Kosambi function.

Results

Isolation and characterization of barley grain LoxB and LoxC cDNAs

A cDNA library from developing barley grains was screened with a barley lipoxygenase probe, consisting of the 1.5 kb 3' part of the LoxA cDNA (Genbank accession: L35931, nucleotide position: 1287-2818). Out of 100.000 recombinant phages two types of LOX cDNAs, LoxB and LoxC, differing from LoxA, were isolated. The partial cDNA sequences of barley LoxB and LoxC had more than 80% identity with the barley LoxA cDNA sequence.

Two identical partial cDNA clones of 1264 bp in length (Fig. 1, LoxB nucleotide position: 1739-3003) encoding the 3' part of a lipoxygenase and a 231 bp untranslated region were isolated. From the same library more than ten partial cDNA clones, coding for 3' fragments of a lipoxygenase, were isolated. The longest insert was approximately 2.0 kb (Fig. 2, LoxC nucleotide position: 920-2910).

In order to clone the complete coding regions of LoxB and LoxC two strategies were used. First, upstream regions were amplified by rt-PCR making use of mRNA isolated from two days germinated barley, since mRNA from this stage is expected to be contain both Lox-transcripts abundantly. Secondly, lacking 5' sequences were amplified by RACE-PCR making use of the same mRNA pool.

Sense-primer sequences used for rt-PCR were based upon conserved regions within cereal grain lipoxygenases. The antisense primers were derived from known gene-specific sequences of LoxB and LoxC. Primer positions are indicated in Fig. 1 and Fig. 2. Antisense oligo sequences were chosen near the 5' ends of the initially isolated partial clones. Rt-PCR products obtained with the usage of the sense primer position 725-745 (Fig. 1) and the LoxB specific antisense primer position 1923-1900 covered an size of approximately 1200 bp as could be expected from comparison with the barley LoxA sequence. Sequence analysis revealed 161 nucleotides perfect overlap with the initially isolated LoxB cDNA clone. Rt-PCR with use of the sense primer position 136-156 (Fig. 2) and the LoxC specific antisense-primer position 1102-1123 (Fig. 2) resulted in the amplification of a product of approximately

```
       *-> RACE-PCR clone
  1    GCCGCCCTACCACCACCACTGCCCTCCTTGTGCCAAGTGAATAAGCTAGGGGACAGGCAC   60

 61    CCACCGGTTGTGTTCCTCGTGCTCTGCTCTGCCCTGCCTCCGCTGCAGAACCAGAGAGCA   120

121    GAGGAGAGGAGAGATGCTGCTGCACGGGCTGGTGGACCGGCTAACGGGGAAGAACAAGGA   180
  1                  M  L  L  H  G  L  V  D  R  L  T  G  K  N  K  E   16

181    GGCGTGGAAGGAGGGCAAGATCCGGGGCACGGCGGTGCTGGTCAAGAGCGACGTGCTCGA   240
 17      A  W  K  E  G  K  I  R  G  T  A  V  L  V  K  S  D  V  L  D   36

241    CCTCGGCGACTTCCACGCCTCCCTCCTCGACGGCGTCCACAAGATCCTCGGCAAGGACGA   300
 37      L  G  D  F  H  A  S  L  L  D  G  V  H  K  I  L  G  K  D  D   56

301    CGGCGTCTCCTTCCGCCTCGTCAGCGCCACCGCCCCTGACCCACAGAATGGGAGCCGGGG   360
 57      G  V  S  F  R  L  V  S  A  T  A  P  D  P  Q  N  G  S  R  G   76

361    CAAGGTGGGGAAGCCGGCGCACCTGGAGGAGATGGTGGTGACGATGAAGTCGACGGCGGC   420
 77      K  V  G  K  P  A  H  L  E  E  M  V  V  T  M  K  S  T  A  A   96

421    GGGGGAGTCGGTGTTCAAGGTCACCTTCGAGTGGGACGATTCGCAGGGCGTCCCCGGCGC   480
 97      G  E  S  V  F  K  V  T  F  E  W  D  D  S  Q  G  V  P  G  A   116

481    CGTCATCGTCCGCAACACCTACCGCTCCGAGTACCTGCTCAAGACGCTCACCCTCCACGG   540
117      V  I  V  R  N  T  Y  R  S  E  Y  L  L  K  T  L  T  L  H  G   136

541    CGTCCCCGGCAAGGGCACCGTCGTCTTCGTCGCCAACTCCTGGATCTACCCCAACGTCGA   600
137      V  P  G  K  G  T  V  V  F  V  A  N  S  W  I  Y  P  N  V  D   156

601    CCGCCTCTTCTTCGCCAACGACACCTATCTGCCCAGCAAAATGCCTGCACTTTTGGTGCA   660
157      R  L  F  F  A  N  D  T  Y  L  P  S  K  M  P  A  L  L  V  Q   176

661    ATACCGGCAAGATGAACTCAACAATCTCCGAGGCGACGACACGACCGGAGAGTACAAGGA   720
177      Y  R  Q  D  E  L  N  N  L  R  G  D  D  T  T  G  E  Y  K  E   196
           * ->  rt-PCR clone
721    GGCTGACCGCGTGTACCGCTATGACTACTACAATGACCTCGGTGAGCCAGACAACGACAA   780
197      A  D  R  V  Y  R  Y  D  Y  Y  N  D  L  G  E  P  D  N  D  N   216
```

Fig. 1.. The LoxB cDNA sequence and the deduced amino acid sequence. Nucleotides 1-878 are obtained by RACE-PCR. Nucleotides 746-1900 are obtained by rt-PCR. Nucleotides 1738-3003 originate from the initially isolated LoxB cDNA clone. Sequences corresponding with oligo nucleotide primers used for PCR and/or cloning are underlined and printed in bold. Border positions and fragment direction are indicated by an arrow with an asterics.The numbers on each line correspond with the nucleotide or amino acid positions. Start and stopcodons are printed in bold. Putative polyadenylation sequence is underlined.

```
781     TCCCCGCCCAGTTCTCGGCGGCACCCAAGAACTCCCCTATCCCCGTCGTTGCAGAACTGG   840
217      P  R  P  V  L  G  G  T  Q  E  L  P  Y  P  R  R  C  R  T  G    236
                                                  RACE-PCR clone  <-
841     CCGACCACCAACAGAAACGGACCCTAGATCGGAGAGCAGAATTCCTAAGTACAAGATTAA   900
237      R  P  P  T  E  T  D  P  R  S  E  S  R  I  P  K  Y  K  I  K    256
        *                      RACE oligo 1      <-*
901     GGAGGCCCTGAACATCTATGTCCCCCGCGATGAGCGCTTTGGGCACCTCAAGTTGTCTGA   960
257      E  A  L  N  I  Y  V  P  R  D  E  R  F  G  H  L  K  L  S  D    276

961     CTTCCTTGGGTACTCTCTCAAGGCCATCACAGAGGCCATTCTTCCGATAACCAGGACCTA  1020
277      F  L  G  Y  S  L  K  A  I  T  E  A  I  L  P  I  T  R  T  Y    296

1021    TGTTGACAGTACACCCAAGGAGTTTGATTCGTTTCAAGACATCTATAATCTCTATGATGG  1080
 297     V  D  S  T  P  K  E  F  D  S  F  Q  D  I  Y  N  L  Y  D  G    316

1081    TCTTCTGAAAGTGCCAGATAACCAACACCTAAAGGAGCTCAAGAATAAATCCCTTCAGTT  1140
 317     L  L  K  V  P  D  N  Q  H  L  K  E  L  K  N  K  S  L  Q  F    336

1141    TATTAAAAAGTCTTTAGCAGTTGCTGGCGATGACTCTAAACTTGCCCTTCCACATGTTAT  1200
 337     I  K  K  S  L  A  V  A  G  D  D  S  K  L  A  L  P  H  V  I    356

1201    CAAATCAGATCAGTATGCTTGGAGGTCAGATGAGGAGTTTGCACGGGAGATGCTCGCAGC  1260
 357     K  S  D  Q  Y  A  W  R  S  D  E  E  F  A  R  E  M  L  A  A    376

1261    CGTTGACCCGGTTTGCATCAGACGTCTGACGAAGTTCCCTGTAAAAAAGTACCTGGATCC  1320
 377     V  D  P  V  C  I  R  R  L  T  K  F  P  V  K  K  Y  L  D  P    396

1321    CAGTGTGTACGGGGACCAGTCAAGCACTATCACTGAGGATCAAATTCAGCTGAACCTGGA  1380
 397     S  V  Y  G  D  Q  S  S  T  I  T  E  D  Q  I  Q  L  N  L  E    416

1381    AGATGGCCTCACAGTGAGACAGGCAATGGACAAGAAGAGGCTCTTCATTCTAGATCACCA  1440
 417     D  G  L  T  V  R  Q  A  M  D  K  K  R  L  F  I  L  D  H  H    436

1441    CGATAACTTCATGCCATTCCTTGACCGCATCAACAAGCTGGAAGGCAACTACATCTATGC  1500
 437     D  N  F  M  P  F  L  D  R  I  N  K  L  E  G  N  Y  I  Y  A    456

1501    TTCAAGGACCTTGCTGTTCCTGAAGGCCGATGGCACGCTGAAGCCCTTGGCCATCGAGCT  1560
 457     S  R  T  L  L  F  L  K  A  D  G  T  L  K  P  L  A  I  E  L    476

1561    GAGCCAGCCGCACCCCGATGGGATCCAGCATGGCGCAAAGAGCACGGTGTACCTTCCAGC  1620
 477     S  Q  P  H  P  D  G  I  Q  H  G  A  K  S  T  V  Y  L  P  A    496

1621    TGACATTAACTCTGGCGTTGATGGCCAGATCTGGCAGCTTGCCAAGGCTTACGCCTCTGT  1680
 497     D  I  N  S  G  V  D  G  Q  I  W  Q  L  A  K  A  Y  A  S  V    516
                                            initial 3'cDNA clone *->
1681    ACCGTTCGTGATTGCGACGAACCGGCAACTCAGTGTGGTGCACCCGGTGCACAAGCTGCT  1740
 517     D  D  S  A  W  H  Q  L  I  S  H  W  L  N  T  H  A  V  I  E    536
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Fig. 1. (Continued).

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1741   ACCGTTCGTGATTGCGACGAACCGGCAACTCAGTGTGGTGCACCCGGTGCACAAGCTGCT   1800
537     P  F  V  I  A  T  N  R  Q  L  S  V  V  H  P  V  H  K  L  L    556

1801   GAGCCCGCACTACCGCGACACGTTGAACATCAATGCCCTGGCGCGGACCACCCTCATCAA   1860
557     S  P  H  Y  R  D  T  L  N  I  N  A  L  A  R  T  T  L  I  N    576
                                                  rt-PCR clone
1861   CGCCGGTGGCGTGTTCGAGCTGACCGTTTTCCCTGGGCAATACGCGCTTGAGATGTCTGC   1920
577     A  G  G  V  F  E  L  T  V  F  P  G  Q  Y  A  L  E  M  S  A    596
       <-*
1921   TGTTGTCTACAAGAACTGGAAGCTCACTGAGCAGGGCCTCCCTGACGATCTCGTCAAGAG   1980
597     V  V  Y  K  N  W  K  L  T  E  Q  G  L  P  D  D  L  V  K  R    616

2001   GGGCATGGCTGTGCCGGACGAATCGAGCCCATACGGCATCCGTCTGCTGATCAAGGACTA   2040
617     G  M  A  V  P  D  E  S  S  P  Y  G  I  R  L  L  I  K  D  Y    636

2041   CCCGTACGCGGTGGACGGACTGGTGATCTGGTGGGCGATCGAGCGGTGGGTGAACGAGTA   2100
637    P  Y  A  V  D  G  L  V  I  W  W  A  I  E  R  W  V  N  E  Y    656

2101   CCTGGCCATCTACTACCCAAATGACGGCGTGCTCCGGGCCGACAAGGAGCTGGAGGAGTG   2160
657     L  A  I  Y  Y  P  N  D  G  V  L  R  A  D  K  E  L  E  E  W    676

2161   GTGGAAGGAGGTGCGCGAGGTCGGGCACGGCGACCTCAAGGACGCCGACTGGTGGCCCAA   2220
677     W  K  E  V  R  E  V  G  H  G  D  L  K  D  A  D  W  W  P  K    696

2221   GATGGTGACCGTCCAGGAGCTGGCCAAGACGTGCACCACCATCATCTGGGTGGCGTCGGC   2280
697     M  V  T  V  Q  E  L  A  K  T  C  T  T  I  I  W  V  A  S  A    716

2281   GCTGCACGCGGCGGTCAACTTTGGGCAGTACCCGTACGCGGGGTACCTCCCGAACCGGCC   2340
717     L  H  A  A  V  N  F  G  Q  Y  P  Y  A  G  Y  L  P  N  R  P    736

2341   GACGGTGAGCCGTCGCCCAATGCCGAAGGAGGGCGACGAAGAGTACGAGCAGCTGAAGGA   2400
737     T  V  S  R  R  P  M  P  K  E  G  D  E  E  Y  E  Q  L  K  E    756

2401   GGGCGGGGAGGCGGCCGACATGGTGTTCATCCACACCATCACCAGCCAGTTCCAGACCAT   2460
757    G  G  E  A  A  D  M  V  F  I  H  T  I  T  S  Q  F  Q  T  I     776

2461   CCTCGGCATCACGCTCATCGAGATCCTGTCCAAGCACTCCTCCGACGAGGTGTACCTGGG   2520
777     L  G  I  T  L  I  E  I  L  S  K  H  S  S  D  E  V  Y  L  G    796

2521   GCAGCGCGACACGCCGGAGTGGACGTCGGACGCCAAGGCGCTCGAGGCGTTCAAGAGGTT   2580
797     Q  R  D  T  P  E  W  T  S  D  A  K  A  L  E  A  F  K  R  F    816

2581   CGGCAGCCGGCTGGTGGAGATCGAGAAGCGGATCCTCGACATGAACAAGGACCCGGCGCT   2640
817     G  S  R  L  V  E  I  E  K  R  I  L  D  M  N  K  D  P  A  L    836
```

Fig. 1. (Continued).

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2641  CAAGAACCGGAACGGGCCCGTGAAGATGCCCTACATGCTGCTGTACCCCAACACGTCGGA  2700
 837   K  N  R  N  G  P  V  K  M  P  Y  M  L  L  Y  P  N  T  S  D    856

2701  CGCCAACGGGGAGAAGGCGCTGGGGCTCACCGCCATGGGCATCCCCAACAGCGTCTCCAT  2760
 857   A  N  G  E  K  A  L  G  L  T  A  M  G  I  P  N  S  V  S  I    876

2761  CTGAGCTGAGCATGGTCTTTGTTTCCTTCTTTCGGTAGTCGTCCGTCCGCTGGTCATGGA  2820
 877   *

2821  TCGGTTCGTGTCTCGCAAGGTGTATTGTGTTACTTTTCATTGGCTTCTGGGTGGTTGCGG  2880

2881  TAATTAGTTGCCAGGGATTAGTGGATGATGGAAAATGATGATTGTGTCGTAGATGATGTT  2940

2941  GATAGGGTGAGGACAATGAGGCATGAATAAGCACTCGTGTGCGCATTCTTTTCAAAAAAA  3000
```

Fig. 1. (Continued).

950 base pairs, which was in size comparable with the corresponding region of LoxA. Sequence analysis revealed 180 nucleotides perfect overlap with the initially isolated LoxC cDNA fragment.

Second, a RACE-PCR system was used for amplification of fragments containing the lacking 5'-ends of LoxB and LoxC. Prior to PCR, first strand cDNA products were added with an oligo d'G tail by terminal transferase reaction. PCR fragments containing the 5'-end of LoxB were obtained by two rounds of amplification, while fragments containing the 5'-end of LoxC were obtained by three rounds of amplification. The products obtained in the final PCR were cloned. The approximately 900 base pairs insert obtained with LoxB specific antisense primers (Fig. 1: position 879-901) showed 180 nucleotides perfect overlap with the insert of the rt-PCR clone of LoxB. The final PCR product obtained with LoxC specific antisense primers (Fig. 2: position 312-335) resulted in the cloning of a fragment containing the 5'-end of LoxC which revealed 180 nucleotides perfect overlap with the insert of the rt-PCR clone of LoxC.

Comparison of Lox cDNA coding sequences

The deduced protein sequences of LoxB and LoxC were compared to those of barley LoxA, rice L2, the novel-Lox from rice, the JA responsive *LOX2:Hv:1* from barley and soybean L1 (26, 30, 40, 46, 47). Sequence comparison, using the Wisconsin BESTFIT program, indicated that the barley lipoxygenases reported in this paper can all be classified as *LOX1*-type proteins. They exhibit high homology with each other and with the rice L-2 from grain.

Comparison with these previously reported LOX sequences indicated that the clones for LoxB and LoxC cover the complete coding sequences of respectively 876 amino residues (LoxB) with a predicted molecular mass of 98.977 kDa and 864 amino residues (LoxC) with a predicted molecular mass of 96.612 kDa. For both sequences in-frame stopcodons were found preceding the putative start codon indicating that both coding-regions are complete. LoxA, LoxC and rice L-2 showed percentage homology to each other above the 80% on DNA level and approximately 75% on amino acid level, whereas LoxB showed an homology of approximately 74 % on DNA level 65% on amino acid level with the grain lipoxygenases.

```
        *-> RACE PCR clone
   1    GTCACACAGCACACCGCAGCGAGCTAACTTAGAAGCGTGCCACACTCCCAGAGATCGAGC     60

  61    AAGATGCTTGGCGTCGGCGGCATCGTGAGCGACTTGACGGGGGGCATCCGGGGCGCCCAC    120
            M  L  G  V  G  G  I  V  S  D  L  T  G  G  I  R  G  A  H      19
                    *-> rt-PCR clone
 121    CTCAAGGGCTCCGTCGTCCTCATGCGCAAGAACGCGCTCGACTTCAACGACTTCGGCGCC    180
  20     L  K  G  S  V  V  L  M  R  K  N  A  L  D  F  N  D  F  G  A      39

 181    CACGTCATGGACGGCGTCACCGAGCTCCTCGGCCGCGGCGTCACCTGCCAGCTCATCAGC    240
  40     H  V  M  D  G  V  T  E  L  L  G  R  G  V  T  C  Q  L  I  S      59

 241    TCCACCAACGTCGACCACAACAACGGTGGGCGCGGGAAGGTGGGCGCGGAGGCGAACCTG    300
  60     S  T  N  V  D  H  N  N  G  G  R  G  K  V  G  A  E  A  N  L      79
         RACE-PCR clone <-*
 301    GAGCAGTGGCTCCTGCCGACGAACCTGCCGTTCATCACCACCGGCGAGAACAAGTTCGCC    360
  80     E  Q  W  L  L  P  T  N  L  P  F  I  T  T  G  E  N  K  F  A      99

 361    GTCACCTTCGACTGGTCGGTGGACAAGCTGGGGGTGCCGGGGGCCATCATCGTCAAGAAC    420
 100     V  T  F  D  W  S  V  D  K  L  G  V  P  G  A  I  I  V  K  N     119

 421    AACCACGCCTCCGAGTTCTTCCTCAAGACCATCACCCTCGACAACGTGCCCGGCCGCGGC    480
 120     N  H  A  S  E  F  F  L  K  T  I  T  L  D  N  V  P  G  R  G     139
       RACE-oligo 2 <-*
 481    ACCATCGTCTTCGTCGCCAACTCATGGGTCTACCCGCAGGCCAAGTACCGCTACAACCGC    540
 140     T  I  V  F  V  A  N  S  W  V  Y  P  Q  A  K  Y  R  Y  N  R     159

 541    GTCTTCTTCGCCAACGACACGTACCTGCCGCACCAGATGCCGGCGGCGCTGAAGCCGTAC    600
 160     V  F  F  A  N  D  T  Y  L  P  H  Q  M  P  A  A  L  K  P  Y     179

 601    CGCGACGACGAGCTCCGGAACCTGAGGGGCGACGACCAGCAGGGGCCCTACCTGGACCAC    660
 180     R  D  D  E  L  R  N  L  R  G  D  D  Q  Q  G  P  Y  L  D  H     199

 661    GACCGCGTCTACCGTTACGACGTCTACAACGACCTCGGCGACTCCCGCGACGTCCTCGGC    720
 200     D  R  V  Y  R  Y  D  V  Y  N  D  L  G  D  S  R  D  V  L  G     219

 721    GGCTCCAAGGACCTCCCCTACCCGCGCCGCTGCCGCACCGGCCGGAAGCCCTCGGACAGC    780
 220     G  S  K  D  L  P  Y  P  R  R  C  R  T  G  R  K  P  S  D  S     239
```

Fig 2. The LoxC cDNA sequence and the deduced amino acid sequence. Nucleotides 1-311 are obtained by RACE-PCR. Nucleotides 155-1101 are obtained by rt-PCR. Nucleotides 920-2910 originate from the initially isolated LoxC cDNA clone. Sequences corresponding with oligo nucleotide primers used for PCR and/or cloning are underlined and printed in bold. Border positions and fragment direction are indicated by an arrow with an asterics.The numbers on each line correspond with the nucleotide or amino acid positions. Start and stopcodons are printed in bold. Putative polyadenylation sequence is underlined.

