MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF THREE LIPOXYGENASES IN MAIZE

A Dissertation

by

ANDRIY NEMCHENKO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Michael Kolomiets
Committee Members,	Hisashi Koiwa
	Won Bo Shim
	James Starr
Head of Department,	Dennis Gross

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ABSTRACT

Molecular and Biochemical Characterization of Three Lipoxygenases in Maize.

(August 2006)

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Chair of Advisory Committee: Dr. Michael Kolomiets

Most plant oxylipins, a large class of diverse oxygenated polyunsaturated fatty acids and their derivatives, are produced through the lipoxygenase (LOX) pathway. Recent progress in dicots has highlighted the biological roles of oxylipins in plant defense responses to pathogens and pests. In contrast, the physiological function of LOXs and their metabolites in monocots is poorly understood. We cloned and characterized three maize LOXs *ZmLOX10* *ZmLOX11* and *ZmLOX12*. Both ZmLOX10 and ZmLOX11 apeared to be 13-LOX, whereas ZmLOX12 is a unique 9-LOX. Whereas leaf was the preferential site of *ZmLOX10* expression, *ZmLOX11* was strongly expressed in silks. Induction of these *ZmLOX10* and *ZmLOX12* by wounding and defense-related compounds suggested their role in plant resistance mechanisms against pests and pathogens. Abscisic acid, however, was the only inducer of *ZmLOX11* in leaves. Higher increase in *ZmLOX10* transcripts in maize infected by fungus *Cochliobolus carbonum* implicated this gene in resistance responses to

necrotrophic pathogens. In addition, *ZmLOX10* was shown to be the first reported LOX to be regulated by a circadian clock. It was found that *ZmLOX10* was also inducible by low temperatures. Phenotypical studies of wild type and mutant near isogenic lines showed that expression of *ZmLOX12*, specific to underground organs, was required for pathogenesis of *F. verticillioides* on maize mesocotyls.

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CHAPTER I

GENERAL INTRODUCTION

Plants are constantly exposed to a variety of biotic and abiotic stresses in the field environment. Therefore, mechanisms evolved to efficiently adjust plant metabolism to meet the challenges of an ever-changing environment, where the presence of pathogens can be detrimental. The metabolism of polyunsaturated fatty acids (PUFA) via LOX-mediated catalysis and the subsequent downstream reactions, are collectively called the LOX pathway. The lipoxygenase (LOX) pathway produces a magnitude of lipid-derived molecules called oxylipins, many of which are implicated in plant-pathogen interactions (Feussner and Wasternack 2002; Howe and Schilmiller 2002; Shah 2005). Some oxylipins are able to induce or repress sets of defense-related genes, others possess direct anti-microbial activity (Shah 2005). Most knowledge of oxylipins was obtained from experiments conducted on dicotyledonous plants. Although molecular responses to pathogens and pests involving LOXs and their products in monocot species have been overlooked, it is known now that defense responses in monocots differ from those of dicots (Pratt et al. 2005).

LOXs comprise a large gene family of nonheme containing dioxygenases widely distributed among eukaryotes. Divided into two major classes according to their regio-specificity, plant 9- and 13-LOXs execute

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This dissertation follows the style of Planta.

oxygenation of free or esterified PUFA. The latter can exist in the form of phospholipids or neutral lipids such as triglycerides (Feussner and Wasternack 2002;Shah 2005). Among plant PUFA, linoleic (18:2) and linolenic (18:3) acids are most abundant (Shah 2005). Intracellular compartmentalization of LOX proteins can provide clues as to their physiological function. Plant LOXs were found in chloroplasts, vacuoles, lipid bodies, microsomal and plasma membranes. LOXs were isolated from nearly all plant organs (Feussner and Wasternack 2002). Exposure to different forms of substrates, spatial and temporal separation of different LOX isoforms by plant organs and cellular compartments creates a global network that can be regulated and adjusted to variety of exo- and endogenous stimuli (Blee 1998;Howe and Schilmiller 2002). In a first approach to study physiological role of 9- as well as 13-LOXs in monocots, the entire LOX gene family consisting of twelve members was identified in maize. After phylogenetic analysis, one LOX out of each group of closely-related maize LOXs was cloned and overexpressed in *E. coli*, revealing seven 9-LOXs and five 13-LOXs. Recent data on monocot oxylipins prompted a hypothesis that 13-LOX and their products contribute to maize resistance, whereas 9-LOX-derived products are susceptibility factors in maize-pathogen interactions.

In order to test this hypothesis two closely related 13-LOXs (*ZmLOX10* and *ZmLOX11*) and one *9-LOX* (*ZmLOX12*) were chosen. This study represents isolation, cloning and biochemical characterization of these three LOX genes in

maize. In addition, possible mechanisms of functioning for *ZmLOX10* and *ZmLOX11* are discussed, as well as phenotypic analysis of lox12-1 mutants and wild type plants.