```
781   AAGCCCGACCACGAGAGCCGGCTGCTGCTGCTGGTGCAGAACGTCTACGTGCTGCGCGAC   840
240   K  P  D  H  E  S  R  L  L  L  L  V  Q  N  V  Y  V  L  R  D     259

841   GAGCTCTTCGGCCACCTCAAGCAGTCGGACTTGCTGGGCTACACGCTCAAGGGCTGGTTG   900
260   E  L  F  G  H  L  K  Q  S  D  L  L  G  Y  T  L  K  G  W  L     279
                        *-> initial cDNA clone
901   GACGGGATCATACTGGCCATCCGCACCTACGTCGACCTCTCCCCCGGCGAGTTCGACTCC   960
280   D  G  I  I  L  A  I  R  T  Y  V  D  L  S  P  G  E  F  D  S     299

961   TTCGCCGACATCCTCAAGCTCTACGAGGGCGGCATCAAGCTGCCCAACATCCCGGCCCTC   1020
300   F  A  D  I  L  K  L  Y  E  G  G  I  K  L  P  N  I  P  A  L     319

1021   GAGGAGGTGCGCAAGCGCTTCCCGCTCCAGCTCGTCAAGGACCTCATCCCCAAGGGCGGC  1080
 320   E  E  V  R  K  R  F  P  L  Q  L  V  K  D  L  I  P  K  G  G   33
  RACE oligo 1 <-*              rt-PCR clone  <-*
1081  GACTTCCTCCTCAAGCTCCCCAAGCCGGAGATCATCAAGGTAGACCAGAAAGCGTGGATG   1140
340   D  F  L  L  K  L  P  K  P  E  I  I  K  V  D  Q  K  A  W  M     359

1141  ACTGACGAGGAGTTCGCCAGGGAGATGCTCGCCGGCGTCAACCCCATGATGATCAAACGC   1200
360   T  D  E  E  F  A  R  E  M  L  A  G  V  N  P  M  M  I  K  R     379

1201  CTCACCGAGTTCCCTCCCAAGAGCACTCTGGATCCGAGCAAGTACGGCGACCACACCAGC   1260
380   L  T  E  F  P  P  K  S  T  L  D  P  S  K  Y  G  D  H  T  S     399

1261  ACCATGACCGAGGAGCACGTGGCCAAGAGCCTGGAGGGCCTCACCGTGCAGCAGGCGCTC   1320
400   T  M  T  E  E  H  V  A  K  S  L  E  G  L  T  V  Q  Q  A  L     419

1321  GCCGGCAACAGGCTCTACATCGTAGACCAGCACGACAACCTGATGCCGTTCCTGATCGAC   1380
420   A  G  N  R  L  Y  I  V  D  Q  H  D  N  L  M  P  F  L  I  D     439

1381  ATCAACAACCTCGACGCCAGCTTCGTGTACGCCACAAGGACGCTGCTCTTCCTGCGAGGG   1440
440   I  N  N  L  D  A  S  F  V  Y  A  T  R  T  L  L  F  L  R  G     459

1441  GACGGCACGCTGGCGCCGGTCGCCATCGAGCTGAGCTCGCCGCTGATCCAGGGCGAGCTG   1500
460   D  G  T  L  A  P  V  A  I  E  L  S  S  P  L  I  Q  G  E  L     479

1501  ACCACCGCCAAGAGCGCCGTGTACACGCCGCAGCACGCCGGCGTGGAGGGCTGGATATGG   1560
480   T  T  A  K  S  A  V  Y  T  P  Q  H  A  G  V  E  G  W  I  W     499

1561  CAGCTCGCCAAGGCCTACGCCTCCGTGAACGACTACGGGTGGCACCAGCTCATCAGCCAC   1620
500   Q  L  A  K  A  Y  A  S  V  N  D  Y  G  W  H  Q  L  I  S  H     519

1621  TGGCTCAACACGCACGCCGTCATGGAGCCCTTCGTCATCGCCACCAACAGGCAGCTCAGC   1680
520   W  L  N  T  H  A  V  M  E  P  F  V  I  A  T  N  R  Q  L  S     539

1681  GTCACCCACCCGGTCTACAAGCTCCTGCACCCGCACTACCGCGACACCATGAACATCAAC   1740
540   V  T  H  P  V  Y  K  L  L  H  P  H  Y  R  D  T  M  N  I  N     559
```

Fig. 2. (Continued).

```
1741  GCGCGGGCGCGCGGGCTGCTCATCAACGCCGGCGGCGTCATCGAGATGACCGTGTTCCCG  1800
 560  A  R  A  R  G  L  L  I  N  A  G  G  V  I  E  M  T  V  F  P     579

1801  CACAAGCACGCCATGCCCATGTCCTCCATGGTCTACAAGCACTGGAACTTCACCGAACAA  1860
 580  H  K  H  A  M  P  M  S  S  M  V  Y  K  H  W  N  F  T  E  Q     599

1861  GCTCTCCCCGCCGATCTAATCAAGAGGGGCATGGCGGTGGAGGACGCATCGAGCCCGCAC  1920
 600  A  L  P  A  D  L  I  K  R  G  M  A  V  E  D  A  S  S  P  H     619

1921  AAGGTGCGGCTGCTGATCAAGGACTACCCGTACGCGACCGACGGGCTGGCCGTGTGGGAC  1980
 620  K  V  R  L  L  I  K  D  Y  P  Y  A  T  D  G  L  A  V  W  D     639

1981  GCCATCGAGCAGTGGGTGTCGGACTACCTGACCATCTACTACCCCAACGACGGCGTGCTG  2040
 640  A  I  E  Q  W  V  S  D  Y  L  T  I  Y  Y  P  N  D  G  V  L     659

2041  CAGGGCGACGTGGAGCTGCAGGCGTGGTGGAAGGAGGTGAGGGAGGTCGGGCACGGCGAC  2100
 660  Q  G  D  V  E  L  Q  A  W  W  K  E  V  R  E  V  G  H  G  D     679

2101  CTCAAGGACGCGGCGTGGTGGCCAAAGATGCAGACGGTGGCGGAGCTGATCAAGGCGTGC  2160
 680  L  K  D  A  A  W  W  P  K  M  Q  T  V  A  E  L  I  K  A  C     699

2161  GCCACCATCATCTGGACCGGGTCGGCGCTCCACGCGGCCGTCAACTTCGGGCAGTACCCC  2220
 700  A  T  I  I  W  T  G  S  A  L  H  A  A  V  N  F  G  Q  Y  P     719

2221  TACTCGGGCTACCACCCCAACAAGCCGTCGGCGAGCCGGAGGCCGATGCCGGTGCAGGGG  2280
 720  Y  S  G  Y  H  P  N  K  P  S  A  S  R  R  P  M  P  V  Q  G     739

2281  AGCGAGGAGTACGCGGAGCTGGAGCGAGACCCGGAGAAGGCCTTCATCCGCACCATCACC  2340
 740  S  E  E  Y  A  E  L  E  R  D  P  E  K  A  F  I  R  T  I  T     759

2341  AGCCAGTTCCATGCCCTGGTGGGCATCTCGCTCATGGAGATCCTCTCCAAGCACTCCTCC  2400
 760  S  Q  F  H  A  L  V  G  I  S  L  M  E  I  L  S  K  H  S  S     779

2401  GACGAGGTCTACCTGGGCCAGCACGACACGCCGGCGTGGACGTCGGACGCCAAGGCGCTG  2460
 780  D  E  V  Y  L  G  Q  H  D  T  P  A  W  T  S  D  A  K  A  L     799

2461  GAGGCGTTCAAGCGGTTCGGGGCGAAGCTGGAGGGCATCGAGAAGCAGGTGGTGGCCATG  2520
 800  E  A  F  K  R  F  G  A  K  L  E  G  I  E  K  Q  V  V  A  M     819

2521  AACTCGGACCCGCAGCTAAAGAACCGCACCGGGCCGGCCAAGTTCCCATACATGCTGCTC  2580
 820  N  S  D  P  Q  L  K  N  R  T  G  P  A  K  F  P  Y  M  L  L     839

2581  TACCCAAACACCTCCGACCACACGGGACAGGCCGAGGGGCTCACCGCCAGGGGCATCCCG  2640
 840  Y  P  N  T  S  D  H  T  G  Q  A  E  G  L  T  A  R  G  I  P     859
```

Fig. 2. (Continued).

```
2641   AACAGCATATCCATCTGATCTGAGCACGTCCGTCGTGGAGACCTCATGCCTTTGTGCTAT   2700

 860   N  S  I  S  I  *                                                864

2701   CCTTGCTTGCTTACACCGGAGCTAGCTTGTGTGTGTGTGTGTGACAGAGAGTTGTGCCGG   2760

2761   TGCATGAATAATTAAGGCAATAAATAAGGGTGCGTGCACGACGGGTCTGATCGGCATGCG   2820

2821   GAAGCTCAGCTCAGCTGGCTACCGGTCAATGCATCGTCGTGTGCAGAAAAAATCCCTTGT   2880

2881   TTTGCCCAATAATGTCGATGGTGTGATGTTCCCTCCAAAAAAAAAAAAAA             2930
```

Fig. 2. (Continued).

However, these four monocot lipoxygenases showed less homology with *LOX2*-type sequences associated with chloroplasts from barley (*LOX2:Hv:1*) and rice (novel-LOX) of approximately 60% on DNA level and 45% on amino acid level. Barley LoxA, B and C and the rice lipoxygenase L-2 are predominantly expressed in kernel and seedling, whereas two distinct isoforms from barley leaf segments (LOX-92; LOX-100 encoded by *LOX2:Hv:1*) were localised in the chloroplasts (47). The novel-LOX from rice is expressed in leaf especially in response to pathogen attack (30). This rice novel-LOX contains an amino-terminal extension of approximately 50 amino acids, which forms part of a 110 amino acids long putative transit peptide for chloroplast targeting (Fig. 3).

```
           1                                                     50
barLoxA    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~ML
barLoxC    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~ML
riceL-2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~ML
barloxB    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~ML
soyLox1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~MF
barLOX2    ML.TATKPLV GGACAAPSSS ARRRTFVVPE ARRKPGNGRR TSVSKVGSTS
riceL-N    MLRPQLNPSS HTTTTSSSSS TQLFASSSCI ASLRRPSSSS SSVVAAARRT

           51                                                   100
barLoxA    .LGGLIDTLT GANKSA.... RLKGTVVLMR KNVLDLNDFG ATIIDGIGEF
barLoxC    GVGGIVSDLT GGIRGA.... HLKGSVVLMR KNALDFNDFG AHVMDGVTEL
riceL-2    ..GGIIGGLT G.NKNA.... RLKGSLVLMR KNALDINDFG ATVIERISEF
barLoxB    .LHGLVDRLT GKNKEAWKEG KIRGTAVLVK SDVLDLGDFH ASLLDGVHKI
soyLox1    SAG....... .........H KIKGTVVLMP KNELEVNPDG SAVDNLNAFL
barLOX2    TSTTTTTTTT LSADSNGAAV GTVTRPDVHV QDRTHATEMK ATVTVHMSKA
riceL-N    RGQGSSRVVV VCASSSATAS RGDSSSDMAA AAAVRVKAV. ATIKVTVGEL
```

Fig 3. Comparison of the amino-terminal ends of six cereal lipoxygenases and soylox-1. Comparison was done with the complete sequence, but only 5'-ends are shown. The cDNA alignements shown were automatically arranged to barley LoxA by the Wisconsin GCG-pileup program, following sequences with highest similarity to LoxA first. barLOX2, JA inducible *LOX2:Hv:1* from barley (47); riceLoxN, Novel lipoxygenase from rice (30); riceL-2, rice lipoxygenase 2 (26); soyLox1, soybean lipoxygenase 1 (40).

Similar to the novel-LOX from rice, the barley *LOX2:Hv:1* contains an amino terminal extension. The LOX-isoforms reported in this paper do not contain such a putative amino-terminal targeting signal. The low homology found between grain kernel and chloroplast associated lipoxygenases may indicate differences in physiological functions for proteins belonging to each distinct group. Comparison of the barley lipoxygenases with the dicot soybean L1 (*LOX1*-type sequence) showed an average homology of 58% on DNA level and 55% on amino acid level.

LoxB and LoxC contain a number of conserved regions known among plant lipoxygenases (Fig. 4). A stretch of 38 amino acids located on amino acid region 512 - 549 of the deduced protein sequence of LoxA cDNA, includes 5 histidines which are highly conserved in lipoxygenases (42).

```
a)
            512  517   522                 540       549
             *          *
             o +  o+ + +o   ++   +   + ++   o+   ++ + o       ++
barLoxA   SGWHQLVSHW LNTHAVMEPF VISTNRHLSV THPVHKLLSP HYRDTMTINA
barLoxC   YGWHQLISHW LNTHAVMEPF VIATNRQLSV THPVYKLLHP HYRDTMNINA
riceL-2   YCWHQLISHW LNTHAVMEPF VIATNRQLSV AHPVHKLLLP HYRDTMTING
barLoxB   SAWHQLISHW LNTHAVIEPF VIATNRQLSV VHPVHKLLSP HYRDTLNINA
soybL-1   SCYHQLMSHW LNTHAAMEPF VIATHRHLSV LHPIYKLLTP HYRNNMNINA
barLoxN   AGVHQLVSHW LRTHACTEPY IIAANRQLSQ MHPVYRLLHP HFRFTMEINA
riceL-N   AGHHELITHW LRTHCAVEPY IIAANRQLSE MHPIYQLLRP HFRYTMRINA
```