CHAPTER II

MAIZE 13-LIPOXYGENASE DUPLICATED GENES ARE DIFFERENTIALLY REGULATED BY CIRCADIAN RHYTHM, COLD STRESS, WOUNDING, PATHOGEN INFECTION AND HORMONAL TREATMENTS

Introduction

Oxylipins, a large class of diverse oxygenated polyunsaturated fatty acids and their derivatives, are important components in plant defense responses to pathogens and pests (Rosahl and Feussner 2005). Recent inroads to the elucidation of the biological roles of these compounds have occurred in dicot systems. The majority of oxylipins are produced through the lipoxygenase (LOX) pathway, which is comprised of at least seven multi-enzyme pathway branches (Feussner and Wasternack 2002). The starting point of the LOX pathway is the incorporation of molecular oxygen into either the 9- or 13-position of the carbon chain of C18 fatty acids. This reaction is mediated by regio-specific 9- or 13-LOXs that use linoleic (18:2) and linolenic (18:3) acids as substrates. Therefore, depending on preferential formation of either 9- or 13-polyunsaturated fatty acids LOXs are categorized as 9- or 13-LOXs, respectively. According to the classification based on their primary structure and

overall sequence similarity, plant LOXs also can be grouped into two gene subfamilies, *type 1* and *type 2* LOXs (Shibata et al. 1994). Enzymes designated *type 1*-LOXs have a high sequence similarity (>75%) to one another and lack plastid transit peptide. There are 9- as well as 13-LOXs in this class. However, the *type 2* enzymes show relatively low overall sequence similarity (<35%) to one another and carry a putative chloroplast targeting sequence. To date, *type 2*-LOXs consist exclusively of 13-LOXs (Feussner and Wasternack 2002). Primary products of LOX enzymatic activity fatty acid hydroperoxides are highly reactive and can form free radicals causing the membrane damage and hypersensitive cell death usually associated with incompatible plant interactions with pathogens (Blee 2002). However, in healthy cells LOX-mediated 9- and 13-hydroperoxides are generally quickly transformed into an array of more stable oxylipins with diverse physiological functions (Feussner and Wasternack 2002;Howe and Schilmiller 2002).

Products of the 13-LOX enzymatic activity can be utilized by several downstream pathway branches, however, currently the best characterized are allene oxide synthase (AOS) and hydroperoxide lyase (HPL) pathways. AOS-mediated or so called octadecanoid pathway produces jasmonic acid (JA) and its precursors and derivatives with signaling properties, collectively known as jasmonates. This pathway is ubiquitously found to occur in plastids, where the phytohormone 12-oxo-phytodienoic acid (OPDA) is formed. OPDA is then transferred into the peroxisome to be converted into JA (Feussner and

Wasternack 2002). A different set of oxylipins is produced by the chloroplast-localized HPL-mediated pathway. The best studied HPL-derived oxylipins C_6 green leafy volatiles (GLV) have been shown to possess both anti-microbial (Prost et al. 2005) and defense signaling activities (Bate and Rothstein 1998). GLVs were shown to enhance the production of anthocyanins in Arabidopsis (Bate and Rothstein 1998), phytoalexins in cotton (Zeringue Jr 1992), and systemin precursor in tomato (Sivasankar et al. 2000). Furthermore, GLVs induce a number of oxylipin and phenylpropanoid biosynthetic genes in Arabidopsis (Bate and Rothstein 1998), bean (Arimura et al. 2000) and citrus (Gomi et al. 2003). C_6-volatiles have been suggested to be essential in activation of wound-related pathways some of which are JA-independent (Bate and Rothstein 1998). This notion was supported by the analysis of downstream effects of separately silencing AOS and HPL branches of the 13-LOX pathway (Duan et al. 2005). Existing data also demonstrate that AOS- and HPL-derived signaling compounds execute cooperative defense signaling via molecular cross-talk (Halitschke et al. 2004). To date, the conclusive role of oxylipins derived from other 13-LOX-dependent enzymatic pathways is still poorly understood.

LOXs are widely distributed among eukaryotes and in most plant species studied are encoded by multiple gene families. Because different LOX isoforms initiate synthesis of functionally diverse products it is not surprising that LOXs have been proposed to play a role in such diverse physiological processes as

plant growth, development, resistance to a wide range of herbivorous insects as well as fungal, viral and bacterial pathogens (Blee 2002). For example, antisense-mediated depletion of JA-producing 13-LOXs in *Arabidopsis* (Bell et al. 1995), potato (Royo et al. 1999) and tobacco (Halitschke and Baldwin 2003) resulted in increased performance of insect pests. A role for a 9-LOX in resistance to fungal pathogens was revealed in antisense knockout experiments in tobacco (Rance et al. 1998a). In that study deficiency in *LOX1* transcripts resulted in loss of the hypersensitive response-mediated resistance towards *Phytophthora parasitica*. Despite some progress made in the functional analysis of LOXs in diverse dicot species, very little is known about the physiological significance of these enzymes in monocots. Several members of the LOX gene families were cloned or analyzed at the protein level from major crop monocot species such as barley, wheat and rice (Agrawal et al. 2004;Bohland et al. 1997;Mauch et al. 1997;Mizuno et al. 2003;Peng et al. 1994;Vörös et al. 1998). In the only reported functional analysis of a monocot LOX, overexpression of rice 13-LOX gene *RCI-1* resulted in increased levels of pathogenesis related protein PR-1 transcripts (Zabbai et al. 2004). This study and another study from barley (Weichert et al. 1999) suggested that 13-LOXs may be involved in the activation of acquired resistance.