```
b):                                          c):
                708                                         862
                 *                                           *
          +      o+++++++++   +   +                   +++++ ++
barLoxA   WIGSAL HAAVNFGQYP YAGFLPN              GLTAKGIPNSISI 862
barLoxC   WTGSAL HAAVNFGQYP YSGYHPN              GLTARGIPNSISI 864
riceL-2   WIGSAL HAAVNFGQYP YAGYLPN              GLSARGIPNSISI 865
barLoxB   WVASAL HAAVNFGQYP YAGYLPN              GLTAMGIPNSVSI 876
soybL-1   WIASAL HAAVNFGQYP YGGLIMN              GLTFRGIPNSISI 838
barLoxN   WVTSGH HAAVNFGQYP MAGYIPN              TDEKMGIPNSISI 937
riceL-N   WVAAAH HAAVNFGQYD FGGYFPN              MVMEMGIPNSTSI 923
```

Fig 4. Comparison of barley, rice and soybean 1 lipoxygenase, showing highly conserved regions (a, b) and the C-terminal isoleucine residue (c) known to be important for plant lipoxygenase. Numbers refer to amino residue positions in LoxA. Residues identical in all sequences are indicated by [+}; conserved histidine residues are indicated by [o}.
Residues important for iron-binding are indicated by [*].

These histidines are also found in the deduced protein sequences of LoxB and LoxC. Two of these histidines, corresponding to His_{517} and His_{522} of barley LoxA, are required for the iron binding (45). The third histidine residue required for iron binding and catalytic activity, His_{708} was also present in the deduced protein sequences of LoxB and LoxC. Finally, the 8 C-terminal amino acid residues deduced from LoxA, LoxB and LoxC are identical to those of most other known plant lipoxygenases (GIPNSISI [42]), except for the conservative substitution of the second isoleucine by valine in the LoxB sequence.

The 3' untranslated regions of these three types of barley cDNA clones did not show any significant homology and were therefore used as a probe in Southern hybridization for copy number detection and in Northern hybridization.

Barley lipoxygenase isoenzyme 1 (LOX 1) is encoded by LoxA, while isoenzyme 2 (LOX 2) is encoded by LoxC

Amino acid sequences from two different peptide fragments determined from LOX1 protein were compared with the deduced sequences of LoxA, LoxB and LoxC. The amino acid sequences deduced from LoxA were identical to those of these peptide fragments (10). Contrary, the predicted amino acid sequences of LoxB and LoxC located in the corresponding region were not completely homologous. The finding that LoxA is encoding LOX1 was supported by western analysis of E.coli extracts. We examed the cDNA products with antibodies raised with high specificity towards the LOX1 or LOX2 protein (22). The LoxA gene product was preferentially recognized by the monoclonal antibodies with a pronounced specificity toward LOX1, whereas the LoxC product showed highest affinity with the antibodies with a pronounced specificity toward LOX2 (data not shown). Furthermore, it has been shown that during grain development mRNA levels corresponding with LoxC correlate with the occurrence of LOX2 protein until 20 DAF, while mRNA levels corresponding with LoxA and LOX 1 protein are predominantly found after 30 DAF (36). These observations strongly supports our findings that LoxC is encoding LOX2. However, since the affinity of the monoclonals for a possible gene product of LoxB has not been tested, it may not fully be excluded that a third isoenzyme is co-detected at low abundance in developing and germinating grain. So far, no distinct enzymatic properties differing from LOX1 or LOX2 could be determined in these grains (10, 19, 36).

Southern analysis

To study the number of these LOX genes in barley Southern blots were hybridized with probes containing 1.2 to 1.5 kb of the 3' part of LoxA, LoxB or LoxC. Due to sequence homology several identical bands were observed with the distinct probes indicating the presence of a small gene family (data not shown).

To verify the existence of a low copy number for LoxA, LoxB and LoxC, Southern hybridizations were repeated with small specific cDNA probes (163-283 bp) containing the 3' untranslated region of each type (Fig. 5). The Southern analysis showed unique bands corresponding to LoxB and LoxC genomic fragments digested with *Bgl*I or with *Bam*HI,

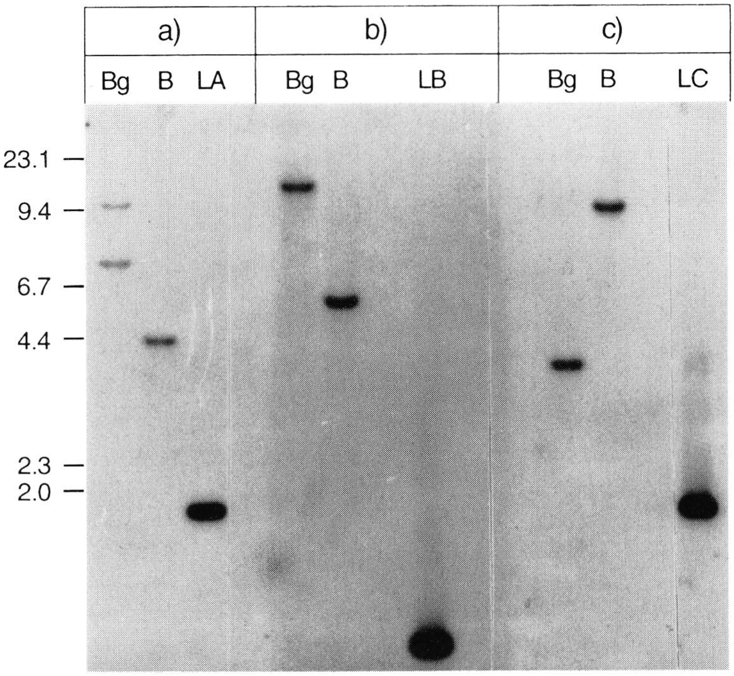

Fig. 5. Southern analysis of barley genomic DNA (10mg/lane) digested with *BglI* [Bg] or with BamHI [B] and hybridized with ^{32}P-Labelled 3' untranslated regions of the different lipoxygenase cDNA types. Southern analysis are showing single copy detection of each gene. The last lane of each panel represents 5 pg of plasmid DNA-digest of resp. LoxA, LoxB and LoxC, as described in fig. 4.
Size markers are indicated in kilobases.

indicating single copy numbers for these genes. In contrast Southern analyses probed with LoxA showed two distinct bands of approximately 10 kb and 8 kb hybridizing with *BglI* digested genomic fragments indicating an allelic variation or a gene duplication of LoxA in the variety Triumph.

RFLP Mapping

To determine the linkage groups and the chromosomal positions of individual Lox genes in relation to a set of already mapped RFLP markers covering nearly the entire barley genome (15), RFLP Mapping was carried out for the mapping parents (Vada, 1B-87). Genomic DNA was digested with six different restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I, *X*baI). Southern hybridization were probed with the 3'untranslated region (UTR) fragment of LoxA, LoxB and LoxC to genomic DNA of the mapping parents (Data not shown). Genetic analysis revealed three loci, whose allelic segregation patterns fitted the expected 3:1 (LoxA, LoxC) and 1:2:1 (LoxB) ratio, indicative for single locus segregation.

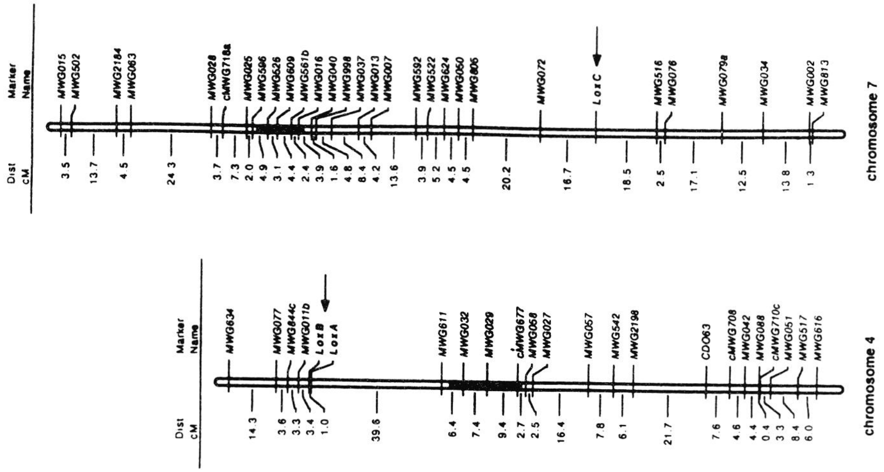

Fig. 6. Chromosomal map with the positions of three barley grain lipoxygenases (LoxA, LoxB and LoxC). Lipoxygenase genes are indicated by arrows.

The LoxC gene maps on chromosome 7 in a chromosomal region covered only sparsely by RFLP markers, while LoxA and LoxB map on chromosome 4, where the two loci are only 1.0 cM apart and form a cluster together with three additional RFLP markers (Fig. 6). The common location of LoxA and LoxB on chromosome 4 has been further substantiated by hybridization of the respective probes to *Hind*III digested DNA of wheat-barley addition lines (21). Here each probe revealed a different major fragment, which could be assigned to this chromosome.

Analysis of lipoxygenase transcripts in barley

To study the transcript levels of lipoxygenase in barley, RNA was isolated from grain during development and germination and from stem and leaf of mature plants (Fig. 7). Northern blots were hybridized with probes containing the 3' untranslated part of each lipoxygenase cDNA. For each distinct type 5 pg of plasmid DNA was also hybridized in the same experiment. The intensities of the hybridizing plasmid bands were used to compare the signal intensities found in northern analysis. Northern blots (Fig. 7) showed highest level of mRNA during germination. LoxB mRNA levels were less abundant in comparison with those of LoxA and LoxC.

In developing grains only transcripts corresponding to LoxA and LoxC were detected. Whereas LoxA transcripts were observed in yellow ripe and with lower levels in fully ripe grains, while LoxC transcripts were present during early period of grain development.

Transcripts corresponding to LoxB and LoxC were also detected in mature stem tissualthough LoxB showed a very low level of expression which was only observed after a long exposure of the autoradiograph (data not shown).

To study the spatial lipoxygenase mRNA levels grains were dissected for a period of day two until day five after germination. Roots, scutellum and leaves (Fig. 8a) and from a separate experiment, aleurone tissue was analysed by northern (Fig. 8b). Most abundant lipoxygenase transcript levels were found in scutellum and leaf. In those tissues transcripts corresponding with LoxC and LoxA were significantly present, while transcripts corresponding with LoxB were poorly detected. In root tissue, lipoxygenase transcripts were observed until day 2 of germination, whereas the levels decline in later stage. LoxA transcripts were also found with considerable level in comparison with loxC, although mRNA level seemed slightly less pronounced. In contrast with the observations from undissected grain (Fig. 7), mRNA levels of LoxB were significant less intense in all tissues and declined fast during early germination (Fig. 8a.).

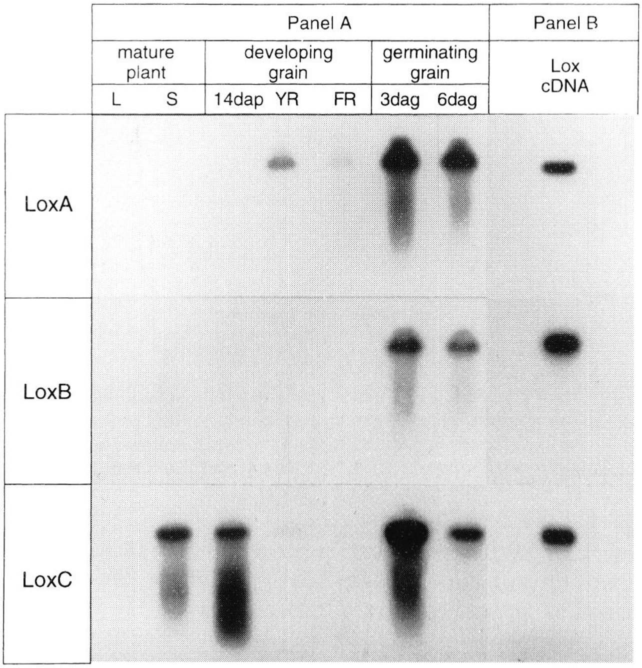

Fig. 7. **Panel A**: Northern blot analysis of RNA extracted from the indicated organs. Each lane contains 5 mg of total RNA. ^{32}P-Labelled 3' untranslated regions of LoxA, LoxB or loxC were used for probing. Mature tissue: L, leaf from 2 months grown plants; S, stems from 2 months grown plants. Grains: 14 dap, 14 days after pollination; YR, yellow ripe (40 dap); FR, fully ripe (quiescent grain); 3 dag, 3 days after germination; 6 dag, 6 days after germinating.
Panel B: Southern analysis of 5 pg linearized plasmid DNA containing fragments of resp.; LoxA, LoxB and LoxC hybridized in the same experiment.

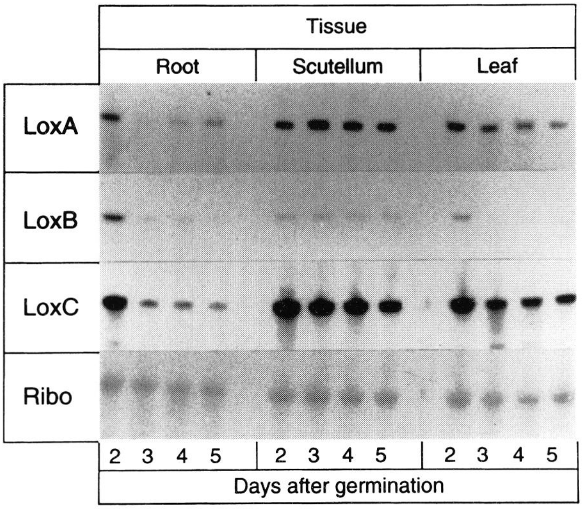

Fig. 8a. Northern blot analysis of RNA extracted from root, scutellum and leave tissue of germinating grain. Time points indicated in days after germination. Blots were hybridized with specific probes for LoxA, LoxB and LoxC. Each lane contains 5 mg of total RNA. Ribosomal RNA probe was used for control on loading.

Considerably high level of LoxB transcripts were observed in root tissue. It must be noted that the observed relatively low levels of LoxB transcript in dissected grain are differing from the data observed in northern analyses of intact grains (Fig. 7). Results strongly suggest that small differences in germination conditions may lead to difference in temporal and spatial expression. From wheat it has been shown that wind turbulence in a phytotron was able to trigger LOX expression in a dose dependent fashion (24). No LOX transcripts could be detected in the aleurone of germinated grain (Fig 8b.).

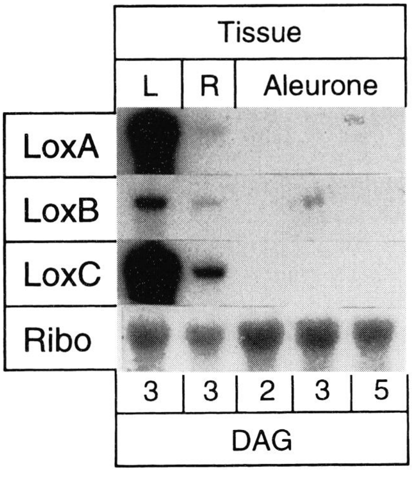

Fig. 8b. Northern blot analysis of RNA extracted from leave, root and aleurone tissue of germinating grain. Timepoints indicated in days after germination. Blots were hybridized with specific probes for LoxA, LoxB and LoxC. Each lane contains 5 mg of total RNA. Ribosomal RNA probe was used for control on loading.

Lipoxygenase expression in dormant and non-dormant developing grains

To study a possible involvement of lipoxygenases in the occurrence of dormancy, genetically identical plants were grown in phytotrons under differing climatic conditions, either to give rise to high levels of dormancy or to low levels of dormancy (38). Two varieties of barley with known genetical differences in susceptibility for grain dormancy were chosen. The variety Kristina for being insensitive to the induction of grain dormancy and the variety Triumph for being susceptible to induction of dormancy. The growth conditions applied resulted in a difference in the length of developmental period. Grains developed from flowering to full maturity over a period of approximately 75 days under non-dormant conditions, whereas under dormant conditions the period lasted approximately 130 days. LOX mRNA levels were studied in yellow ripe grain (mid-maturation stage) and fully ripe grain by northern analyses (Fig. 9). Hybridization was done with a probe consisting of 1500 bp of the 3' part of the LoxA cDNA. Highest levels of LOX mRNA are found in the yellow ripe stage of both varieties (Kristina and Triumph). Non-dormant grown grains did show a slightly higher level of LOX mRNA in the yellow ripe stage. Low levels of LOX transcripts could be detected in mature grains from both varieties, which indicates that LOX transcript levels decline during grain ripening. No significant differences in LOX mRNA levels were observed between the non-dormant variety Kristina and the dormant variety Triumph for both tested growth conditions, suggesting that LOX is not directly involved in the induction of grain dormancy.

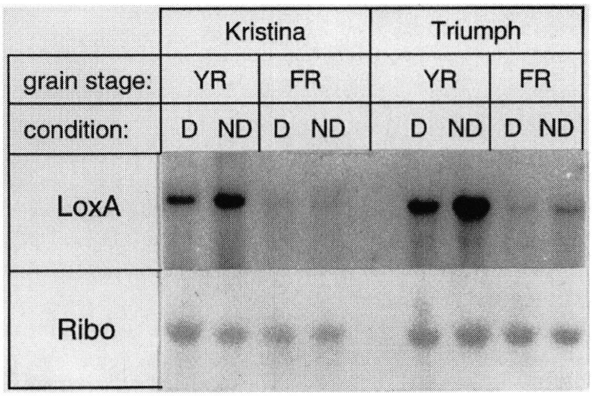

Fig. 9. Northern blot analyses of RNA extracted from developing grains of the varieties Kristina and Triumph. The blot was hybridized with a 1500 bp cDNA fragment of LoxA covering the 3'half of the coding sequence.
A ribosomal RNA probe was used for control on loading.
D, dormant condition; ND, Non-dormant condition;
YR, yellow ripe (mid-mature); FR, fully ripe (mature grain).

Discussion

This is the first report of the cloning and characterization of three full length cDNA fragments coding for distinct cereal lipoxygenases. In most plant species characterized so far, lipoxygenases are encoded by gene families whose members show complex expression patterns. From barley grains only one lipoxygenase encoding cDNA (LoxA) has been reported (46). In order to obtain more information about the appearance of lipoxygenases in barley grain, we have cloned two new full length LOX encoding cDNA sequences and we studied their spatial and temporal expression.

The deduced amino sequences of the three distinct barley cDNAs share known conserved sequences among plant lipoxygenases. Conservation of amino acid sequence has been noted for the regions containing the histidine residues and the C-terminal part, all known to be important for iron binding (Fig. 3). The three deduced LOX types do not contain an amino-terminal extension characteristic for chloroplast targeting and can therefore be catagorized as cytosolic LOX (LOX1-type) encoding sequences (Fig. 4). Contrary the lox2-type proteins from barley and rise contain amino terminal targeting signals, which consist of relative high proportions of serine and threonine residues (47, 30). Also for dicots like *Arabidopsis* (3), potato (34) and tomato (17) chloroplast associated lipoxygenases have been determined. Lipoxygenases catagorized as chloroplast-targeted lipoxygenases (LOX2-types) are mostly involved in the generation of 13 hydroperoxide fatty acids. If the substrate is α-linolenic acid 13-(*S*)-HPOT will be formed, which can further be used in the pathway leading to JA (47, 30, 3, 34). JA play an important role in stress responses, like pathogen attack. These types of lipoxygenase are involved in mediating plant resistance by induction of diverse plant defence genes (5). Barley seedling LOX2 is, like the chloroplast associated LOX types, involved in the formation of 13-hydroperoxide. Although barley LOX 2 can be assigned as a cytosolic type, it might be possible that during germination LOX2 takes part in the *de novo* synthesis of JA. On the onset of germination high levels of JA are found in soybean coleoptiles (4). During seedling growth the JA levels decline rapidly in the hypocot zones after elongation. In barley, LOX2 activity is measured after 24 h of grain imbibition, followed by an increase of activity until 48 h. After this time point the activity is gradually increasing until day seven (19). However, to our knowledge existence of JA in barley grain or seedlings has not been reported so far.

Southern analysis indicated that the three Lox genes are single copy members of a small gene family of lipoxygenases (Fig. 5). Although with the short specific probe for LoxA, two bands were found in the *Bgl*I digest, the appearance of a single hybridizing band of approximately 3 kb in the *Bam*HI digest with a comparable intensity as found for the hybridizing fragments for LoxB and LoxC, indicates that LoxA is single copy and not duplicated. The genetic evaluation of Lox-derived banding patterns revealed three individual loci, carrying the LoxA, LoxB and the LoxC gene (Fig. 6). In contrast to other gene families, e.g. those encoding the storage proteins of the barley grain, the Lox genes are not clustered on a single locus. Remarkably, the chloroplast associated LOX2:Hv:1 and a closely related gene were both mapped on distinct chromosomes (47). Although the occurrence of sequence duplications both within individual chromosomes and between different chromosomes has been observed in barley (18, 15) more data are required to shed light on the evolution of this gene family. The observed duplications of a Lox ancestor gene might be due to either the occurrence of unequal cross over, accounting for the duplication on chromosome 4, or a translocation resulting in the duplication between chromosome 4 and 7. To test both hypotheses, more RFLP markers, tightly linked to Lox genes are required. The availability of such markers would help to identify chromosomal segments, which should have been duplicated in the course of the distribution of these genes within the genome.

Of these three cDNA types the amino acid sequence encoded by LoxA was complete identical to the amino acid sequences of two lipoxygenase 1 peptides. Furthermore, we have expressed cDNAs encoding loxA and LoxC in *E.coli.* The LoxA product showed highest affinity with antibodies with a pronounced specificity towards LOX1, while the product of loxC showed highest affinity with antibodies with a pronounced specificity toward LOX2. It must be noted that LoxA mRNA and LOX1 protein were predominantly detected in quiescent barley grains (10, 38, 19). We speculate that LoxC cDNA codes for LOX2, since LoxC mRNA and LOX2 protein are both abundant during early development, while expression of LoxB during early development is extremely low, which could only be detected by northern analysis after very long period of autoradiographic exposure. However, three distinct lipoxygenase transcripts have been observed, while only two distinct lipoxygenase isoenzymes have been detected during grain development and during germination (10, 19). The third isoenzyme might posses biochemical properties like LOX1 or LOX2. The protein might also be very instable, which could be an explanation for the absence of a third isoenzyme during purification (10).

Northern analysis showed the occurrence of transcripts corresponding with all three types of LOX in grain and seedling. Transcripts coding for LoxB and LoxC were not restricted to grain, but were also observed in stem tissue, although LoxB transcripts were only visible after a long period of autoradiographic exposure. Lox C, which encodes isoenzyme Lox-2, might be involved in the novo-synthesis of JA via the octadecanoid pathway. Thereby it might be implicated in stress-signal mediation. It has been reported that LoxB and LoxC are induced after application with sorbitol and after application with JA in barley leaf segments (13). Intensive hybridizations were shown with the LoxB and LoxC probes for both applications. However LOX activity could only be measured from the JA treated extracts.

In our experiments, transcripts corresponding to LoxA were not detected in mature vegetative tissue. Analysis of the LoxA promoter element showed that 10 days old barley seedlings were responsive to JA (33). So far, it may be concluded that all three grain lipoxygenases are expressed in vegetative tissues. Northern analysis performed with RNA isolated from dissected grains, showed that LoxA and LoxC are predominantly expressed in scutellum, leaf and root, while LoxB is expressed in relatively low abundance in those tissues. No LOX transcripts were detected in aleurone during germination.

Furthermore, we studied the possible involvement of LOX in the induction of grain dormancy. The level of grain dormancy is determined by environmental conditions and genotype constitution. In oat, water stress and high temperatures during grain maturation reduce the level of dormancy, while shortening of photoperiod increases dormancy (2, 44). Also in barley the level of dormancy varies widely in response to temperature changes during maturation of the grain (31). Water deficit barley, close to period of anthesis, increases the level of dormancy, while drought during period of grain ripening reduces dormancy. We have evaluated LOX expression in the barley variety Kristina, a variety which lacks dormancy induction and in Triumph, a variety which is highly susceptible for dormancy induction. Both varieties were grown under conditions known to cause dormant or non-dormant grains from susceptible genotypes (38). A slight difference in mRNA levels was found for both varieties