The long-term goal of our research program is to elucidate the function of LOXs and their derivatives in maize (*Zea mays*) interactions with two economically most important maize fungal pathogens *Aspergillus flavus* and

Fusarium verticillioides. These filamentous fungi colonize corn seed and cause tremendous economic loss and significant health problems due to their ability to produce potent carcinogenic mycotoxins, aflatoxins and fumonisins, respectively. Recently published data demonstrated that 13-LOX-derived oxylipins inhibit mycotoxin production, suggesting that 13-LOXs may be a part of resistance mechanisms to seed contamination with mycotoxins (Burow et al. 2000;Calvo et al. 1999b). Supporting this hypothesis, expression of two closely related 13-LOXs was dramatically suppressed in response to *A. flavus* infection of peanut seed that accumulate high levels of aflatoxins (Tsitsigiannis et al. 2005). The genome of maize harbors at least 12 LOX genes (Kolomiets 2004). Only two of these genes have undergone molecular and biochemical characterization (Kim et al. 2003;Wilson et al. 2001). *cssap92* (designated as ZmLOX3 in Fig. 3), a predominantly 9-LOX, is upregulated during seed germination and infection with, *A. flavus* and *F. verticillioides* (Wilson et al. 2001). The other published maize LOX gene is reportedly a mixed function LOX that possesses both 9- and 13-LOX activity (Kim et al. 2003) and is a member of the *type 1*-LOX subfamily (designated ZmLOX1 in Fig. 3).

As a first step in achieving our long-term goal of better understanding the function of 13-LOXs and their metabolites in maize interactions with mycotoxigenic fungi, here we report the isolation and molecular and biochemical characterization of two novel 13-LOX genes, designated *ZmLOX10* and *ZmLOX11*. Although these genes share more then 90% amino acid sequence

identity, their expression is differentially regulated in diverse tissues, in response to wounding, chilling, treatments with defense-related hormones, and to infection with *Cochliobolus carbonum.* Moreover, expression of *ZmLOX10* in leaves is regulated by circadian clock, the first reported case of circadian rhythm-regulated plant LOX.

Materials and methods

Isolation of genomic DNA and Southern blot analysis

Freshly harvested leaves from two-week old maize (*Zea mays L.*) seedlings were used for extraction of genomic DNA as described (Zhang et al. 2005a). For Southern blot assay genomic DNA (10 µg) from several different inbred lines was cut with various restriction enzymes: *Eco*RI, *Bam*HI, *Hind*III, *Xho*I and *Xba*I. Digested DNA was separated on a 0.8% agarose electrophoresis gel, transferred (0.025 M phosphate transfer buffer) and then cross-linked to the nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN) by UV Stratalinker^{TM} 2400. The blots were hybridized overnight at 42 °C with the ^{32}P-labeled *ZmLOX10* specific probe. The probe was 428 bp long and consisted of the 3'-portion of the gene including 402 bp before and 24 bp after the stop codon. The membrane was hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX). Washes were performed:

first with low stringency buffer (2×SSC and 0.1% SDS) at 42 °C twice for 5 min, followed by an additional wash with high stringency buffer (0.2 M SSC and 0.1% SDS) twice at 42 °C for 15 min. The blots were exposed to X-ray film (Kodak, Rochester, NY) in cassettes at -80 °C for 72 h unless otherwise indicated.

Plant material and treatments

Maize plants were grown in a growth chamber in 7 cm pots in Strong-Lite® potting soil (Universal Mix, Pine Bluff, AZ), temperature was maintained from 22ºC to 30ºC under a 16 h day length, 50% average relative humidity, 560 to 620 µE of light. For all of the organ-specific expression studies (except for the ear, the tassel and the silk tissues), wounding and hormonal treatments we used two-week-old maize seedlings of inbred line B73 at the V3 developmental stage. Tassel tissue was harvested at the time of pollen shedding, ears at the stage of silk emergence, and unpollinated silks were harvested from B73 plants grown in the field, and used for total RNA extraction and mRNA blot analyses. For wounding experiments, the second fully expanded leaves at V3 stage were wounded by crushing the lamina with the hemostat. Cold treatment of plants at V2 stage was conducted by shifting the seedlings to 4 °C for 24 h, whereas, control plants were kept at room temperature (22 °C). For hormonal treatments, the seedlings were cut at the soil level and were incubated with the cut end placed in 100 mL of either 200 µM JA, 2.5 mM SA, 100 µM ABA and 0.01%