Kristina and Triumph in yellow ripe grains compared for two differential growth conditions (Fig. 9). However, since Kristina is not susceptible for the induction of dormancy the observed differences between growth conditions might rather be a consequence of differences in total gene activity correlated with grain developmental stage or maturation velocity, than being a key factor in determination of dormancy. Therefor it must be concluded that no significant difference in levels of LOX expression was observed in RNA extracted from both varieties during grain development under both growth conditions, indicating no correlation between LOX expression during grain development and the induction of dormancy.

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Chapter 4

Expression of lipoxygenase isoenzymes in developing barley grains*

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Abstract

Expression of lipoxygenase was studied in whole developing barley grains from 5 DAF to full maturity. Lipoxygenase showed two distinct peaks of activity. The first peak of activity occurred in the early stages of grain development, from 5 until 20 DAF, whereas the second peak occurred at the later stages, from 30 DAF to the full grain maturity. The temporal expression of both LOX-1 and LOX-2 was studied at the protein and mRNA levels using specific probes and by analyzing the hydroperoxides formed during grain development. These experiments showed that a LOX-2-type isoform is present at early stages of development, located in the envelopes of the grain, whereas a LOX-1 isoform accumulates in the embryo in the late stages of development. Additionally, the spatial and temporal pattern of hydroperoxide-metabolizing activity was shown to be comparable to that of lipoxygenase activity. We demonstrate that two lipoxygenases appear during barley grain development, differing in their spatial and temporal expression. These two enzymatic pathways give rise to a distinct set of end-products indicating that the lipoxygenases may play different physiological functions during grain development.

1. Introduction

Lipoxygenases (E.C. 1.13.11.12) are a class of iron-containing enzymes that catalyze the oxygenation of fatty acids containing a *cis,cis*-1,4-pentadiene structure to form hydroperoxides of fatty acids. In plants, linoleic and linolenic acids are the two most abundant fatty acids of this type. Although the role of these enzymes is not fully elucidated in plant cells, several functions have been postulated. Lipoxygenases may be involved in growth and development, defense against wounding or pathogens and senescence [1,2]. The lipoxygenase pathway has also generated special interest because the ultimate products formed by this enzymatic cascade, e.g. jasmonate or methyl jasmonate and traumatin, are regulatory molecules and have important physiological roles. Lipoxygenases have been characterized in many species and plant organs. In barley, lipoxygenase has generated particular interest due to its possible involvement in the production of off-flavors in beer on ageing [3].

Two lipoxygenases, LOX-1 and LOX-2, have been purified and characterized from the embryo of germinated barley [4,5]. LOX-1 and LOX-2 differ in the product they form from linoleic acid (9-HPOD and 13-HPOD, respectively), their isoelectric point, and the pH range over which the enzymes are active. Previously, a cDNA (LoxA clone), encoding the LOX-1 protein was isolated [6], while two additional clones, LoxB and LoxC, have been identified recently. LoxC probably encodes LOX-2, whereas LoxB encodes a sofar unidentified lipoxygenase isoform [Van Mechelen, *unpublished data*]. Substantial research has been conducted in barley on the characterization of lipoxygenase during germination [7,8]. In a recent study [9], the temporal and spatial expression pattern of LOX-1 and LOX-2 in barley embryos was shown to be very similar during the first days of germination. The only major difference in their expression pattern was observed in the mature grain, where LOX-1 accounted for most of the total lipoxygenase activity, indicating that only LOX-1 accumulates in the embryo during grain development. However, lipoxygenase expression patterns in the developing barley grain have not been reported.

The products of lipoxygenase activity are fed into three distinct enzymatic pathways [2,10], leading to a set of lipid-breakdown products that probably fulfill different physiological roles *in vivo*. In the first pathway, hydroperoxides of fatty acids are cleaved by hydroperoxide lyase into an aldehyde and a keto-acid, which may play a role as defensive agents against pathogenic micro-organisms.

In a second pathway, allene oxide synthase activity, previously called hydroperoxide dehydrase or hydroperoxide isomerase, converts hydroperoxides of fatty acids into allene oxides, that can be spontaneously converted to a- and g-ketols. The sequential catabolism of the 13-hydroperoxide of linolenic acid by allene oxide synthase and allene oxide cyclase, constitute the first enzymatic steps in the biosynthesis of jasmonic acid.

The third main route for hydroperoxide conversion is the peroxygenase cascade and catalyzes hydroperoxide-dependent hydroxylation, sulfoxidation and epoxidation [11].

Allene oxide synthase has been partially purified and characterized from barley [12]. The activity of this enzyme has also been demonstrated in extracts of quiescent grains incubated with linoleic acid, where the only products formed were ketols [7]. Lyase activity however, has never been clearly demonstrated in barley. A recent study of peroxygenase activity in quiescent grains of different cereals, revealed a very low peroxygenase activity in barley grain homogenates [13]. In conclusion, in quiescent grains of barley, most hydroperoxide-metabolizing activity can be ascribed to allene oxide synthase activity.

In this study, we followed the temporal and spatial expression of lipoxygenase isoforms in the developing barley grain and showed the presence LOX-2-type lipoxygenase in the grain envelope at early stages of development. Furthermore, the spatial and temporal pattern of hydroperoxide-consuming activity is shown to be comparable to the lipoxygenase expression pattern, whereby two distinct LOX metabolism routes may occur in developing barley grains.

2. Materials and methods

2.1. *Plant Material*

Barley grains (*Hordeum vulgare* L. cv. Caruso, harvest 1993) were grown in a phytotron under the following conditions: alternately 16 hours light (300 mmol.m-^{2}.s^{-1}) at 15 °C and 8 hours darkness at 12 °C, with a relative humidity of 80 %. The temperature was increased to 18-20 °C for grain ripening. Spikes were tagged on the day of flowering and collected at different stages of development, up to physiological full maturity (FM) approximatively 75 days after flowering. The harvested grains were stored at -80 °C prior to assay. Grain samples used for RNA extraction were immediately frozen in liquid nitrogen prior to storage at -80 °C.

Grains harvested at 12 (± 2) days after anthesis were dissected by peeling away the outer layers (glumes), the inner layers (pericarp/nucellus/testa) with forceps and separating the rest of the grains into the embryo and the endosperm parts.

2.2. *Extraction and Enzyme Assays*

All steps were performed at 4 °C. For LOX-activity determination, crude extracts were prepared by homogenizing 10 grains in 20 mM Tris-HCL buffer at pH 7.5 (1:10, $^{W}/_{V}$) in the absence or presence or 0.1 % ($^{V}/_{V}$) NP-40 (BDH, England). The resulting suspension was centrifuged for 20 min at 15,000g. The extracts prepared in the presence of NP-40 were incubated for 20 min on ice with gentle shaking prior to centrifugation. Hydroperoxide-consuming enzymes were extracted with 50 mM sodium phosphate buffer, pH 6.5, containing 0.1% ($^{V}/_{V}$) Triton X-100 (1:10, w /v) . After 20 min of gentle shaking on ice, the extracts were centrifuged for 20 min at 15,000g. LOX-activity was measured by polarography using a Clark-type oxygen electrode. HPOD-consuming activity was measured spectrophotometrically at 234 nm in 50 mM sodium phosphate buffer (pH 6.5) containing 15 mM 13-HPOD. 13-HPOD was prepared by incubating soybean LOX-1 (10 units/ml) with 0.1 mM of linoleic acid in oxygen-saturated 0.1 M sodium borate buffer pH 9. 13-HPOD was extracted on an octadecyl solid-phase extraction column (Bakerbond, Baker) and analysed by RP-HPLC as described in the hydroperoxide analysis section. 13-HPOD and 9-HPOD standards supplied by Cayman Chemical Company, USA were used for product identification.

2.3. Volatile aldehydes determination

Crude extracts were prepared from grains homogenised with 50 mM sodium phosphate, containing 0.1 % of NP-40, as indicated above. Incubations were performed in 100 mM of oxygen-saturated sodium phosphate buffer (pH 6.5) in the presence of 200 mM linoleic acid. After acidification to pH 3.5, the incubation mixture (10 ml) was concentrated on a Sep Pak C18 cartridge (Baker) and the products were eluted with 2 ml of methylene chloride. Aldehydes were measured by GC/FID on a capillary column (HP, 25 m x 0.32 mm i.d., 1.05 mm). Temperature was held at 50°C for 6 min, increased to 250°C at 10°C/min and finally held at 250°C for 15 min. Hydrogen was used as carrier gas and the column pressure was set at 50 KPa. Hexanal and nonenal concentrations were calculated from the concentration of the internal standard, 1-heptanal. The detection limit in the incubation media was determined to be approximately 0.2 mM for hexanal and *trans*-2-nonenal.

2.4. Hydroperoxide Analysis

Crude extracts were prepared from grains (or parts of the grain) as described above in the absence of detergent and were incubated in 100 mM of oxygen-saturated sodium phosphate buffer (pH 6.5) in the presence of 200 mM of linoleic acid with gentle agitation at room temperature. Isolation and identification of 9- and 13-hydroperoxide of linoleic acid using reverse-phase HPLC were performed as previously described [9,14].

2.5. RNA Extraction and Northern Blotting

RNA was isolated from whole grains according to [15]. Northern blotting, preparation of the labelled LoxA, LoxB or LoxC specific probes and hybridization of the blots were performed as previously described [9]. 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). Plasmid controls (10 pg of each) were hybridized and exposed in one experiment with the Northern analysis in order to compare the intensity of the observed transcripts. Equal loading of the RNA samples was checked by rehybridization of the blots with a ribosomal probe.

2.6. Gel Electrophoresis and Western Blotting

SDS-PAGE and Western blotting were performed as previously described [9]. After blotting, the membranes were incubated with LOX-1 or LOX-2-specific monoclonal antibodies (5D2 and 4.2, respectively) [9].

3. Results

3.1. Lipoxygenase activity in developing grains

Lipoxygenase activity was poorly extracted from whole barley grains by a standard extraction buffer devoid of detergent (Fig. 1). However, addition of non-ionic detergents, such as Triton X-100 or NP-40 increased the amount of extractable lipoxygenase activity, particularly at early stages of grain development (from 5 to 20 DAF). The concentration of NP-40 giving maximum extractable activity was determined to be 0.1% (data not shown). This enhanced activity is due to an increased extraction and solubilization of the lipoxygenase rather than a micelle influence, since lipoxygenase activity did not increase when the detergent was added to an enzyme sample extracted without detergent.

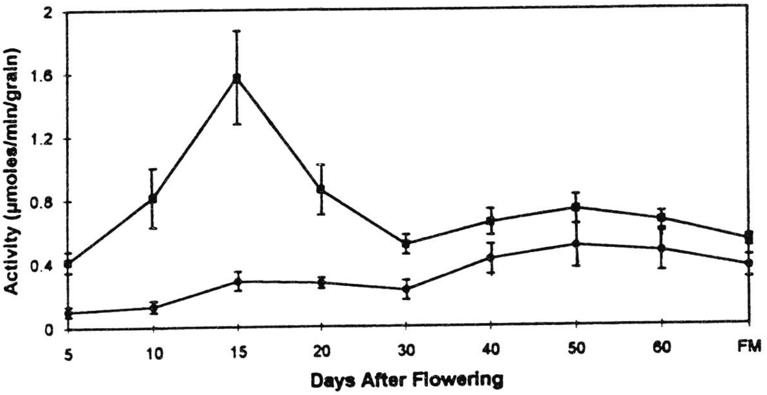

Fig. 1. Total lipoxygenase activity in whole barley grains during development. The extracts were performed in the presence (n) or absence (l) of 0.1 % NP-40 in the extraction buffer.
Values were the means ± SE of three independent experiments.

Lipoxygenase activity in detergent extracts of whole grains appeared at an early stage of development, with a peak of activity 15 DAF, and then declined (Fig. 1). After 30 DAF, the activity increased again, reached a maximal value at 50 DAF and remained high until full grain maturity.

To identify the LOX isoforms active during development, we analysed the hydroperoxides formed by whole grain extracts incubated with the substrate linoleic acid (Fig. 2). During the early stages of development, from 5 to 20 DAF, 13-HPOD was the main product formed, whereas at later stages, from 30 DAF until full grain maturity, 9-HPOD was the principal product. This suggests that the lipoxygenase activity seen at early and late stages of principal product. This suggests that the lipoxygenase activity seen at early and late stages of grain development (Fig.1), are a 13- and a 9-LOX, respectively. The percentages of 9- and 13-HPOD seen in Fig. 2 were similar when lipoxygenase extraction was performed with or without NP-40. Since the presence of NP-40 most strongly increased the extraction of lipoxygenase activity from early developing barley grain, it suggests that detergent is particularly important for the solubilization of the 13-LOX isoform. The HPOD level measured in assays of 0.1% NP-40 extracts of grain at later stages of development was very low, indicating that active HPOD-metabolizing enzymes were also extracted from the grain.

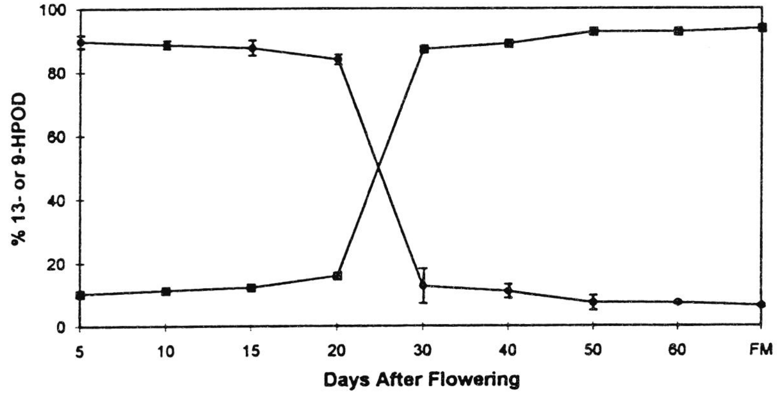

Fig. 2. Relative proportion of 13-HPOD (l) and 9-HPOD (n) formed during incubation of linoleic acid with enzymatic extracts of whole barley grains, prepared in the absence of detergent, throughout grain development. Values were the means ± SE of three independent experiments.

The levels of LOX-1 and LOX-2 isoforms were determined in whole developing grains by Western blot analysis using monoclonal antibodies specific for LOX-1 and LOX-2 isoforms [9]. Fig. 3A shows that LOX-1 protein levels were very low at the early stages of development, namely from 5 to 15 DAF, but thereafter accumulated to remain at high levels up to full grain maturity. Significant levels of LOX-2 protein were detected at the onset of grain development from 5 DAF, but after 20 DAF LOX-2 protein could no longer be detected (Fig. 3B).

Three lipoxygenase mRNAs, LoxA, LoxB and LoxC [6; Van Mechelen et al., *Chapter 3*], are expressed in the embryos of germinated barley grains. However, only two LOX-isoforms have been detected in germinating barley [4,5,9]. LoxA and LoxC encode the LOX-1 and the LOX-2 proteins, respectively, but the product of the *LoxB* gene has not been identified [Van Mechelen et al., *unpublished results*].

Using gene-specific probes, corresponding to the 3' untranslated region of LoxA, LoxB, and LoxC, their respective mRNA levels were determined in whole grains during development (Fig. 4).

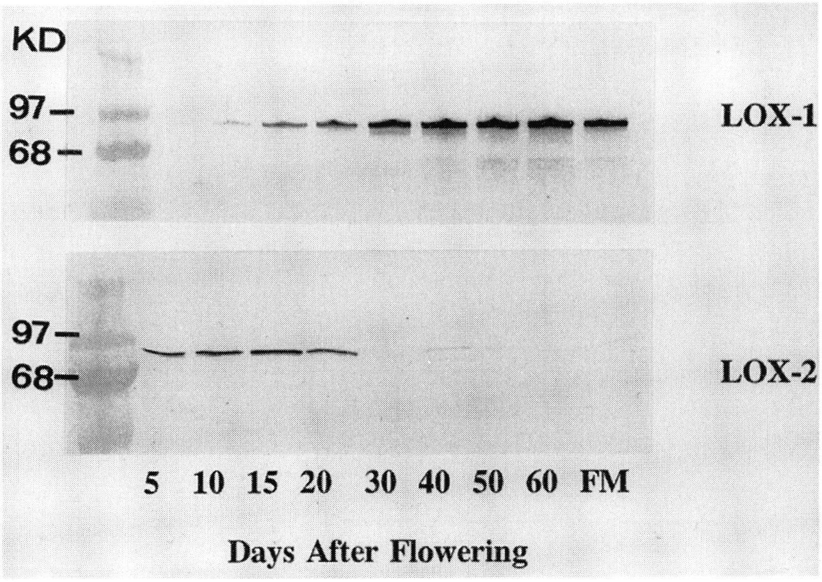

Fig. 3. Western Blot analysis of LOX-1 (A) and LOX-2 (B) in developing whole barley grain. Proteins were extracted at different stages of grain development in the presence of 0.1 % NP-40 and separated by SDS-PAGE. After blotting, lipoxygenase was immunodetected with specific monoclonal antibodies against LOX-1 or LOX-2. Each lane corresponded to the same number of developing grains.

LoxA mRNA was first detected 30 DAF, the level increasing up to 40 DAF and from this time-point remaining constant until a late stage of grain maturation (Fig. 4A). After lengthy autoradiographic exposure of the Northern, LoxA mRNA was also detected at 5 and 10 DAF (data not shown), indicating a very low expression of LoxA at early stages of development. LoxC transcripts were only detected early in grain development (Fig. 4C), namely from 5 to 20 DAF. Since expression levels of LoxA and LoxC transcripts were closely correlated with levels of LOX-1 and LOX-2 respectively, expression of both isoforms may primarily be regulated at the pretranslational level.

Although levels of LoxB mRNA were extremely low during grain development (Fig. 4B), the LoxB expression pattern was rather similar to that of LoxC mRNA, the highest transcript levels being detected at the onset of development.

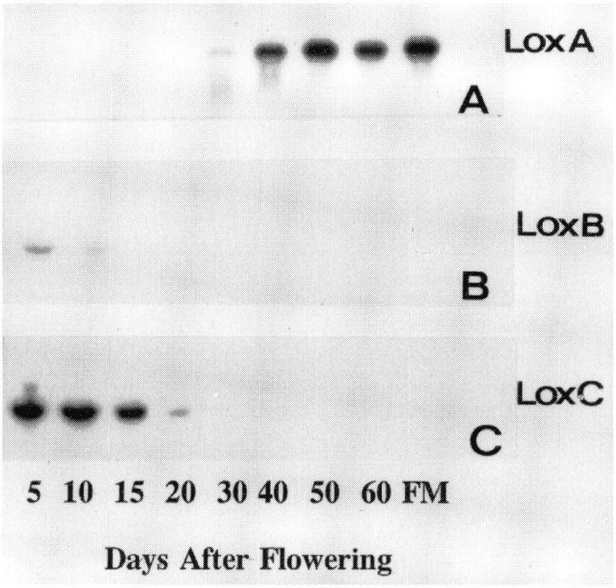

Fig. 4. Northern analysis of LoxA (A), LoxB (B) and LoxC (C) mRNAs in developing whole barley grains.

3.2. *Localization of the LOX-2 type isoform present at the early stages of development*

Localization of lipoxygenase activity in early developing grain was performed with material harvested at 12 DAF, when the grain could easily be separated into outer and inner envelopes, endosperm and embryo fractions. By light microscopy, the outer envelopes fraction was found to correspond to the glumes formed by the palea and lemma tissues, whereas the inner envelope fraction consisted of pericarp (outer and inner pericarp), testa and nucellus. At this developmental stage, only the glumes and the outer pericarp contained chloroplasts. Aleurone tissue was mainly present in the endosperm tissue, although it was present to a lesser extent in the inner envelope fraction as well.

Table 1 shows that at 12 DAF, LOX-activity was localized in the grain envelope, distributed between the outer and inner envelope fractions, in a ratio of 1:2. Thus, at this stage, LOX was located in the glumes and in the pericarp (outer or inner pericarp) and/or testa and/or nucellus tissues. Occurence of LOX-activity in aleurone tissue could be excluded since most of the aleurone was present in the endosperm fraction, which did not exhibit LOX activity. When extracts of the different grain parts were incubated with linoleic acid, 13-HPOD was the main product, confirming the presence of a LOX-2 type enzyme in the outer and inner envelopes.

	Outer envelopes	Inner envelopes	Aleurone	Embryo
LOX-activity (%)	34	60	1	4
% [13-HPOD/ (13- + 9-HPOD)]	88	90	n.d.	n.d.

Table 1: LOX-activity characterization in dissected parts of developing grains harvested 12 DAF. Parts of the grain were dissected and assayed for LOX-activity. LOX-activity was expressed as percentage of the total lipoxygenase activity measured in the whole grain. The percentage of 13-HPOD *versus* total HPOD was determined during incubation of extracts with linoleic acid. n.d.: not detected.

Furthermore, using a panel of monoclonal antibodies specifically recognizing LOX-1 or LOX-2 isoforms [9], only LOX-2 was detected in the envelope fraction. The isoelectric point and the apparent molecular mass in SDS-PAGE of the detected isoform corresponded to those of LOX-2 found in germinating barley grains (data not shown).

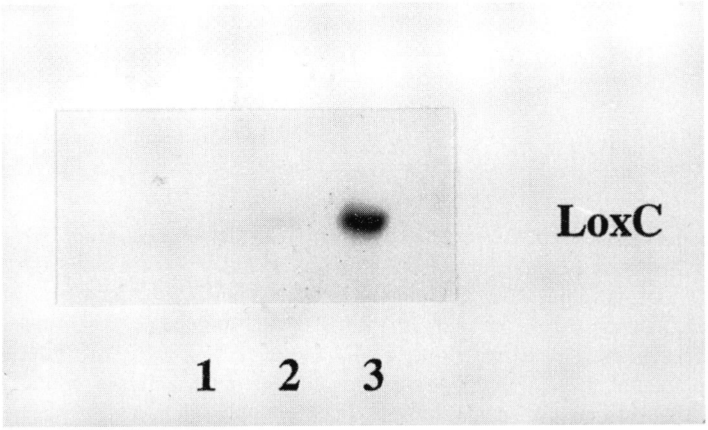

Fig. 5. Northern blot of LoxC mRNA in the dissected parts of a12 DAF barley grain. 1: embryo and endosperm; 2: glumes; 3: pericarp, testa and nucellus.

Using the LoxC-specific probe, the highest levels of LOX-2 transcripts were detected in the inner envelopes, whereas only low levels were found in the glumes (Fig. 5). LoxB mRNA showed a similar pattern to LoxC, except that LoxB transcript levels were very low and could only be detected after long exposure times (data not shown).

The *Km* value for the LOX-type present in the envelopes was determined to be 70 (± 10) mM and 48 (± 8) mM for linoleic and linolenic acid, respectively. The apparent *Km* for LOX-2 from germinated grains was previously found to be 18 and 23 mM, respectively [4,5]. Additionally, the location of the lipoxygenase activity at late stages of development was determined by separating the embryo from the rest of the grain, showing that from 40 DAF to the full maturity stage, lipoxygenase activity was a LOX-1 isoform and was localized in the embryo (Table 2).

	Embryo part	Embryoless part
LOX-activity (%)	99	1
% [9-HPOD/ (13- +9-HPOD)]	92	n.d.

Table 2: LOX-activity characterization in dissected parts of developing grains harvested 60 DAF. Barley grain was separated in embryo and embryoless grain. Both parts were assayed for LOX-activity. LOX-activity was expressed as percentage of the total lipoxygenase activity measured in the whole grain. The percentage of 9-HPOD *versus* total HPOD was determined during incubation of extracts with linoleic acid. n.d.: not detected.

3.3. Hydroperoxide-metabolizing enzymes in developing grains

HPOD-metabolizing activity was followed at different stages of grain development (Fig. 6). Low levels of activity were detected early in grain development, namely from 5 to 15 DAF, but increased strongly from 20 DAF, reaching a maximum level at 50 DAF. The activity was localized to the inner and outer envelopes of the grain, in the early stages of development from 5 to 15 DAF, while at the late stages, i.e. 50 DAF to full maturity, HPOD-metabolizing activity was restricted to the embryo (data not shown). The level of HPOD-metabolizing activity in the early grain envelope was circa 6% of the activity present in the embryo in late development, while it represented only 3% of the lipoxygenase activity present in the envelopes at the same developmental stage. The rate of 9-HPOD or 13-HPOD metabolizing activity in the outer envelopes of the grain (glumes) harvested 10 DAF were similar, suggesting that this pathway metabolizes both substrates. The nature of the HPOD-metabolizing activity in the envelopes and in the embryo of the developing grains was analysed with the lyase-specific spectrophotometric assay described by Vick [16]. No lyase activity could be detected, although the assay is not very sensitive. Furthermore, using gas-chromatography analysis, we could not detect significant levels of nonenal in embryo extracts from quiescent grains incubated with linoleic acid (data not shown).

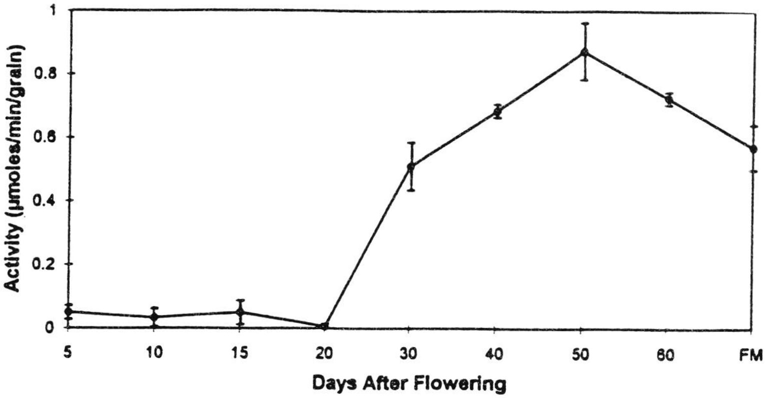

Fig. 6. Hydroperoxide-consuming activity in developing whole barley grains. Values are the means ± SE of three independent experiments.

Peroxygenase activity, an HPOD-degrading activity which was not assayed in our extracts, was shown to be very low in quiescent barley grains [14]. These data confirm previous results, showing that allene oxide synthase activity accounts for the majority of total HPOD-metabolizing activity found in embryos of quiescent grains [7].

4. Discussion

While LOX-1 activity was previously described in the embryos of quiescent barley grains [9,14], we have shown that LOX-1 is synthesized and accumulates in the embryo of the developing grain, from approximately 20 DAF. We have also shown that at the onset of grain development, a LOX-2-type lipoxygenase is active in the peripherical tissues of the kernel. High activities were detected in the glumes (photosynthetic tissues) and in a fraction containing the pericarp (non-photosynthetic inner and photosynthetic outer pericarp), testa and nucellus tissues, although it was not possible to localise LOX-2 activity more precisely within the later fraction. This activity remained until 20-30 DAF, at which stage the grain envelope begun to turn yellow, indicating chloroplast degradation and senescence in these parental peripherical tissues. In subsequent grain development, degradation of the LOX-2 type protein was shown by Western analysis (Fig. 3B), whereas synthesis of LOX-1 in the embryo, is initiated. The appearance of lipoxygenase isoforms during grain development, described here and during germination, described in a previously publication [9], demonstrate the complex temporal and tissue-specific expression of the *Lox* genes.

In our study, LOX-2 isoform and transcripts were not detected in grains at full maturity in contradiction to previous data showing a low level of LOX-2 isoform and mRNA in embryos of the quiescent grain [9]. This apparent discrepancy may be explained by a less efficient extraction of mRNA and protein from whole grain, since in this study the embryos were not dissected out prior to extraction. It is also possible that growth conditions during grain development may influence the level of LOX-2 in embryos, since the plant material was cultivated in the field in one case [9], and in a phytotron for this study.

The LOX-2 type enzyme was found to have very similar properties to the LOX-2 extracted from germinating barley grains, described previously [4,5]. The enzyme formed 13-hydroperoxides of fatty acids and exhibited the same isoelectric point and apparent molecular mass. Furthermore, the LOX-2 type enzyme cross-reacted with the LOX-2 antibodies, and synthesis of this enzyme coincided with the appearance of LoxC transcripts, which also encode the LOX-2 expressed during germination [9]. The only differences observed between the LOX-2 enzymes concerned their extractibility and kinetic properties. Thus, LOX-2 in the grain envelope could only be extracted efficiently in presence of NP-40, whereas LOX-2 from germinating barley grains was routinely extracted with aqueous buffer [9]. The release of lipoxygenase by non-ionic detergents has been reported previously [17], indicating that in envelopes, LOX-2 may be membrane-associated and have a different subcellular localization from the LOX-2 found in embryos. However, a non-specific absorption of the enzyme during extraction cannot be excluded. The LOX-2 enzymes also differed in their K_m values, whereby the LOX-2 activity extracted from the envelopes of the early developing grain exhibited a slightly lower K_m for linolenic acid than for linoleic acid, the reverse of that seen for the germination expressed LOX-2. This kinetic property could be characteristic for lipoxygenases from green leaves, where the linolenic content is higher than the linoleic content [18], suggesting that LOX-2 in glumes could be associated with chloroplast membranes. Recent studies have demonstrated that chloroplasts are the site of hydroperoxide formation and metabolism [19,20] emphasizing the role of chloroplasts in the synthesis of signalling molecules.

Few studies have been conducted on lipoxygenase during cereal grain development and the function of LOX-activity in developing barley embryos remains unclear. Nevertheless, a physiological role during the maturation of the grain has been suggested for these enzymes [21-23]. In the embryo of developing corn seeds, two periods of high lipoxygenase activity were shown to correspond to periods of synthesis and action of abscisic acid, namely induction and maintenance of dormancy [23]. Hildebrand et al. [21] reported that seed coats of soybean had a much higher lipoxygenase activity than embryo tissue during early stages of development, indicating that the presence of this enzyme activity in external tissues of developing seeds could be a general phenomenon.

During grain development, hydroperoxide-metabolizing activity was localised to the same tissues as lipoxygenase activity. Although the activity was low, it was clearly shown that HPOD-consuming activity was present at the early stages of development in the outer layers of the grain, indicating that 13-hydroperoxide may be metabolized further. Furthermore LOX and HPOD-degrading activity disappeared from the envelopes (between 20 and 30 DAF) simultaneously with the senescence of these tissues. Later in development, an HPOD-metabolizing activity was detected in the embryo. Whereas allene oxide synthase was probably responsible for all or nearly all the HPOD metabolism in barley embryos at late developmental stages, the nature of the HPOD-metabolizing activity in the envelopes of the grain is unknown. If this activity is due to a lyase, C_6-aldehydes, hexanal and hexenal would be expected, while ketols/jasmonic acid are the expected products of allene oxide synthase activity. In the former case, the presence of hexanal- and hexenal-biosynthetic routes in the external exposed tissues of the grain may constitute an efficient defense system against pathogens [24]. In the second case, the synthesis of jasmonic acid may occur in the external layers of the grain, as has been demonstrated in the pericarp and seed coat of soybean [25]. It will be interesting to establish whether regulatory molecules formed in the peripherical tissues of the grain such as jasmonic acid might be delivered in the embryo and aleurone to act as transduction signals involved in the control of dormancy and germination.

Acknowledgements

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Chapter 5

The Untranslated Leader Sequence of the Barley Lipoxygenase 1 Gene (Lox1) Confers Embryo-Specific Expression*

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*Plant Journal: 15(3), 435-440 (1998).

Chapter 5

Summary

The barley lipoxygenase 1 gene encodes a protein expressed in embryos during grain development and germination and in leaves after methyl-jasmonate (MeJA) treatment. Transient gene expression assays in germinating barley embryos were used to identify cis-regulatory elements involved in the embryo-specific expression of the Lox1 gene. Analysis of transcriptional or translational fusions between Lox1 5' upstream sequences and the gusA reporter gene indicated that the 5'-untranslated leader sequence was involved in embryo-specific expression. Replacement of the leader sequence from the aleurone-specific Chi26 gene (Leah et al., 1994) with the Lox1 leader sequence resulted in a chimeric gene expressed at high levels in embryo as well as in aleurone cells. Insertion of the Lox1 leader sequence between the 35S minimum promoter (A domain -90/+8) and the gusA reporter gene greatly enhanced promoter activity in a tissue-specific manner. Deletion/replacement analysis of the Lox1 leader sequence, combined with transient expression in germinating embryos and in vitro transcription/translation assays, suggests that the Lox1 leader sequence contains cis-elements regulating qualitative (tissue-specific) and quantitative gene expression.

Introduction

Lipoxygenases are enzymes found in almost all eukaryotes (Hildebrand et al., 1988 ; Siedow, 1991), which catalyze the dioxygenation of polyunsaturated fatty acids containing a cis, cis-1,4-pentadiene structure to produce conjugated cis, trans-diene hydroperoxy derivatives. Lipoxygenases are involved in a number of diverse aspects of plant physiology including senescence, plant defense reactions, plant growth and development, mobilization of lipid reserves during seed germination (Siedow, 1991 ; Slusarenko et al., 1993), and are proposed to play a role as vegetative storage proteins (Tranbarger et al., 1991).

In barley, lipoxygenases (LOX1 and 2) were first purified from germinating grain (Doderer et al., 1992) and their respective cDNA clones (LoxA and LoxC) were subsequently screened from a developing grain cDNA library (van Mechelen et al., 1995). LOX1 and LOX2 protein have been localised in the embryos of quiescent or germinating grain (Holtman et al., 1996). While LOX2 may play a role in the mobilization of storage lipids in the germinating embryo and the generation of energy (Holtman, 1997) as has been shown in cucumber seedlings (Feussner et al., 1995), the physiological role of LOX1 in barley remains a matter of speculation. LoxA transcripts are also detected in leaves in response to MeJA and to wounding (Rouster et al., 1997). A detailed analysis of the promoter of the corresponding LoxA gene (Lox1) has localised a MeJA responsive element -331 to -291 nt upstream of the transcription start (Rouster et al., 1997). The present study was aimed at identifying the cis-acting elements controlling Lox1 expression in the barley grain, to provide additional insight into the regulation of this enzyme.

Our investigations clearly demonstrate that cis-acting elements within the untranslated leader sequence of the Lox1 gene are essential for its embryo-specific expression and that it is possible to modify the tissue-specificity of other genes by insertion of this leader sequence. We also show that the Lox1 leader sequence contains an enhancer element which increases the activity of upstream promoter fragments in vivo. The molecular mechanism by which the Lox1 leader sequence enhances reporter gene expression is investigated.

Results and Discussion

The barley Lox1 gene is expressed during grain development and germination

Lox1 gene transcript levels in the developing and germinating grain were followed by Northern blot analysis (Fig. 1). During grain development, the Lox1 gene is expressed at low levels in aleurone/endosperm from 20 until 40 days after anthesis. In embryos, the level of Lox1 expression is significantly higher, reaching a maximum at around 30 days after anthesis. Upon germination, the steady state level of Lox1 mRNA in embryo tissues increases, indicating *de novo* transcription.

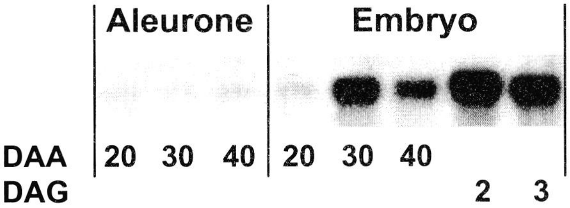


Fig. 1 : Steady state Lox1 mRNA levels in barley grains during grain development and germination.Twenty micrograms of total RNA extracted from aleurone/endosperm and embryo tissues were used to prepare a northern blot, which was hybridized with a full length Lox1 cDNA probe. DAA: days after anthesis, DAG: days after germination.

Lox1 5' upstream region-GUS constructs are expressed in germinating embryos

In order to identify regions of the Lox1 promoter involved in regulating embryo-specific expression, a 910 bp Lox1 promoter fragment (Rouster et al., 1997) and several 5' or 3' deletions were fused to the GUS reporter gene, 23 nucleotides upstream of the ATG start (Fig. 2). The constructs were introduced into 3 day old embryos by particle bombardment, and the GUS activity measured after 48 h incubation.

Lox1 promoter fragments 5' deleted up to position -129 (constructs 1 to 5) retained most of the promoter activity (approx. 80%). Further deletion to -102 (construct 6) and -75 (construct 7) resulted in a drastic reduction of promoter activity. However the residual activity in construct 7, containing the TATA box and downstream sequences, was still significant, equivalent to (10 % of the full-length promoter activity. This indicates that essential promoter or enhancer element(s) involved in Lox1 expression in embryos are located between -129 and -75.

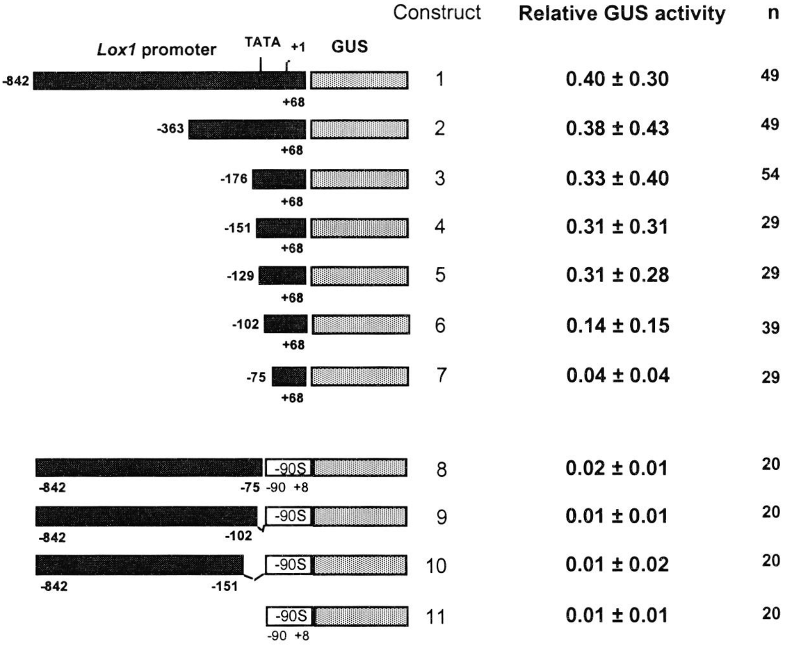


Fig. 2 : Lox1-GUS expression in 3 days germinated embryos.
5' deleted Lox1 promoter fragments were fused to the GUS reporter gene, while 3' Lox1 promoter deletions were fused to the CaMV35S A domain (-90S) -GUS reporter gene. Numbers below the open rectangles indicate the distance, up or downstream, from the Lox1 transcription start site. The relative GUS activity ± SE given for each numbered construct is an average of several independent transient expression assays (n).

The Lox1 promoter fragment (-842 to -75), located upstream of the TATA box, was deleted sequentially from the 3' end and then fused in front of the -90S-GUS (35S promoter A domain (-90/+8) containing the 35S TATA box in front of the GUS reporter gene, constructs 8 to 10). None of these Lox1 promoter fragments were able to drive GUS expression in germinating embryos, although they show strong promoter activity in barley leaves treated with methyl-jasmonate (Rouster et al., 1997). The importance and location of elements in the Lox1 promoter for embryo-specific expression, lying 3' of the TATA box, were investigated further.

The untranslated Lox1 leader sequence is crucial for embryo-specific expression

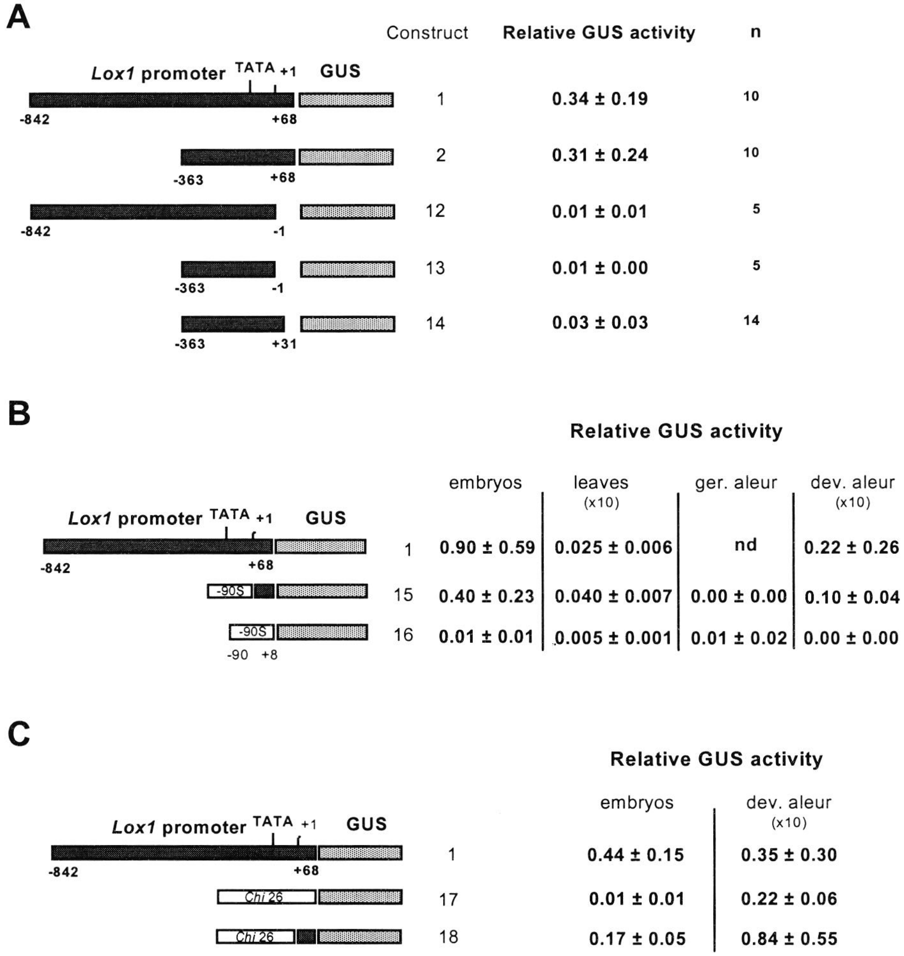
The full-length Lox1 promoter (-842/+68) or 5′ deleted (-363/+68) promoter fragments fused to the GUS gene (constructs 1 and 2), were found to drive GUS expression in germinating embryos (Fig. 3A). However, a transcriptional fusion of either the full-length or 5′ deleted Lox1 promoter (-842/-1 and -363/-1) to the GUS gene (constructs 12 and 13), failed to drive GUS expression. Inclusion of half of the Lox1 leader sequence (-363/+31) in the promoter fusion (construct 14), did not restore GUS activity. We therefore concluded that the Lox1 leader sequence may be required for translation of the GUS transcripts or alternatively may contain promoter elements essential for embryo-specific Lox1 expression.

When the untranslated leader sequence of the aleurone-specific chitinase gene Chi26 (Leah et al., 1994) was inserted between the Lox1 promoter (-842/-1) and the GUS reporter gene, expression of this construct could not be detected in germinating embryos (data not shown). This indicates that the addition of a leader sequence per se at the 5′ end of the GUS transcript could not account for the ability of the Lox1 promoter to drive GUS expression in embryos.

The expression of several plant genes are known to be regulated by elements located in the transcribed part of the gene (Bate et al., 1996; Bolle et al., 1994, 1996; Caspar and Quail, 1993; Helliwell et al., 1997). In order to test its promoter function, the Lox1 leader sequence (+1,+68) was inserted between the -90S promoter and the GUS reporter gene (construct 15, Fig. 3B). This construct drove high levels of GUS activity in germinating embryos, when compared to -90S-GUS (construct 11). The data support the hypothesis that embryo-specific expression of the Lox1 gene is directed by an enhancer element located in the 5' leader sequence. The leader sequence cannot function as a promoter per se, as was seen from the inactivity of a Lox1 leader-GUS construct (data not shown), but only in combination with a promoter eg. the minimal 90S (A domain of the 35S promoter).

The 5′ untranslated leader sequences of three nuclear genes (PetH, PetE and PsaF), encoding chloroplast thylakoid proteins in spinach, have been shown to contain elements essential for quantitative control of gene transcription in conjunction with their respective upstream promoter (Bolle et al., 1994). In contrast, the Lox1 5′ leader sequence contains elements responsible for the qualitative regulation of Lox1 gene expression. The tissue-specificity of the enhancer element of the Lox1 leader closely mirrored the tissue specificity of the full length promoter, namely maximal expression in germinating embryos and low or very low expression in developing aleurone layer and leaves, respectively (Fig. 3B).

The ability of the Lox1 5' leader to drive embryo-specific expression within the context of a gene with differing tissue-specificity, was tested by substituting the leader sequence in the Chi26 gene with the Lox1 leader sequence. While the Chi26 gene is selectively expressed in aleurone cells of the developing grain (Leah et al., 1994), the chimeric promoter not only directed GUS expression in germinating embryos, but also enhanced GUS expression in developing aleurone cells (compare construct 17 vs construct 16, Fig. 3C).



A

Construct	Relative GUS activity	n
1	0.34 ± 0.19	10
2	0.31 ± 0.24	10
12	0.01 ± 0.01	5
13	0.01 ± 0.00	5
14	0.03 ± 0.03	14

B

Relative GUS activity

	embryos	leaves (x10)	ger. aleur	dev. aleur (x10)
1	0.90 ± 0.59	0.025 ± 0.006	nd	0.22 ± 0.26
15	0.40 ± 0.23	0.040 ± 0.007	0.00 ± 0.00	0.10 ± 0.04
16	0.01 ± 0.01	0.005 ± 0.001	0.01 ± 0.02	0.00 ± 0.00

C

Relative GUS activity

	embryos	dev. aleur (x10)
1	0.44 ± 0.15	0.35 ± 0.30
17	0.01 ± 0.01	0.22 ± 0.06
18	0.17 ± 0.05	0.84 ± 0.55

Fig. 3 : The activity of the Lox1 promoter or chimeric promoters containing the Lox1 leader sequence.
A) The Lox1 promoter, with or without its leader sequence, was fused upstream to the GUS reporter gene and introduced into 3 day germinating embryos.
B) The Lox1 leader sequence was inserted between the -90S promoter and the GUS reporter gene and introduced into 3 day germinating embryos, 8 day old leaves, or aleurone tissue of either developing grains (dev. aleur) or 3 days germinating grains (ger. aleur).
C) Chimeric Chi26 promoter-GUS reporter genes, with or without the Lox1 leader sequence were introduced into 3 day germinating embryos or into the aleurone layer of developing grains (dev. aleur).
The relative GUS activity ± SE given for each construct is an average of n independant transient expression assays.