Tween-20 as a control (Sigma, St. Louis, MO). Treatment with 10 µL/L ethylene was conducted in hermetically-sealed 5.6 L dessicators. Entire ethylene treated seedlings (two seedlings per replicate, three replicates) except for roots and mesocotyls were harvested at different time points after treatment, frozen immediately in liquid N_2, and stored at -80 ºC. For studies of circadian rhythm-regulated expression, seedlings of B73 line were initially grown in a growth chamber at 22 °C with 12 h photoperiod of 36.000-37.000 lux with the lights turned on at 08:00 CT. When the seedlings reached V2 stage they were divided into three groups: first group was maintained at 12 h/12 h light/dark photoperiod; second group was transferred to a growth chamber with constant light; and third group was transferred to constant darkness beginning at 02:00. All treatments were carried out in separate but identical growth chambers (Percival Scientific, Inc., Perry, IA). During the next 48 h the second fully expanded leaves from two plants in each light treatment were harvested every 4 h and immediately frozen for subsequent RNA extractions.

Infection of plants with *Cochliobolus carbonum*

To study the LOX gene expression in a single genetic background during either compatible or incompatible we used near-isogenic strains of *C. carbonum* race 1 that either did (Tox2+) or did not (Tox2-) produce its pathogenicity factor, HC-toxin, kindly provided by Dr. Guri Johal at Purdue University. For inoculation

with *C. carbonum,* we used two-week-old seedlings of inbred line Pr which is susceptible to *C. carbonum* Tox2+ wild type strain but resistant to Tox2- mutant strain (Multani et al. 1998). Conidial suspensions containing 10^5 conidia per mL in 0.1% Tween-20 were prepared as described in (Meeley et al. 1992). Control plants were inoculated with sterile water (mock-inoculated). Plants were inoculated by spraying the leaves to imminent run-off with conidial suspension. Following inoculation, plants were immediately placed under a plastic cover and were incubated for selected times in the greenhouse. After each incubation period, infected leaves were collected and frozen immediately in liquid N_2 and stored at -80 °C until used for total RNA isolations.

RNA extraction and northern and dot blot analysis

Total RNA from corn tissues was extracted by using TRI reagent (Molecular Research Center Inc., Cincinnati, OH) according to the protocol provided by the manufacturer. After extraction 10 µg of total RNA from each experiment was separated using electrophoresis 1.5% (w/v) formaldehyde agarose gels in 1×MOPS buffer and then transferred onto a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA). To confirm equal loading of RNA samples into an agarose gel and uniform transfer onto a nylon membrane for all experiments (except the *C. carbonum* inoculation study) rRNA was stained with ethidium bromide and was visualized by UV light. For *C. carbonum* inoculation

study, equal loading of total RNA was visualized by hybridization to the probes derived from 18S rRNA or tubulin. Because *ZmLOX10* and *ZmLOX11* genes share extremely high sequence identity within their coding sequence, the gene-specific probes were amplified by PCR from the 3' untranslated region of the cDNAs where they share only limited identity. PCR primers for amplification of gene-specific probes were: lox10utrF1 5'-ATC CTC AGC ATG CAT TAG TCC A-3', lox10utrR1 5'-AGT CTC AAA CGT GCC TCT T-3', lox11utrF1 5'-ACC CGT CCG TCC TCT CC-3', and lox11utrR3 5'-ATC CTA ATG GTA ACT CAA A-3'. The *ZmLOX10* template was the *Sal*I/*Not*I insert of the Pioneer p0006.cbysd84 EST clone. Genomic DNA extracted from B73 inbred line was used as a template to PCR-amplify the probe for *ZmLOX11*, which then was sequenced to ensure its identity as the *ZmLOX11* gene. To ensure gene-specificity of the probes, dot blot assay was performed by hybridization of the PCR fragments to denatured DNA of either entire *ZmLOX10* cDNA insert of p0006.cbysd84 plasmid or the genomic fragment of 3'UTR of *ZmLOX11* generated by PCR as described above to the Hybond-N+ nylon membrane (GE healthcare, Piscataway, NJ) according to the manufacturer's protocols. Northern blot membranes were hybridized with ^{32}P-labeled gene-specific probes using UltraHyb hybridization solution (Ambion, Austin, TX) under conditions described in the manufacturer's protocol. Membranes were washed in 1 x SSC, 0.1% SDS at room temperature for 15 min, in 0.1 x SSC, 0.1% SDS at room temperature for 30 min, followed by a final wash in 0.1 x SSC, 0.1% SDS at 42 °C for 30 min.

For autoradiography, RNA blots were exposed to a BioMax X-ray film (Kodak, Rochester, NY) for two to seven days at –80 ºC. Blots presented in this manuscript are representative examples of at least two independent experiments.