The Lox1 leader sequence contains an embryo-specific element and a general enhancer element

In order to gain additional evidence for the presence of a general enhancer in the Lox1 leader sequence, constructs 15 and 11 were introduced into 8 day old barley leaves. In this tissue, the -90S promoter, construct 11, is constitutively but weakly expressed (Rouster et al., 1997). The presence of the Lox1 leader in construct 15 was also seen to enhance expression of the downstream GUS gene (by 8-fold), in leaf tissue (Fig. 3B).

In order to localize the enhancer or embryo-specific element within the Lox1 leader sequence, a series of deletion/replacements with a random 20 nucleotide sequence [denoted . ΔR(8,27), ΔR(28,47), ΔR(48,67)], were introduced into the leader sequence in the context of the -90S-Lox1(1,68)-GUS construct. These changes did not significantly affect the stability of the transcript leader sequence secondary structure, as seen from their respective ΔG values (Fig. 4). The constructs were introduced into 3 day old embryos, and the resulting GUS activity determined (Fig. 4).

	Construct	Relative GUS Activity		ΔG (kcal/mol)
		Embryos	Leaves (x10)	
-90S-GUS	16	**0.001** ± 0.000	**0.005** ± 0.001	
-90S-Lox1-GUS	15	**0.099** ± 0.043	**0.040** ± 0.007	**-19.7**
-90S-ΔR(8,27)-GUS	19	**0.069** ± 0.019	**0.034** ± 0.003	**-20.6**
-90S-ΔR(28,47)-GUS	20	**0.009** ± 0.008	**0.015** ± 0.003	**-20.2**
-90S-ΔR(48,67)-GUS	21	**0.006** ± 0.004	**0.008** ± 0.002	**-21.3**

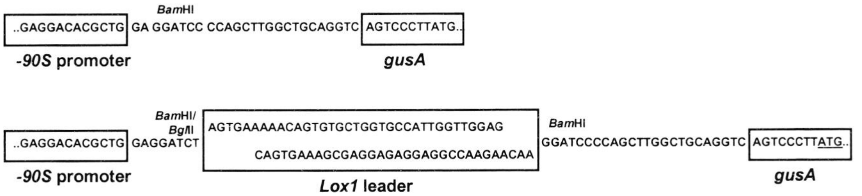


Fig. 4 : The effect of mutations in the Lox1 leader on the activity of a chimeric -90S-Lox1 leader promoter construct. Constructs containing mutated Lox1 leader sequences, cloned between the -90S promoter and the GUS reporter gene, were introduced into 3 day germinating embryos or 8 day old leaves. The relative GUS ± SE activity given for each construct is an average of n independant transient expression assays. The effect of the mutations on the secondary structure of the mRNA 5′ leader was assessed using the program RNAfold of the GCG package and the corresponding energy value is given (ΔG). The sequence used for the ΔG determination, shown beneath, starts with the beginning of the Lox1 leader and mutated forms and ends at the GUS start methionine (ATG) site.

Replacement of the region (8,27) in construct 18, did not greatly reduce promoter activity. However, replacement of the region (28,47, construct 19) or (48,67, construct 20) resulted in a drastic reduction in GUS activity. To be able to differentiate between embryo-specific expression and a general enhancer element within the Lox1 leader, the same constructs were introduced into 8 day old barley leaves. In this case, the level of GUS activity driven by ΔR(48,67) was similar to the GUS activity of the "leaderless" construct -90S-GUS, indicating that nucleotides between 48 and 67 may contribute to a general enhancer function. Construct 19 (-90S- Δ(28,47)-GUS), while barely driving GUS expression in embryos, gave a low level of GUS activity in leaves, suggesting that the nucleotides between 28 and 47 may contain part or all of an embryo-specific element.

A search for sequence motifs similar to these Lox1 leader elements within the Genbank sequence database and for transcription factor binding sites (Transfac 3.2 database) with the Matinspector program (Quandt et al., 1995) did not reveal any homologies to known genes or transcription factor binding motifs.

Enhancer elements within the Lox1 leader may regulate expression at the transcriptional or translational level

The 5′ leader sequences of the spinach PetH, PetE and PsaF genes were all shown to contribute to gene regulation at the transcriptional level, in vivo (Bolle et al., 1994). However, the 5′ leader of the late pollen transcript lat52, regulates gene expression at the translational level, by enhancing the translational yield of a downstream coding sequence in a tissue-specific manner (Bate et al., 1996). Since the role of the Lox1 5′ leader in gene regulation was analysed by transient expression assays, it was not possible to measure its effect on steady-state transcript levels or transcription rates. However, it was possible to examine the effect of the leader sequence on translation efficiency in vitro. The native and mutated Lox1 5′ leader-fragments from constructs 15, 18, 19, and 20 were cloned in front of the GUSpA construct (Michelet et al., 1994), which comprises the GUS coding region fused to a poly(A) stretch of 117A residues at the 3′end, which were transcribed in vitro. The effect of the Lox1 leader sequences on translational activity was investigated in the reticulocyte in vitro translation system (data not shown). While the addition of any of the 5′ leader sequences to the GUS transcript enhanced translation efficiency by (2 - 3 fold, mutations in the 3′ region of the leader had no significant effect on translation. Since the mutations in the Lox1 leader did not greatly increase their potential for secondary structure, which was low in all cases, it is unlikely that the secondary structure of the Lox1 leader can account for enhanced gene expression in vivo. Although we have no evidence that the Lox1 leader regulates gene expression at the translational level, it is possible that tissue-specific translation initiation factors (in the barley embryo) selectively upregulate translation of Lox1 leader transcripts in vivo, as has been proposed for the lat52 transcript (Bate et al., 1996).

An additional feature of translational enhancement is its independence of the upstream promoter, as observed for lat52 (Bate et al. 1996). However, the ability of the Lox1 leader sequence to regulate the GUS reporter gene was clearly influenced by the promoter context, such that GUS expression in embryos was more strongly enhanced by the Lox1 leader in the context of the CaMV -90S promoter than the Chi26 promoter.

In conclusion, our analysis of Lox1 gene expression in barley has revealed a highly complex regulation mechanism, which directs expression in barley grain in a temporal and tissue-specific manner as well as being responsive to both the plant hormone MeJA and to wounding in leaf tissue. The cis-acting sequences which control the different grain-specific and as well as leaf expression patterns of the Lox1 gene have now been localized to the 5′ leader sequence and a MeJA responsive enhancer upstream of the TATA box.

Experimental procedures

Materials

Barley grains (Hordeum vulgare cv. Triumph) were surface-sterilized in 0.1% sodium hypochloride and germinated at 17oC for 3 days in the dark. Embryos were then dissected from the germinating grains for subsequent transient expression experiments. The aleurone layer of 3 day germinated grains was exposed by removing the outer layers (seed coat and pericarp). Developing grains were harvested 24 days after anthesis, from plants grown according to Leah et al., 1994, and subsequently surface sterilized as described above. Leaf tissue was harvested from seedlings grown at 25 oC for 8 days under constant illumination.

DNA and RNA protocols

All DNA manipulations were performed according to standard protocols (Sambrook et al., 1989). The sequences of all DNA constructs were confirmed by dideoxynucleotide chain termination sequencing. Total RNA was prepared from barley embryos or isolated aleurone/endosperm tissue using aurintricarboxylic acid as RNAse inhibitor (Leah and Mundy, 1989). Northern blots (on Hybond N membranes, Amersham) were hybridized with the Lox 1 cDNA probe according to Amersham's protocol. The specificity of the probe for Lox A (Lox 1 transcripts) was confirmed from selective hybridization to Lox A and not Lox B or Lox C cDNA clones (Holtman et al., 1996).

Reporter gene constructs

Lox1 promoter sequences in the 5' Lox1 genomic clone were amplified by PCR with specific linker/primers (30 mers) containing the restriction sites (HindIII 5', BamHI 3'). The fragments were directionally cloned into a BamHI site, 23 nucleotides upstream of the ATG start codon (GGATCCCCAGCTTGGCTGCAGGTCAGTCCCTTATG) of a ß-glucuronidase (GUS) reporter gene, having an rbcS-3C terminator (Fang et al., 1989). 3' end deletions of the Lox1 promoter, synthesized by PCR as HindIII-XhoI fragments, were fused 5' to the 35S A domain (-90/+8)-GUS reporter gene (-90S-GUS) to produce chimeric promoters. To facilitate this fusion, a polylinker was introduced upstream of the 35S A domain, the -90/+8 region was synthesized by PCR using a forward primer containing HindIII, NcoI, KpnI, XhoI, XbaI, SpeI, BglII restriction sites and a reverse primer with a BamHI restriction site. The A domain was then cloned, as a HindIII-BamHI fragment, upstream of the GUS reporter gene. The Lox1 leader sequence was obtained by PCR as a BglII-BamHI fragment and cloned into a BamHI site 23 nt upstream of the translation start in the -90S-GUS (see Fig. 4) or the Chi26(-394/+1)-GUS (Leah et al., 1994). Lox1 leader fragments with internal deletion/replacements were generated by PCR with the deleted replaced fragment included in the primers. The 20 bp replacement sequence, CGGCACTACTAGTGGTATCC, was not related to the Lox1 leader sequences it replaced.

Transient expression and reporter gene assays

Lox1 promoter-GUS reporter genes were mixed in a 2:1 molar ratio with a Ubiquitin-luciferase control plasmid (pAHC18, Bruce et al., 1989), precipitated onto gold particles (~1.6 µm Ø) and introduced into 3 day old embryos by particle bombardment according to Klein et al. (1988). Approximately 1 µg of DNA was delivered in the bombardment of each sample of 6 embryos, or 6 grains from which the husk/pericarp/testa layers had been removed. The tissue samples were then incubated in water for 48 hours at 17oC in the dark and GUS and luciferase activities were then measured as described by Leah et al. (1994). The relative GUS activity was calculated by dividing the GUS fluorescent unit value by the luciferase light value obtained for each sample. Total luciferase light units per sample ranged from 2.104 - 7.105 in germinating embryos, 7.105 - 7.106 in developing aleurone and 2.105 - 6.105 in leaves. GUS and luciferase values are based on five replicate bombardments per construct within each set of transient expression assays.

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Chapter 6

Jasmonate mediated lipoxygenase expression in barley embryonic suspension cultures*

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Abstract

Expression of three distinct lipoxygenase genes (*LoxA*, *LoxB* and *LoxC*) was studied in barley embryo-derived suspension cells (*Hordeum distichum L.* cv. Igri) after cold (4 °C), or methyl-jasmonate, ABA or mannitol treatment. Transcripts corresponding to *LoxB* were present at constant level, whereas transcripts corresponding to *LoxC* encoding barley LOX2 were solely observed in response to methyl-jasmonate. Levels of LoxA transcript encoding barley LOX1 were lower that those of LoxB and LoxC. The jasmonate responsive *RIP60* gene was used as a marker for jasmonate-mediated gene expression. Increase of RIP60 transcripts coincided with increase of LoxC mRNA levels. Lipoxygenase transcript remained constant after treatment with ABA. Extracts of suspension cells converted linoleic acid mainly into 9-HPOD, but extracts of cells treated with MeJA formed mostly 13-HPOD. Western analysis indicated an increase in LOX2-type protein after jasmonate treatment. Our results indicate the presence of a third isoenzyme possibly encoded by the *LoxB* gene in barley.

Chapter 6

1. Introduction

Lipoxygenase (LOX, linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a non-heme iron-containing dioxygenase that catalyses the oxidation of polyunsaturated fatty acids containing a (1*Z*,4*Z*)-pentadiene system to form hydroperoxydiene derivatives. A function for lipoxygenase in plants during development and germination has not fully been established. In cucumber seedling, lipoxygenase activity has been implicated with the mobilization of storage lipids (Feussner et al., 1995a). Storage acyl glycerols can be converted into 13-hydroperox lipids which are subsequently reduced to 13-hydroxy lipids, to be used as a substrate for *b*-oxidation, thereby providing an energy source for seedling growth. Similar data have been obtained with barley, where LOX2 encoded by the *LoxC* gene was shown to be capable of utilizing directly storage lipids as a substrate for hydroperoxidation (Holtman et al., 1997). However, an important function for lipoxygenase in plants involves signal mediation in stress-response through the synthesis of jasmonate (jasmonic acid; JA) or its derivative methyl-jasmonic acid (MeJA) [see for reviews: Creelman and Mullet, 1997; Wasternack and Parthier, 1997]. In general it is assumed that JA is synthezised via the octadecanoid pathway in which the conversion of a-linolenic acid into 13(*S*)-HPOT is directed by a LOX2-type protein (Creelman and Mullet, 1997). JA induces the production of proteinase inhibitor proteins (Peña-Cortés et al., 1993) and several other defence related genes such as thionins (Andresen et al., 1992), ribosomal inactivating proteins (Reinbothe et al., 1994a and 1994b), polyphenol oxidases (Constabel et al., 1995) and phytoalexins (Gundlach et al., 1992). Expression of *Lox* genes during germination has also been associated with the existence of JA in seedlings. Highest levels of JA are found in soybean coleoptiles during early stage of germination, whereas much lower levels are found in the hypocotyl after elongation (Creelman and Mullet, 1995). Similar data were obtained for oat, where JA level also showed a rapid decrease after the initition of germination (Ueda et al., 1994). JA inhibits the IAA-induced cell wall elongation by repressing the synthesis of cell wall polysaccharides in monocotyledons seedlings (Ueda et al., 1994; Miyamoto et al., 1997). Increasing levels of lipoxygenase activity after seed imbibition correlated with increasing levels of JA has been reported for wheat and soybean (Dathe et al., 1994; Creelman and Mullet et al., 1995). Functions for lipoxygenase during germination might be related to involvement in the octadecanoid pathway leading to the synthesis of JA.

From barley, three distinct lipoxygenase encoding cDNAs have been cloned and characterized (Schmitt and Van Mechelen, 1997). However, the existence of only two distinct isoenzymes have been shown in barley grains (Doderer et al., 1992); LOX1 is encoded by the *LoxA* gene while LOX2 is encoded by the *loxC* gene. LOX1 and 2 have been purified and characterized (van Aarle et al., 1992; Doderer et al., 1992; Yang et al., 1993). LOX2 is expressed during early stage of grain development, whereas LOX1 accumulates in late stage of grain development (Schmitt and Van Mechelen, 1997). LOX1 is present in both quiescent and germinating barley kernels, whereas LOX2 is not detectable before 24 h of germination (Holtman et al., 1996). The major difference between the isoenzymes is the product they form when linoleic acid is used as a substrate. LOX1 produces 9-HPOD, whereas LOX2 forms 13-HPOD. Both isoforms show an optimum activity at pH 6.5 (Doderer et al., 1992). The product related to the third gene (*loxB*) has not been identified so far.

Studies of lipoxygenase involvement in mediating JA, ABA or stress signals have been restricted to vegetative parts from barley (Feussner et al., 1995b; Rouster et al, 1997; Vörös et al, 1998). LoxA transcripts have been detected in leaves in response to MeJA and in response to wounding, respectively. No LoxA transcripts were found in response to ABA, drought condition or mildew infection (Rouster et al., 1997). To establish a link between the appearance of multiple lipoxygenase isoforms in barley and a function during plant stress we have subjected embryo-derived barley suspension cells (*Hordeum distichum* L. cv. Igri) to mannitol, yeast extracts (elicitors), low temperature (4 °C) or treated with the plant regulators MeJA and ABA, respectively. Suspension cells are well suited systems for studying the effects of environmental changes and application of hormones on gene expression. Lipoxygenase mRNA levels were studied by northern blots and protein levels were determined by western blots. Lipoxygenase activity was analysed in extracts of suspension cells as a function of pH and product formation.

2. Materials and methods

2.1. Plant material.

Suspension cells derived from immature embryo's of *Hordeum distichum* L. cv. Igri were cultured in 250 ml Erlenmeyer flasks containing 50 ml of liquid A2 medium (Jahne et al., 1991), supplemented with 6% ($^{W}/_{V}$) glucose. Suspension cells were subcultured weekly by transferring 10 ml of the seven-day culture to 40 ml fresh medium. Flasks were incubated on a gyratory shaker (120 rpm) at 25 °C, in the dark. Stress conditions were applied from the third day after refreshment of the medium. Cells were harvested after 4 h, 24 h and 48 h of stress or hormonal treatments and collected in volumes of 4 ml per sample. Cells were treated with mannitol to 15% ($^{W}/_{V}$) or with 100 mM jasmonic acid or with 10 mM ABA, respectively. Untreated cells were used for control. For analyzing the effect of low temperature, one flask containing untreated suspension cells was simultaneously placed in a 4 °C growth chamber.

Grains (*Hordeum distichum* L. cv. Igri) were germinated as described previously (Schmitt and Van Mechelen, 1997). Dry grains (of *Hordeum distichum* L. cv. Igri) were used for extraction without any pretreatment.

2.2. RNA isolation and Northern analysis.

Four ml of suspension culture were harvested in 10 ml tubes, following centrifugation at low speed (500 rpm). Supernatant was discarded. Cells were immediately frozen in liquid nitrogen and homogenized after freezing, using a mortar and pestle. RNA was isolated according to the protocol of Slater (1984). Of each sample 5 mg of total RNA was denaturated by glyoxal and dimethylsulfoxide and separated on an agarose gel (Sambrook et al., 1989). Northern blotting was done on gene-screen-plus membranes. LOX specific probes were prepared from fragments containing 3' untranslated parts of pLoxA, pLoxB and pLoxC, respectively. These short fragments were amplified by PCR from their longer templates following the protocol of Sambrook (1989). After PCR and prior to labelling, fragments of pLoxA (Genbank accession: L35931, nucleotide position: 2655-2818; pLoxB (Genbank accession: L37359 nucleotide: position: 2762-3003) or pLoxC (Genbank accession L37358, nucleotide position: 2656-2910) were purified by Sepharose S-200 spin columns. Other probes used consisted of the complete cDNA of RAB21 from rice (Mundy and Chua, 1988), the 200 bp 3' part of pHv-S61 insert encoding RIP60 (Becker and Apel, 1992) and the complete cDNA of GAPDH (Chojecki, J., 1986). Probe labelling was performed with a random labelling kit (Pharmacia) using 32P-*a*-dCTP, according to the manufacturer's protocol. Labelling efficiency was calculated by liquid scintillation analysis. For each analysis equal activity of 5.10^6 dpm was added to 10 ml hybridisation solution.

2.3. Gel Electrophoresis and Western Blotting.

SDS-PAGE was performed on 12.5% homogeneous gels using the multiphor II System (Pharmacia Biotech, Uppsala, Sweden). After electrophoresis, proteins were transferred from gel onto nitrocellulose membrane by semidry blotting, following the protocol as described by the gel manufacturer (Pharmacia). Simultaneously a gel containing identical samples was used for coomassie staining in order to make relative protein contents loaded on gel comparable. The nitrocellulose membranes were incubated in PBS containing 0.05% Tween 20 plus 1% BSA for 30 min, followed by incubation with LOX specific antibodies for 16 h.. The used monoclonal antibodies were selected on their specificity towards LOX1 (5D2) or LOX2 (5.8) or for their cross-detecting affinity (4.9), respectively (Holtman et al., 1996). Subsequently the membranes were washed 3 times 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 addition of 5-bromo-4-chloro-3-indolyl-posphate/nitro blue tetrazolium. Colour reaction was terminated by washing the membranes with distilled water.

Samples for PAGE were prepared from suspension cells either non-treated or treated with MeJA as described in the section Plant Material, from aleurone cells isolated from grain after 3 days of germination, from total grain after 4 days of germination or from dry grain, respectively.

2.4. Extraction and enzyme activity assays.

Extracts were derived from total grain after 4 days of germination; from dry grain; or from suspension cells, either non-treated or treated with or MeJA for 48 h, respectively. Suspension cells were harvested in 10 ml tubes, following centrifugation at low speed (500 rpm) for 5 min. The supernatant was discarded. During extraction samples were cooled on ice. Crude extracts from grain were prepared by homogenizing four grains in 2 ml of extraction buffer per sample (20 mM Tris-HCL buffer at pH 6.5 (1:10 $^{W}/_{V}$) containing 2 mM sodium azide and 0.5 mM PMSF), using an Ultraturrax for one min (Janke & Kunkel, Staufen, Germany). Approximately 2 ml of cells (pellet fraction) were extracted in 2 ml of extraction buffer. Two ml of each homogenate was centrifuged at 15,000 g for 20 min. The supernatant was used for activity analysis. The substrate solution was prepared by adding 35 ml of linoleic acid (from a stock emulsion of 24 mM) to 5 ml of bidistelled water containing 50 ml of Tween 20. The solution was kept at pH 9.0 by adding 0.2 M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 5.5 or 6.5 by adding 0.2 M HCl, 0.1 M phosphate buffer pH 5.5 or pH 6.5 was added to a total volume of 100 ml, respectively. Lipoxygenase activity was determined spectrophotometrically by adding 50 ml of sample to 2.95 ml of substrate solution. The increase in absorbence at 234 nm was measured for 10 min at 25 °C. The activity was expressed in units (mM min^{-1} mg^{-1} protein) hydroperoxyde formed, 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).

2.5. Hydroperoxide Analysis.

Crude extracts (100 mL) prepared from suspensions or grains as described above, were incubated in 10 ml of 100 mM of oxygen-saturated sodium phosphate (buffered to pH 5.5 or 6.5, respectively) in the presence of linoleic acid (200 mM) for 40 min with gentle agitation at room temperature. The lipoxygenase reaction was stopped by lowering the pH to 3.5. As a control on auto-oxidation, 100 mL of extraction buffer was incubated with linoleic acid substrate solution in each experiment. To each reaction mixture 5 mg of prostaglandin B_2 (Cayman Chemical Company, USA) were added as an internal standard. The products of the lipoxygenase reaction were purified using octadecyl solid-phase extraction columns (J.T. Baker, The Netherlands). The columns were each prewashed with 3 column volumes of ice-cold methanol and 2 column volumes of ice-cold water. The incubation mixtures were passed through the columns. Subsequently, the column was washed with 2 column volumes of ice cold water. Lipoxygenase reaction products were eluted with 2 mL of ice cold methanol.

The products were analyzed by reverse-phase HPLC as previously described (Van Aarle et al., 1991) using a ChromSpher C_{18} column (5 mM, 4.6 x 250 mm, Chrompack [Raritan, NJ]), a UV detector (L-4000, Merck-Hitachi, 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 flow rate of 0.5 mL min^{-1}. The pump used was an Intelligent Inert Pump (L-6210, Merck-Hitachi). For standards, pure fractions of 13-HPOD, 9-HPOD (Cayman Chemical Company, USA) were used.

2.6. Lipoxygenase substrate affinity

To study the substrate affinity of lipoxygenase from suspension cell extracts, LOX activity was measured polarographically by determing the oxygen uptake using a Clark-type electrode (YSI Inc., Yellow Springs, Ohio, USA), using substrate dilutions (from stock working emulsion) of linoleic or a-linolenic acid. Stock emulsions (24 mM) of the fatty acids were dispersed in distilled water containing 1 % Tween-20. Measurements were performed in 200 ml of oxygen-saturated borate buffer (0.2 M boric acid, containing 25 mM HEPES, pH 5.5), which has an oxygen-content of 250 mM at 25 °C. The reaction was started by addition of substrate stock solution.

3. **Results**

3.1. Lipoxygenase mRNA levels in embryo-derived suspension cells subjected to 4 °C, MeJA, mannitol or ABA treatment, respectively.

Lipoxygenase mRNA levels in untreated barley embryo-derived suspension cells (cv. Igri) were compared with cells treated with 4 °C, MeJA, mannitol and ABA by northern analyses (Fig. 1). Treatments of suspension cells was started after three days of medium refreshment in order to minimize possible influence of the delusion event on gene expression.

LoxB transcripts were most abundant in suspension cells (Fig. 1), whereas in untreated cells lipoxygenase transcripts corresponding to the LoxB type were found only. LoxB mRNA level showed some decline in untreated cells during period of cultivation. Subjecting cells to low temperature or MeJA resulted in an increase of LoxB transcript levels. Similar to LoxB transcription levels, GAPDH transcription levels also decreased during the cultivation period under standard conditions, or treated with either mannitol or ABA. Ribosomal probing showed that all samples were loaded with equal amounts of total RNA indicating that repression of mRNA transcription occurred during the period of cultivation under the standard condition as well as under mannitol and ABA treated conditions, respectively. Relatively low levels of LoxA transcripts were observed in suspension culture after treatment with cold and MeJA, whereas LoxC transcripts only occurred after 24 h of MeJA application. The Jasmonate responsive *RIP60* gene (Becker and Apel, 1992) was used as a marker for jasmonate mediated gene expression. Induction of the *RIP60* gene correlated with the occurrence of LoxC transcripts.

No difference in LOX transcription levels could be observed after addition of ABA, whereas ABA induced RAB (*R*esponsive to *AB*A) gene expression occurred. RAB transcripts were also found after treatment with mannitol. These experiments indicate that LOX expression in embryo-derived suspension cells is not significantly induced by ABA or mannitol, whereas all three *Lox* genes were more or less found to be induced by application of MeJA. The fact that RIP60 transcript levels were solely detected after MeJA treatment suggests that the other tested conditions probably did not involve a rapid intracellular jasmonate increase.

Previous reported studies done with *Eschscholtzia californica* suspension cells did show a rapid increase of JA in response to yeast extract and fungal elicitor, respectively (Blechert et al., 1995; Mueller et al., 1993). The increase of intracellular JA concentration resulted in expression of several JA responsive genes. Similar responses after addition of fungal elicitor

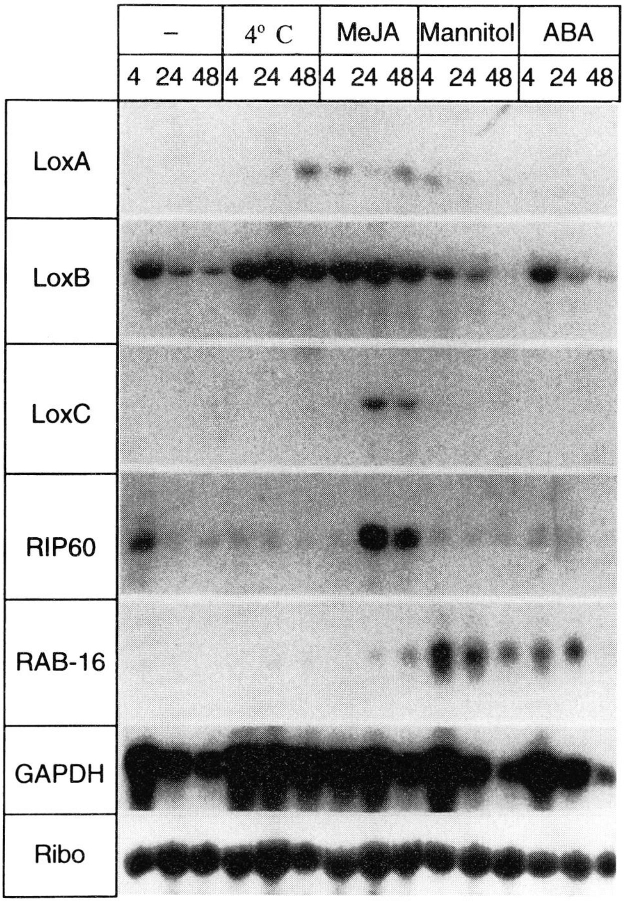

Fig. 1. LoxA, LoxB, LoxC, RIP60, RAB16 and GAPDH mRNA levels in barley embryo derived suspension cells (*Hordeum distichum* L. cv. Igri) after standard treatment, 4° C or incubated in solutions containing MeJA, Mannitol or ABA. Suspension cells were subjected to the different conditions in the dark and harvested after 4, 24 or 48 h, respectively, indicated. A 28S-ribosomal probe was used for control on equal loading.

have been reported for tobacco cell suspension (Rickauer et al., 1997). The embryo-derived barley suspension cells were tested for the ability to cause a similar response by addition of yeast extracts (0.5% $^{W}/_{V}$). Addition of yeast extracts did not lead to a significant induction of RIP60 nor to significant response of one of the *Lox* genes (data not shown), suggesting that jasmonate and lipoxygenase are here not involved in such a form of stress-signalling .

3..2. Lipoxygenase protein levels increase in response to MeJA.

Lipoxygenase protein levels were studied by immunoblot analyses, done with 3 monoclonal antibodies. One Mab (4.9) recognized both previously characterized lipoxygenase isoforms from barley grain, whereas the two other Mabs have pronounced specificity towards either LOX1 (Mab 5D2) or LOX2 (Mab 5.8), respectively (Holtman et al., 1996). Extracts derived from suspension cells, either untreated or MeJA-treated were compared with extracts derived from germinated aleurone tissue, germinated grains and dry (quiescent) grains, respectively. With these antibodies proteins with a molecular mass of approximately 90 kDa were detected in suspension cells and in the control grain samples (Fig. 2.). No LOX protein could be detected in aleurone of germinating grain.

Strongest reactivity was observed with the cross-recognizing antibody (Mab 4.9). Especially untreated suspension cells showed a relatively strong reactivity with the cross-reacting antibody (Mab 4.9). Comparison with the grain samples indicate that the other two antibodies with pronounced specificity towards LOX1 or LOX2 have low reactivity with extracts of untreated suspension cells. Results indicate that Mab 4.9 may also recognize a distinct lipoxygenase isoform in embryo-derived suspension cells.

After MeJA addition an increase of lipoxygenase protein levels was detected with all three distinct antibodies, although again the relatively strongest increase in protein level was observed with Mab 4.9. Taken together the observed increasing amounts of LOX mRNA transcript levels (Fig. 1) and the increasing amounts of LOX protein levels in response to MeJA, suggests the *de novo*-synthesis of LOX1 and LOX2 (Fig. 2).

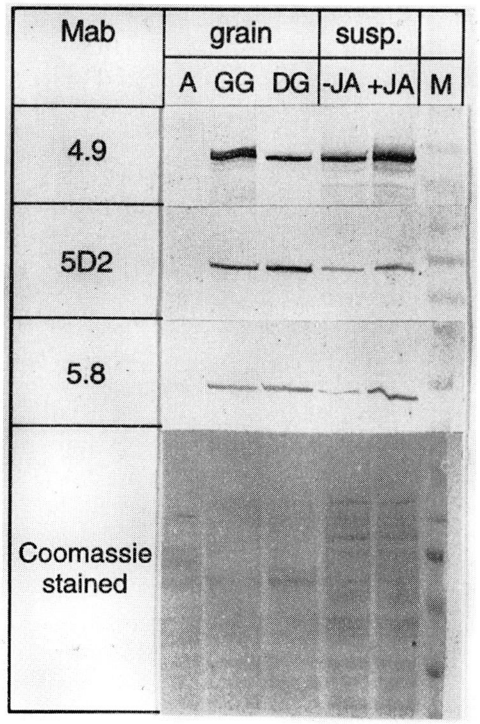

Fig. 2. Western blot analyses and coomassie stained SDS-PAGE gel from barley (*Hordeum distichum* L. cv. Igri): aleurone of 3 days germinating grain`(A); total germinating grain after four days of germination (GG); total dry grain (DG); and embryo derived suspension cells either non-treated (-JA) or MeJA treated (+JA) for 48 h. Protein levels were determined using three distinct Mabs specific for barley lipoxygenase. Mab 4.9 have high specificity for both barley lipoxygenase isoforms (LOX 1 and LOX 2). Mab 5D2 has a pronounced specificity towards barley LOX1. Mab 5.8 has pronounced specificity towards barley LOX2. The coomassie stained gel was used to total compare the amounts of protein loaded per sample on gel.

3.3. Lipoxygenase activity in embryo-derived suspension cells compared with activity in grains.

Lipoxygenase activities were measured in extracts from germinated grain, dry grain and in embryo-derived suspension cells. To compare LOX-activities in grain with activities in suspension cells, total activities were expressed per mg protein. Total lipoxygenase activity in suspension cells showed a distinct pH optimum of 5.5 (Fig. 3). Addition of MeJA to suspension cells did not change the pH optimum for activity. Moreover, the total activity measured in suspension cells did not increase after addition of MeJA. The pH profile found in suspension cells is different from the pH profile found for dry grains and germinating grains, respectively. From both grain-extracts, activities were measured in a broad pH range from 5.0 to 6.5 (Fig. 3).

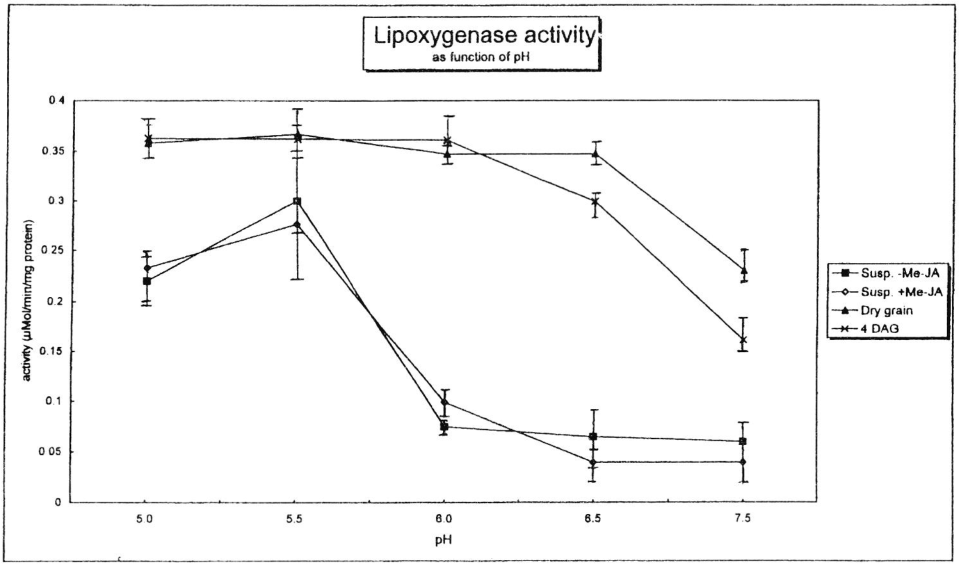

Fig. 3. Lipoxygenase activity as function of pH. Extracts were prepared from suspension cells either non-treated or treated with MeJA for 48 h; dry grain; grains after 4 days of germinating. Activity values are expressed in units per mg total protein. Each data point is the mean of three independent experiments.

To analyse the effect of MeJA on the product specificity of lipoxygenase isoenzymes we have determined the hydroperoxides formed in extracts of barley suspension cells after incubation for 48 h with or without MeJA (Fig. 4). Linoleic acid was used to determine the ratio of 9-HPOD to 13-HPOD. The main product formed in extracts of suspension cells without MeJA addition consisted of 9-HPOD. Addition of MeJA to suspension culture resulted in formation of mostly 13-HPOD. The K_m value for the lipoxygenases from crude suspension extracts (without MeJA treatment) was determined to be 50 (+/-12) mM and 120 (+/- 40) mM for linoleic and α-linolenic acid, respectively.

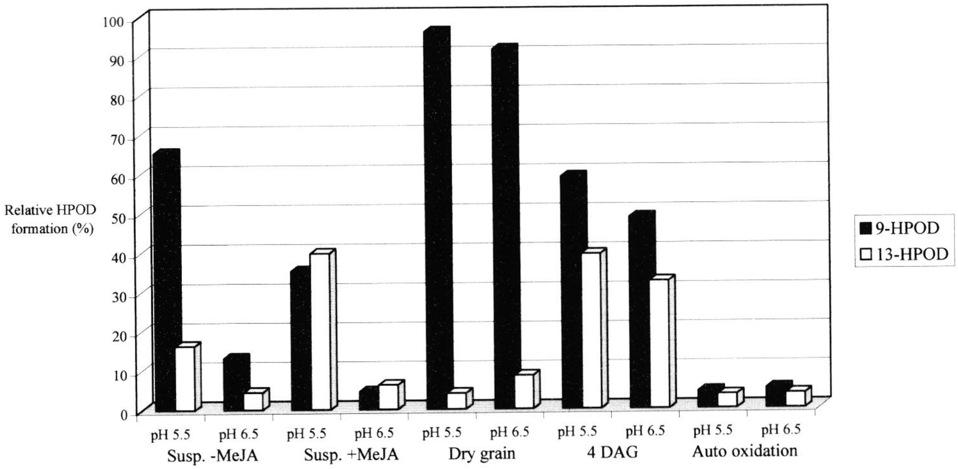

Fig. 4. Relative proportion of 9- and 13-HPOD formed from linoleic acid by extracts from suspension cells either non-treated or treated with MeJA for 48 h; dry grain; and grains after 4 days of germination.

4 . Discussion

The occurrence of distinct lipoxygenase isoenzymes in barley embryo derived suspension cells was studied under varying stress-related conditions on the level of mRNA.

The presence of LoxB transcript levels occurred in all tested conditions, while transcripts corresponding with LoxA and LoxC were not detected under standard cultivation conditions or after ABA treatment or mannitol treatment. Subjecting cell cultures to 4 °C resulted in induction of LoxA. From cultures treated with MeJA transcripts corresponding to all three Lox types were observed (Fig. 1). The *LoxC* gene showed strongest response to MeJA. Expression of three *Lox* genes has previously been shown to be most abundant during germination (Holtman et al., 1996). However, only the occurrence of two isoenzymes, namely LOX1 and LOX2 has been described for developing barley grains and germinated grains (Doderer et al., 1992; Holtman et al., 1996; Schmitt and Van Mechelen, 1997). LOX1 and LOX2 are encoded by LoxA and LoxC, respectively (Holtman et al., 1996; Schmitt and Van Mechelen, 1997).

Western analysis indicates the presence of distinct lipoxygenases in suspension cells (Fig. 2). Remarkably relatively strong reactivity was observed with Mab 4.9, an antibody, which recognizes both lipoxygenase isoenzymes LOX1 and LOX2 (Holtman et al., 1996). The reactivity found in suspension extracts with antibodies, which have pronounced specificity towards either LOX1 (Mab 5D2) or LOX2 (Mab 5.8) were relatively less strong if compared with extracts from grain, indicating that LOX1 and LOX2 are here not representing the majority of LOX-isoenzymes.

The strong reactivity found for Mab 4.9 (LOX1 and LOX2 cross-detecting Mab) can be explained by the presence of a third LOX protein, encoded by the *LoxB* gene. Previous analysis showed the presence of LoxB transcripts in barley, especially during germination (Holtman et al., 1996). Since, the presence of a third isoenzyme has not been demonstrated in germinating barley, it can not be excluded that a third isoenzyme contains biochemical properties very similar to one of the other LOX-isoenzymes making it difficult to be discriminated. The third isoenzyme might as well be present during germination as could be concluded from the presence of significant levels of LoxB transcripts during germination (Holtman et al., 1996; Chapter 3, p 67). Taken together the results obtained by northern and western analysis, we conclude that a third isoenzyme must be present in suspension cells.

In order to obtain more information about the characteristics of the LOX present in suspension cells, we compared LOX activities from suspension culture extracts (with and without MeJA treatment) with those of dry grains and germinating grains. The activity in suspension cells showed a narrow pH optimum of 5.5 (Fig. 3). Addition of MeJA to suspension cells did not alter the pH optimum for activity. In both grain extracts activities were measured in a broad pH range from pH 5.0 to pH 6.5, which is in agreement with results previously obtained with purified LOX1 preparations (Doderer et al., 1992). Different from LOX1, purified LOX2 was active in a narrow pH range with an optimum of 6.5 (Doderer et al., 1992). The total LOX activity in suspension extracts was at least three to five fold lower at pH 6.5 than at pH 5.5. The relative low levels of LOX1 and LOX2, as detected by western analyses with the isoenzyme specific Mabs LOX1 [5D2] and LOX2 [5.8], indicates the presence of the third isoenzyme. Thereby, the pH optimum for LOX activity of 5.5 found for suspension extracts, shows that the LOX which is most abundantly present in suspension culture could neither be LOX1 nor LOX2 (Doderer et al., 1992).

Addition of MeJA to suspension culture resulted in induction of distinct LOX transcripts (Fig. 1) and proteins (Fig. 2), whereas the pH optimum for activity did not alter (Fig. 3). If addition of MeJA would have resulted in the induction of LoxC and subsequently in synthesis of LOX2 in suspension cells, a shift in pH-activity profile might be expected since the optimum activity for LOX2 purified from grain was determined at pH 6.5. Remarkably, the observed increase in Lox transcript and LOX protein levels on western blots after addition of MeJA (Fig. 2) did not result in an increase of total LOX activity (Fig. 3).

Furthermore, we analysed the hydroperoxides formed in product formation assays with the use of linoleic acid as substrate. In untreated cell suspension almost all the formed hydroperoxides consisted of 9-HPODs (Fig. 4). Addition of MeJA to suspension cells resulted in a shift of product ratio towards 13-HPOD. The observed change in product formation can be explained by the synthesis of LOX2 in response to MeJA, since induction of LoxC and synthesis of LOX2 could be concluded from northern and western blot analysis, respectively. The *LoxC* gene product (LOX2) mainly forms 13-HPOD after linoleic acid incubation (Doderer et al., 1992). LOX2 can also form 13-HPOT if α-linolenic acid is used as a substrate, which enables involvement of LoxC in the biosynthesis pathway leading to jasmonate (Holtman et al., 1996). It should be noted that addition of MeJA did result in the presence of LoxC as well as RIP60 (JA responsive gene) in a similar fashion. However, why would a signal molecule like JA trigger *de novo* synthesis of an enzyme (LOX2) which is involved in the biosynthesis of the signal molecule itself.

In conclusion, we showed that LOX of barley embryo derived suspension cells, have enzyme properties different from those found in developing and germinating grains. The enzyme properties found in suspension extracts and the relatively strong reactivity of the LOX-cross recognizing antibody (Mab 4.9) indicates the presence of a third LOX protein, probably encoded by the LoxB gene. Characterization of recombinant LoxB protein, expressed in *E.coli,* will provide a good tool to determine more of the biochemical properties of the third isoform.

Furthermore, in barley embryo derived culture cells *Lox* genes were induced by MeJA, whereas the other tested conditions had hardly any or no effect on Lox expression. The weak response of those *Lox* genes to the other subjected conditions suggests that those genes are not directly involved in stress-signal mediation of suspension culture cells. The altered product formation in response to MeJA and the relatively strong responsiveness of LoxC to MeJA, suggests the *de novo*-synthesis of LOX2. However, the difference in pH optimum for activity of 5.5 found in suspension, compared with the optimum found for purified LOX2 of 6.5 are contradictory. The observed differences in pH optimum for activity might be explained by being a result of differences in purification rate of the used extracts (Doderer et al., 1992). Purification of the LOX from suspension culture will be a next step to obtain more information about the exact nature of the protein.

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Summary

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12, LOX) are non-heme iron enzymes which catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids containing a (1*Z*-4*Z*)-pentadiene system to form optically active fatty acid hydroperoxides. In plants, these hydroperoxides are subsequently converted into a series of significant products, including plant hormones e.g. jasmonic acid (JA).