Construction of overexpression vectors and analysis of ZmLOX10 biochemical properties

The entire open reading frame was PCR-amplified using primers containing *Bam*HI-site adopted for in-frame start-codon (5'-CGG-ATC-CAT-GAT-GAA-CCT-GAA-CCT-GAA-G-3') and *Eco*RI-site located after the stop-codon (5'-CCT-TAA-GTC-AGA-TGG-ATA-TGC-TGT-GGG-G-3'). Following separation and elution from an agarose-electrophoresis gel the PCR fragment was inserted into the pCR2.1 TOPO® vector (Invitrogen, Carlsbad, CA). After restriction digestion with *Bam*HI/*Eco*RI the coding region of *ZmLOX10* was placed under the control of isopropyl thio-*β*-D-galactopyranoside (IPTG)-inducible promoter of an *E. coli* expression vector pET28a (EMD Biosciences, Inc., San Diego, CA) using T4 DNA Ligase (Roche Applied Science, Indianapolis, IN). Following ligation the pET28a expression construct containing *ZmLOX10* was heat-shock transformed into BL21 (DE3) strain of *E. coli* (EMD Biosciences, Inc., San Diego, CA). Two independent *E.coli* BL21 (DE3) cultures containing the expression constructs were grown overnight at 37 °C in LB medium (20 ml) containing 25 µg/mL of

kanamycin and 50 µg/ml ampicillin. When the cell culture reached density value A600 = 0.7, 1 mM IPTG was added to induce expression of the constructs at 15 °C for 48 h. Cells collected from 200 mL of culture were resuspended in 30 mL of lysis buffer (50 mM Tris-HCL, pH 7.5) that contained 10% (v/v) glycerol, 0.5 M NaCl and 0.1% Tween-20. Cells then were disrupted by a sonifier tip with a frequency of 5 pulses per 30 s. In order to remove debris of cellular compartments, samples were centrifuged (12.000×g for 15 min). Supernatant was divided into 1 mL aliquots and stored at -20 °C (Feussner et al. 1998). Oxygenation of linoleic acid was carried out by incubating crude extract of bacteria containing *ZmLOX10* overexpression construct with the substrate (120 µM final concentration) diluted in 1 mL of 0.1 M sodium phosphate buffer (pH 5.7-7.0) or in 0.1 M Tris buffer (pH 7.7–8.6), respectively, for 20 min at room temperature. The reaction was stopped by addition of 100 µL of glacial acid. After centrifugation the organic phase was vacuumed to rid of solvents. Remaining lipids were resuspended in 0.1 mL of HPLC solvent. Detection of LOX products by HPLC was carried out on an Agilent (Waldbronn, Germany) 1100 HPLC system. The absorbances at 234 nm and 210 nm were recorded simultaneously. The enantiomer composition of the hydroxyl fatty acids was analyzed as described in (Feussner and Kuhn 1995).

Bioinformatics analysis

Homology search using ZmLOX10 and ZmLOX11 translated sequences was done using the BLAST search which is publicly available at the National Center for Biotechnology Information (NCBI) web-site (www.ncbi.nlm.nih.gov/BLAST/). Percentage of identity of ZmLOX10 and ZmLOX11 to other plant LOXs was evaluated using ClustalW software available at the European Molecular Biology Laboratory, the European Bioinformatics Institute (EMBL-EBI) (www.ebi.ac.uk/clustalw/). For phylogenetic analysis, deduced protein sequences of ZmLOX10 and ZmLOX11 were aligned with sequences of available plant LOXs. For construction of the phylogenetic tree we selected only published maize LOXs or plant LOXs physiological function of which was established by either antisense suppression or by transgenic overexpression. For all the bioinformatics analyses the default settings were used, unless indicated otherwise, The sequences were aligned using the Muscle program (Edgar 2004a). A phylogeny was reconstructed using the neighbor-joining method implemented in the Phylip package using Kimura's correction for multiple substitutions. The maximum likelihood tree was constructed using the parameters as described (Felsenstein 1989). Subcellular localization was predicted based on the identification of signal peptide sequences by four different programs ProtComp (www.softberry.com/berry.phtml), PSORT (psort.nibb.ac.jp/), TargetP (www.cbs.dtu.dk/services/TargetP/) and ChloroP

(www.cbs.dtu.dk/services/ChloroP/). Presence of conserved domains in the deduced amino acid sequences was determined by NCBI Conserved Domain Search (www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and Pfam software (www.sanger.ac.uk/Software/Pfam/). Analysis of ZmLOX10 promoter region for the presence of *cis*-acting elements and transcription factor binding sites was done using the PLACE program (www.dna.affrc.go.jp/PLACE/) (Higo et al. 1999;Prestridge 1991). To determine the genomic structure and identify promoter sequences of *ZmLOX10* and *ZmLOX11* a homology-based blast search was performed by utilizing the databases of the maize Genome Survey Sequences (GSS) (available at www.ncbi.nlm.nih.gov/BLAST/, www.plantgdb.org/PlantGDB-cgi/blast/PlantGDBblast and http://tigrblast.tigr.org /tgi_maize/index.cgi) as well as Maize Assembled Genomic Island databases (MAGI) (available at www.plantgenomics.iastate.edu/maize/).