In **Chapter 1** mainly an overview of the biological functions of plant lipoxygenases is presented. The presence of multiple lipoxygenase isoenzymes has been reported for many plant species. Lipoxygenase isoenzymes have been characterized and their involvement in diverse physiological processes has been demonstrated. This chapter emphasizes the structural features of lipoxygenase and the current state of biological characterization in plants obtained with molecular and biochemical technologies. Lipoxygenase is also expressed at high levels in barley grain, although the importance of the expression during development and germination has not been established as yet. On the other hand, one of the presumed unfavourable aspects of lipoxygenase in economically important food-crops is the possible involvement in the formation undesired substances, because of unpleasant taste characteristics. The aim of this thesis was to obtain information of the number of lipoxygenase isoenzymes appearing in barley grain and to study the physiological functions of distinct isoenzymes by a combination of molecular and biochemical analyses.

In **Chapter 2** the isolation and characterization of a barley (*Hordeum distichum* cv. L. Triumph) lipoxygenase (LoxA) cDNA from grain is described. The deduced primary structure is presented. It includes a 5' untranslated region of 69 nucleotides, an open reading frame of 2586 nucleotides encoding a protein of 861 amino acid residues and a 3' untranslated region of 142 nucleotides. The molecular mass of the encoded polypeptide was calculated to be 96.440 Da with an isoelectric point of 5.96. Its amino acid sequence shows a high homology with that of other plant lipoxygenases identified to date.

In **Chapter 3** the cloning of two additional full length lipoxygenase cDNA sequences (LoxB and LoxC) is described. The cDNAs were obtained from barley (*Hordeum distichum* cv. L. Triumph) grains. These cDNAs share high homology with the barley LoxA cDNA. Southern blotting experiments show that each corresponding gene is present in a single copy per haploid genome. RFLP mapping confirms the presence of single lipoxygenase loci and shows that *LoxA* and *LoxB* are located on chromosome 4, while *LoxC* is located on chromosome 7. Two isoenzymes, lipoxygenase 1 and 2 have been purified previously from germinating barley and characterized. Lipoxygenase 1 is encoded by *LoxA*, while lipoxygenase 2 is most likely encoded by *LoxC*. The product related to the third cDNA (*LoxB*) has not been identified so far, suggesting a low protein abundance for the corresponding isoform in barley. Steady state levels of these lipoxygenase transcripts have been studied in diverse plant tissues. Transcripts corresponding with these lipoxygenase genes are predominantly observed in grain and/or in seedling. Moreover, transcripts corresponding to *LoxB* and *LoxC* are also observed in mature vegetative tissue. No lipoxygenase mRNA could be detected in aleurone layer of germinating grains. Furthermore, a possible involvement of lipoxygenase in the occurrence of grain dormancy was evaluated, using two barley varieties having differences in susceptibility for

induction of dormancy. Lipoxygenase expression was examined in developing grains from both varieties, grown under climate conditions known to be stimulating or eliminating the occurrence of dormancy. No significant differences in lipoxygenase mRNA levels were observed in developing grains grown under dormant or non-dormant conditions, suggesting that lipoxygenase is not directly involved in induction of grain dormancy.

In **Chapter 4** the expression of lipoxygenase genes in developing barley grains from 5 days after flowering (DAF) to the fully mature grain stage is described. Lipoxygenase shows two distinct peaks of activity. The first peak of lipoxygenase activity occurs at the early stages of grain development, from 5 until 20 DAF, whereas the second peak occurred at the later stages, from 30 DAF to full grain maturity. The temporal expression of both LOX1 (*LoxA*) and LOX 2 (*LoxC*) was studied at the protein level and mRNA level using specific probes. Furthermore, grain extracts were incubated with linoleic acid to analyze the enzyme specificity of LOX during grain development. These experiments showed that LOX 2 is present at early stages of development and that it is located in the envelopes of the grain, whereas LOX 1 accumulates in the embryo during later stages of development. Additionally, the spatial and temporal pattern of hydroperoxide-metabolizing activity is shown to be comparable with that of lipoxygenase activity. In conclusion, two lipoxygenases appear during barley grain development, differing in their spatial and temporal expression. The two enzymatic pathways give rise to a distinct set of end-products indicating that the lipoxygenases may play different physiological functions during barley grain development.

In **chapter 5** identification of *cis*-regulatory elements involved in embryo-specific expression of the *LOX1* (*LoxA*) gene is described. The barley lipoxygenase 1 gene is predominantly expressed in embryos during grain development and germination. Nevertheless, after methyl jasmonate (MeJA) treatment the *LOX 1* gene is also found to be expressed in leaves. The upstream *cis*-regulating element involved in MeJA regulation has previously been identified. From those transient expression assays in germinating barley embryos information was obtained on the presence of *cis*-regulatory elements involved in embryo-specific expression of the *Lox 1* gene. Analysis of transcriptional or translational fusions between *LOX 1* 5' upstream sequences and the *gusA* reporter gene indicated that the 5'-untranslated leader sequence was involved in embryo-specific expression. Replacement of the leader sequence from the aleurone-specific *Chi26* gene with the *LOX 1* leader sequence resulted in a chimeric gene expressed at high levels in embryo as well as in aleurone cells. Insertion of the *Lox 1* leader sequence between the 35S minimum promoter (A domain -90/+8) and the *gusA* reporter gene greatly enhanced promoter activity in a tissue-specific manner. *In vitro* transcription/translation assays of *LOX 1* leader *gusA* gene constructs showed a moderate effect (approx. 2.5 fold increase) on protein synthesis. Deletion/replacement analysis of the *LOX 1* leader sequence combined with transient expression in germinating embryos and *in vitro* ranscription/translation assays suggest that the *LOX 1* leader sequence contains *cis*-elements involved in qualitative (tissue-specific) and quantitative gene expression.

In **chapter 6** the temporal expression of three distinct lipoxygenase genes (*LoxA*, *LoxB* and *LoxC*) in barley embryo-derived suspension cells (*Hordeum distichum* L. cv. Igri) is presented. Cells were subjected to treatment with 4 °C (cold), methyl-jasmonate (MeJA), ABA or mannitol. It is shown that the lipoxygenase genes were most responsive to MeJA, while the other tested conditions had hardly any or no effect on lipoxygenase expression. The lack of response of those *LOX* genes to the other subjected conditions suggests that those genes are not directly involved in stress-signal mediation in embryo-derived suspension cells. Thereby, only transcripts corresponding with *LoxB* were basically observed. A relative strong reactivity of a LOX-cross recognizing antibody (Mab 4.9) in non-MeJA-treated suspension cells indicates the presence of a third LOX protein, most likely encoded by the *LoxB* gene.

Extracts of suspension cells incubated with linoleic acid mainly generated 9-hydroperoxydes, but after MeJA addition the product formed consisted mostly of 13-hydroperoxydes, which is the main product generated by LOX 2. Western analysis indicate an increase in lipoxygenase content after jasmonate treatment, although alteration in total lipoxygenase activity is not observed. Lipoxygenase activity determined from suspension extracts show a narrow pH-optimum of 5.5, which differs significantly from the optima found for the previously characterized LOX 1 and LOX 2 isoenzymes from germinating barley. Although the lack of activity in suspension extracts at pH 6.5 is in contrast with the previously determined activity optimum for LOX 2, the observed expression of *LoxC* together with an increasing reactivity with LOX-Mabs and the 13-HPOD formation suggests the *de novo*-synthesis of a LOX-2-type enzyme after MeJA treatment in embryo-derived suspension cells.

Samenvatting

Lipoxygenases (LOXs, EC 1.13.11.12) zijn ijzer bevattende enzymen, die meervoudig onverzadigde vetzuren omzetten in chirale *Z,E* geconjugeerde vetzuurhydroperoxides. Deze hydroperoxides vormen intermediairen die langs verschillende enzymatische routes kunnen worden omgezet in een scala stoffen, waaronder het planten hormoon jasmonzuur. Lipoxygenase genen uit verschillende plantensoorten vertonen onderling regio's met hoge homologie. De hoge mate van conservering in genetische samenstelling van deze regio's duidt op een voor de plant essentiële enzymfunctie. De biologische functie van lipoxygenases in planten is slechts in beperkte mate bekend. LOX activiteit wordt bijvoorbeeld sterk geïnduceerd in kiemende zaden, maar een biologische functie voor het enzym tijdens ontwikkeling en kieming ontbreekt. In planten bestemd voor consumptie kunnen de produkten van LOX de kwaliteit ongunstig beïnvloeden. Inzicht in de regulatie van LOX kan daarop uiteindelijk leiden tot een kwaliteitsverbetering van plantaardige consumptiegoederen. Granen, zoals gerst, bevatten veel lipoxygenase. Kennis van *LOX* genen levert een hulpmiddel voor het zoeken naar de functies van LOX in gerstenkorrels.

In dit proefschrift wordt onderzoek beschreven over de identificatie en karakterisering van een aantal lipoxygenase enzymen uit gerstenkorrels. De klonering en expressie van een aantal *LOX* genen wordt beschreven en in relatie gebracht met produktspecificiteit van de verschillende LOX enzymen in gerstenkorrels en in jonge gerst zaailingen.

In **hoofdstuk 1** wordt een literatuur overzicht gegeven over biochemisch en moleculair onderzoek dat is uitgevoerd aan lipoxygenases uit planten. Beschreven worden de verschillende typen LOX die kunnen voorkomen in cytosol of chloroplasten en hoe verschillende LOX activiteiten van elkaar onderscheiden kunnen worden. Hierbij worden de biologische functies van lipoxygenases behandeld. Het meest bekend is de betrokkenheid van LOX in de biosynthese van het plantenhormoon jasmonzuur. De rol van lipoxygenase bij de biosynthese van jasmonzuur, alsmede de diverse functies van jasmonzuur worden beschreven.

In **hoofdstuk 2** wordt de klonering van een volledige lipoxygenase cDNA sequentie (LoxA) uit gerst beschreven. Het 2586 nucleotiden tellende LoxA transcript bevat een 5' onvertaald uiteinde van 69 nucleotiden (de zgn. leader) en een 3' onvertaald uiteinde van 142 nucleotiden. Het vertaalde gedeelte codeert voor een eiwit van 96.4 kDa dat hoge homologie vertoont met DNA sequenties van genen coderend voor cytosolische lipoxygenases uit heterologe plantensoorten.

In **hoofdstuk 3** wordt de isolatie van nog twee andere complete cDNA sequenties (LoxB en LoxC), eveneens afkomstig uit gerstenkorrels, beschreven. Beide cDNA sequenties vertonen hoge homologie met LoxA en coderen tevens voor cytosolische lipoxygenases. De drie *Lox* genen worden nader gekarakteriseerd en Southern hybridisaties hebben aangetoond dat deze lipoxygenase genen met één genkopie per haploïd genoom in gerst aanwezig is. Met behulp van RFLP mapping is vastgesteld dat de genen coderend voor *LoxA* en *LoxB* op chromosoom vier voorkomen, terwijl het gen coderend voor *LoxC* op chromosoom zeven gelokaliseerd is.

In eerder onderzoek zijn twee lipoxygenase isoenzymen geïdentificeerd uit kiemende gerstenkorrels, te weten LOX1 en LOX2. Door middel van peptide analyse en expressie van partiële LOX cDNA fragmenten in *E.coli* is bepaald dat LoxA codeert voor LOX1 en dat LoxC codeert voor LOX2. Voor het derde cDNA (LoxB) kon geen corresponderend eiwit aangetoond worden. Mogelijk komt dit genprodukt in geringe hoeveelheid voor in gerstenkorrels en zaailingen of mogelijk is het eiwit zeer instabiel waardoor het in gerst

extracten moeilijk te detecteren is. Uit northern blot analyse blijkt dat expressie van deze drie lipoxygenase genen hoofdzakelijk in gerstenkorrels en zaailingen optreedt. Vooral in scutellum en embryo cellen worden relatief veel lipoxygenase transcripts waargenomen, in tegenstelling tot aleuron cellen waarin geen lipoxygenase transcripten zijn waargenomen. Onderzocht is of er een relatie bestaat tussen lipoxygenase expressie in gerstenkorrels en de aanwezigheid van het verschijnsel dormantie (kiemrust). Hiertoe werden gerstenkorrels van granen met een verschillende gevoeligheid voor dormantie onderzocht op de aanwezigheid van lipoxygenase transcript. Er werden echter geen significante verschillen gevonden tussen de verschillende soorten, waardoor verondersteld kan worden dat lipoxygenase het voorkomen van graandormantie niet beïnvloedt.

In **hoofdstuk 4** wordt de activiteit van lipoxygenases tijdens de zaadontwikkeling beschreven. Gedurende de ontwikkeling werden twee stadia met verhoogde lipoxygenase activiteit waargenomen. De eerste piek werd tussen 5 en 20 dagen na bloei gemeten, terwijl de tweede piek vanaf dag 30 tot en met het volledig volwassen gerstenkorrel stadium (ca. 70 dagen na bloei) gemeten werd. De expressie van *LoxA*, *LoxB* en *LoxC* en de hoeveelheid LOX1 en LOX2 eiwit werden gedurende de korrel ontwikkeling gevolgd door respectievelijk northern en western analyse. LoxC transcript en LOX2 eiwit werden vroeg in de gerstenkorrel ontwikkeling, specifiek in graan envelop, waargenomen. LoxA transcript en LOX1 eiwit werden laat in de gerstenkorrel ontwikkeling waargenomen in het embryo. Het *LoxB* gen kwam niet tot expressie tijdens de korrelontwikkeling. Met behulp van substraat incubatie experimenten werd de specificiteit van de LOX activiteit nader gekarakteriseerd. Linolzuur incubatie van extracten, afkomstig uit gerstenkorrels tijdens de vroege ontwikkeling, resulteerde in vorming van 13-hydroperoxides. In extracten uit het late ontwikkelingsstadium werden overwegend 9-hydroperoxides waargenomen. De gevormde hydroperoxide produkten zijn in overeenstemming met waarnemingen uit eerder onderzoek, uitgevoerd met gezuiverde LOX1 en LOX2, respectievelijk. Tevens bleek het voorkomen van hydroperoxide metabolisme te correleren met het voorkomen van lipoxygenase activiteit. De verschillen in lipoxygenase activiteit en in produkt specificiteit welke optreden tijdens de graanontwikkeling suggereren dat de LOX isoenzymen verschillende fysiologische functies gedurende de ontwikkeling uitoefenen.

In **hoofdstuk 5** wordt de embryo specifieke regulatie van de LoxA promoter activiteit nader onderzocht m.b.v. promoter deletie studies. De promoter blijkt een cis-regulatoir element te bevatten dat embryo specifieke expressie reguleert.

Uit eerder onderzoek is gebleken dat *LoxA*, naast graan specifieke expressie, tevens in bladeren tot expressie komt na behandeling met jasmonzuur. In de LOX1 promoter werden regulatoire sequenties gevonden die betrokken zijn bij inductie door jasmonzuur. Naast deze jasmonzuur responsieve gebieden werden ook aanwijzingen gevonden voor de aanwezigheid van cis-regulatoire elementen in het 5'-onvertaalde gedeelte van LoxA (in de zgn. leader sequentie), welke betrokken zijn bij de regulatie van embryo specifieke expressie. Dit werd o.a. aangetoond door gebruik te maken van de aleuron specifieke *Chi26* promoter. Door uitwisseling van het 5'-onvertaalde leader gedeelte van de *Chi26* promoter met het 5'-onvertaalde LoxA leader gedeelte (in een p*Chi26-gusA*-fusie construct), ontstond een chimeer genconstruct dat zowel in embryo cellen en in aleuron weefsel expressie vertoonde.

Insertie van de 5'-onvertaalde LOXA leader tussen een verkorte 35S-CaMV (-90S) promoter fragment en het *gusA*-gen leidde tot een construct dat een verhoogde GUS expressie in embryo cellen vertoonde. Experimenten met DNA constructen die mutaties in de 5'-onvertaalde LOXA leader bevatten, bevestigden de aanwezigheid van cis-regulatoire elementen voor embryo specifieke expressie.

In **hoofdstuk 6** wordt de expressie van LoxA, LoxB en LoxC onder verschillende stress-omstandigheden in embryo cel suspensie kweken beschreven. De suspensie culturen werden behandeld met respectievelijk, jasmonzuur, abscisine zuur, mannitol of onderworpen aan cultivatie bij 4 °C. Slechts behandeling met jasmonzuur leidde tot een tijdelijke inductie van lipoxygenase gen aktiviteit. Echter een verhoogde totale LOX enzym activiteit kon onder deze condities niet aangetoond worden. Vergelijking van de LOX activiteit in celkweek en in gerstenkorrel extracten laten een verschil zien in enzym specificiteit. Het optimum voor LOX activiteit in suspensie kweek extracten, werd gemeten bij pH 5.5, terwijl bij extracten van dröge en kiemende gerstenkorrels de activiteiten over veel bredere pH trajecten, beiden van 5.0 tot 6.5, gemeten werden. In suspensie kweek extracten werden na linolzuur incubatie voornamelijk 9-hydroperoxides als reactie produkt gevonden, maar na behandeling van suspensie kweek met jasmonzuur werden overwegend 13-HPOD gevonden. De resultaten suggereren dat *LoxC* in embryo suspensie cellen door jasmonzuur wordt geïnduceerd. Het pH-optimum voor activiteit van 5.5 is echter niet in overeenstemming met eerdere resultaten verkregen uit experimenten welke werden uitgevoerd met gezuiverd LOX 2 uit gerstenkiemen, waarbij een pH optimum van 6.5 gevonden werd.

Tevens bleek uit northern blot dat het LoxB transcript, als enige LOX transcript, met een basaal niveau in embryonale gerst suspensie cellen tot expressie komt. Ook werd uit western blot van suspensie kweek extracten proportioneel veel LOX reactivitiet waargenomen met een kruisreagerend antilichaam (Mab 4.9). Deze resultaten suggereren de aanwezigheid van een derde LOX isoenzym dat door LoxB wordt gecodeerd.

Curriculum Vitae

Johannes Reinier van Mechelen werd op 28 augustus 1957 geboren te Amsterdam, alwaar hij ook opgroeide. Het diploma HAVO werd in 1977 behaald aan de Gemeentelijkeschool voor HAVO te Amsterdam.

In datzelfde jaar is hij met een opleiding tot leraar begonnen aan de lerarenopleiding Vrije Leergangen verbonden aan de Vrije Universiteit te Amsterdam. In 1982 studeerde hij af in de vakken Biologie (tweede graad), Kunstgeschiedenis (derde graad) en Tekenen (derde graad). Vervolgens studeerde hij aan de Vrije Universiteit te Amsterdam Biologie. Tijdens zijn doctoraal periode werd het hoofdvak gevolgd aan de vakgroep Moleculaire Genetica (Prof. Dr. J.N.N. Mol), alwaar hij onderzoek heeft gedaan aan de ontwikkeling van planttransformatie methoden met behulp van cocultivatie met *Agrobacterium tumefaciens*. Daarnaast heeft hij zich bezig gehouden met zgn. sitedirected mutagenese op DNA sequenties van het Nopaline Synthase gen uit *A. tumefaciens*. Een bijvak werd gevolgd aan de vakgroep Moleculaire Microbiologie (Prof. Dr. F.K. de Graaf), waar hij de invloed van chemische modificaties op adhesinen van *E.coli* bacterien onderzocht. Een tweede bijvak werd uitgevoerd aan de afdeling Experimentele Oncologie van het Nederlands Kanker Instituut te Amsterdam (Prof Dr. P. Borst), waar hij moleculair onderzoek heeft verricht naar tumorprogressie van lymphoma's.

Na te zijn afgestudeerd kwam hij in 1988 in dienst van TNO-Voeding aan de Afdeling Plantenbiotechnologie dat onderdeel uitmaakt van het Centrum voor Fytotechnologie in samenwerkingsverband met de Rijksuniversiteit te Leiden. Hij was tot medio 1997 werkzaam als moleculair bioloog aan projecten ter verbetering van land en tuinbouw gewassen. Tijdens dit dienstverband werden de werkzaamheden verricht die tot dit proefschrift hebben geleid.

Vanaf maart 1998 tot heden is hij in dienst bij de stichting Technology Consultancy Center van het Academisch Opleidings Centrum (RUG). Hij is vandaar gedetacheerd bij ORIGIN Nederland B.V. te Barneveld, alwaar hij momenteel een opleidingstraject tot systeem analist volgt.