Results

Isolation and sequence analysis of *ZmLOX10* and *ZmLOX11* genes

To identify and clone maize 13-LOX genes potentially involved in biosynthesis of GLVs, the amino acid sequence from GLV-producing potato 13-LOX gene (clone H1) (T07062, (Royo et al. 1996) was used in a BLAST search against the extensive DuPont/Pioneer and publicly available EST collections from Zea

mays. This search identified 241 EST clones that belonged to a single contig and shared 57% identity at the deduced amino acid level with potato LOX H1. Sequence from the longest EST clone, named p0006.cbysd84, revealed that it represents only about 2 kb of the 3'-portion of the gene. Therefore, the 5' end of this gene was cloned by the 5'RACE technique. The combined full-length cDNA sequence contained a complete open reading frame of 2718 bp and was named *ZmLOX10* (GeneBank accession number DQ335768) in the order that reflects its level of homology to *ZmLOX1* as compared to the rest of the twelve maize LOX genes identified (Kolomiets, unpublished data). This sequence encodes a predicted 905 amino acids peptide with the estimated molecular mass of 102 kDa and an estimated pI of 6.57. Deduced amino acid sequence of ZmLOX10 exhibited the highest identity (72%) to the barley *LOX2:Hv:3* gene (Q8GSM2, (Bachmann 2002) and 57% identity to tobacco *NaLOX2* (AAP83137; (Halitschke and Baldwin 2003), both of which are *type2*-13-LOXs.

Southern blot analysis was performed to determine the number of copies of the *ZmLOX10* gene in the maize genome. The initial probe derived from EST consisted of 428 bp from the 3'-portion of the gene, including 402 bp before and 24 bp after stop codon. Under the most stringent conditions, for most of the five restriction enzymes and the three inbred lines two bands of similar intensity were observed (Fig. 1), suggesting the existence of another closely related gene. To identify this missing gene, the newly cloned 5' portion of *ZmLOX10* was used in a new BLAST search of the available public and

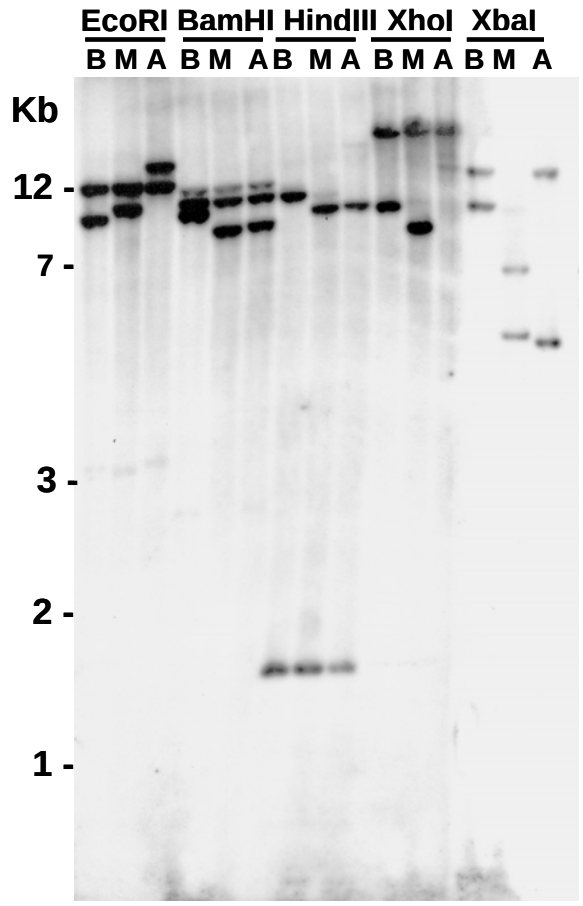


Fig. 1 Southern blot analysis of gene copy number of *ZmLOX10*. Ten µg of genomic DNA extracted from maize inbred lines B73 (B), Mo17 (M) and A632 (A) was digested with *EcoR*I , *BamH*I , *Hind*III, and *Xba*I, separated by electrophoresis, blotted onto nylon membranes and hybridized to the 3'-terminal 1.8 kb fragment derived from *ZmLOX10* cDNA clone cbysd82. DNA size markers in kilobases (kb) are indicated on the left. Presence of second band on the blot indicates the existence of additional highly homologous gene in the maize genome.

Pioneer/DuPont EST collections. This search identified three EST clones that shared over 95% nucleotide identity to the *ZmLOX10* cDNA sequence. The complete sequence of the longest DuPont EST clone cds3f.pk006.d9a contained the 3' portion of the gene but lacked around 215 amino acids of the N-terminus as predicted by comparison to ZmLOX10. This missing part of the gene was first identified by the BLAST search of available maize Genome Survey Sequences (GSS) (http://www.plantgdb.org/cgi-bin/PlantGDBblast) and then cloned by 5'-RACE technique from cDNA derived from silks. Analysis of the combined cDNA sequence proved the presence of a new gene which shares 90% amino acid sequence identity to ZmLOX10 and was designated as *ZmLOX11* (GeneBank accession number DQ335769). The full length cDNA sequence for *ZmLOX11* was 3125 bp long, encoding 911 amino acids and a predicted molecular mass of 103 kDa and estimated pI of 7.11. Despite their high sequence identity *ZmLOX10* and *ZmLOX11* are discrete genes in the maize genome and were mapped to chromosomes, 4 and 7, respectively, by using PCR with gene-specific primers and template genomic DNA from oat-maize chromosome addition lines (data not shown).

Plant LOXs can be found in various cell organelles including chloroplast, microbodies and cytoplasm (Feussner and Wasternack 2002). In order to predict subcellular localization of the LOX proteins we used four different publicly available programs: ProtComp, PSORT, TargetP and ChloroP. Analysis of ZmLOX10 and ZmLOX11 N-terminal sequences revealed the presence of a

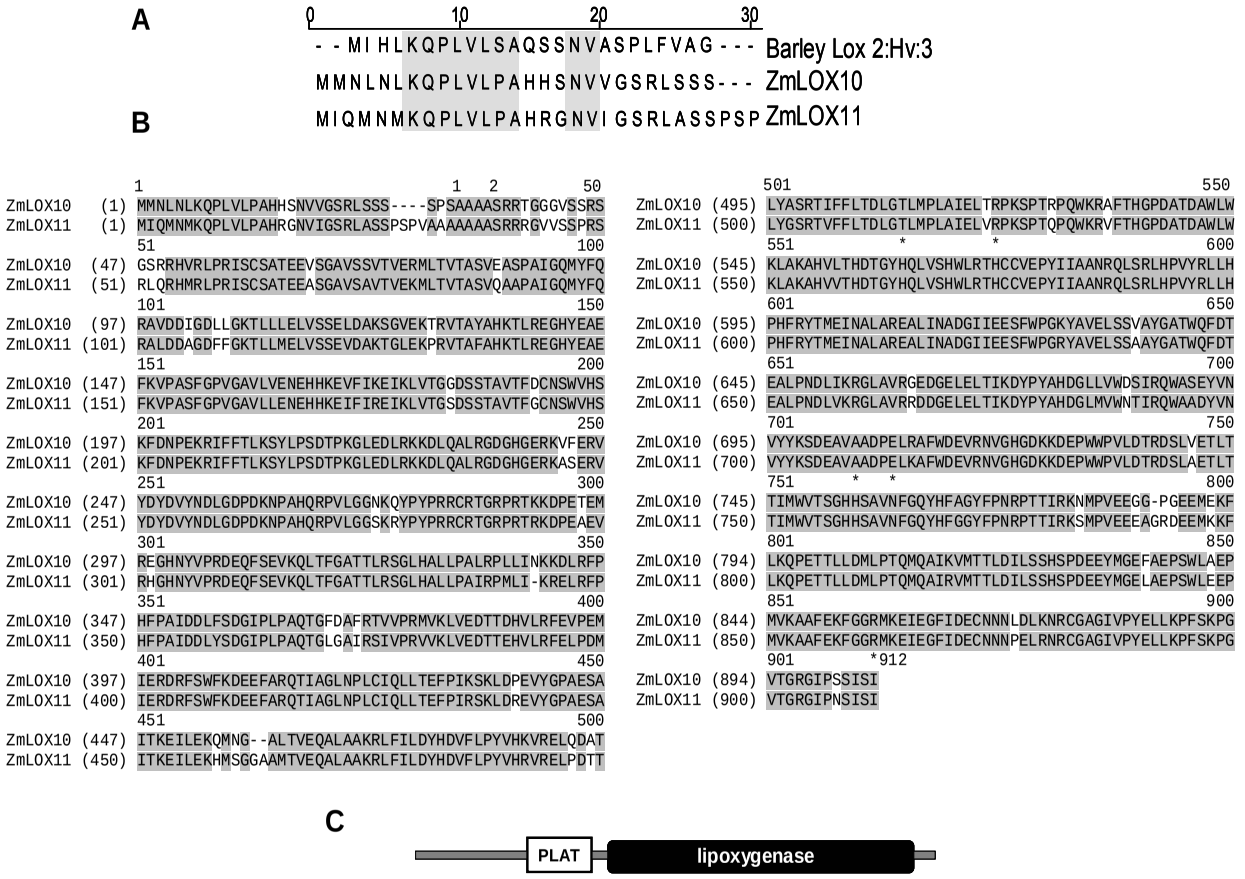


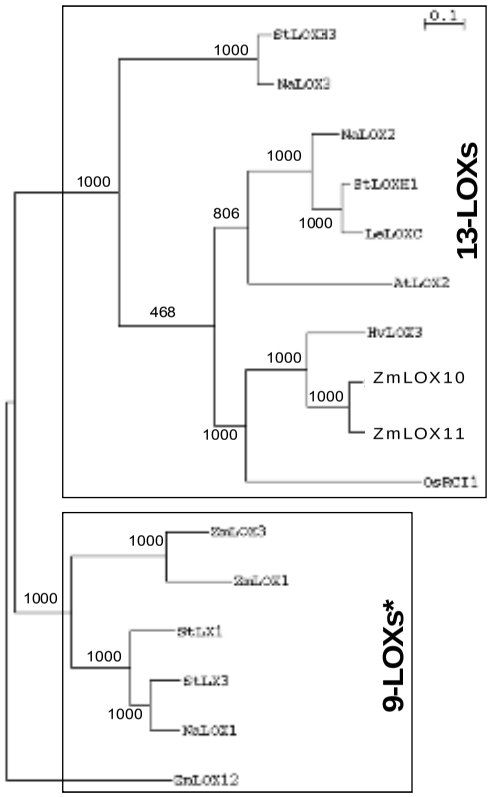
Fig. 2 Sequence analysis of the deduced amino acid sequences of ZmLOX10 and ZmLOX11. (A) Alignment of putative signal peptides of ZmLOX10 and ZmLOX11 to the chloroplast-targeting signal peptide of their closest homolog Lox2:Hv:3 from barley which was reported to be localized to chloroplast. (B) Alignment of the deduced amino acid sequences of ZmLOX10 and ZmLOX11. Numbers 1 and 2 above the sequences indicate predicted cleavage site for signal peptide of ZmLOX11 and ZmLOX10 respectively. Conserved amino acids required for iron binding and catalytic activity indicated by asterix. (C) Analysis of protein domain architecture performed by Pfam program package revealed presence of a typical lipoxygenase domain, and an N-terminal PLAT domain known to mediate membrane attachment via other protein binding partners.

chloroplast-targeting signal peptide with the cleavage site at position 34 and 30, respectively (Fig. 2B). The closest homolog to both maize LOXs, the barley 13-LOX gene *LOX2:Hv:3* that shares more than 70% amino acid identity was conclusively localized to chloroplasts (Bachmann 2002). Fig. 2A is an alignment of the predicted signal peptide sequences of ZmLOX10 and ZmLOX11 with the corresponding sequence of LOX2:Hv:3. The degree of conservation provides strong support for our hypothesis that ZmLOX10 and ZmLOX11 are most likely localized to chloroplasts.

Similar to other known plant LOXs, deduced amino acid sequences of both ZmLOX10 and ZmLOX11 contained residues required for iron binding and enzyme catalytic activity (Prigge et al. 1996): His-559 and His-564, His-569 and His-574, His-754 and His-760, Asn-758 and Asn-764, Ile-905 and Ile-911, respectively (Fig. 2B). Moreover, it contains the Ser/Phe (621/622) conventional motif described to be indicative for plant 13-LOXs (Hornung et al. 1999). Analysis of the domain architecture performed using Pfam program (Fig. 2C) showed that the proteins are comprised of two major domains: the lipoxygenase domain typical for all LOXs, and the PLAT domain known to mediate membrane attachment via other protein binding partners (Marchler-Bauer et al. 2005).

Phylogenetic analysis

We investigated the phylogenetic relationship of ZmLOX10 and ZmLOX11 to other plant LOXs functions of which have been established to inform our predictions of biochemical and biological functions of these maize LOX genes. The maximum likelihood analysis identified two well supported lineages of plant LOX proteins that appear to reflect rather accurately their regio-specificity and subcellular localization, cytoplasm-localized 9-LOXs and chloroplast-localized 13-LOXs (Fig. 3). ZmLOX10 and ZmLOX11 group together, with very strong bootstrap support, with chloroplast-localized 13-LOXs from both monocot and dicot species suggesting that these maize isoforms are most likely 13-LOXs. Within this group the monocot proteins, barley LOX2:Hv:3 and rice RCI1, group together with ZmLOX10 and ZmLOX11 suggesting that they are orthologs and thus, may have a similar, as yet unidentified, function. Several members of the 13-LOX group such as Arabidopsis LOX2 and tobacco LOX3 are known to provide linolenic acid hydroperoxide substrates for the synthesis of JA *in vivo* (Bell et al. 1995;Halitschke and Baldwin 2003) whereas others such as potato H1 and tomato LOXC are involved in the biosynthesis of C6 volatiles (Chen et al. 2004;Leon et al. 2002). Proteins with either predominant C9-positional specificity such as ZmLOX3 (Wilson et al. 2001) or a mixed C13/9 regio-specificity such as ZmLOX1 (Kim et al. 2003) clustered together with reasonable bootstrap support and are more distantly related to the group of 13-LOXs.



biosynthesis of	physiological functin	trivial name	NCBI acces. #	citation
not JA	insect resistance	LOX H3	T07065	Royo et al. 1999
JA	insect resistance	NaLOX3	AAP83138	Halitschke and Baldwin 2003
N/D	N/D	NaLOX2	AAP83137	Halitschke and Baldwin 2003
C6-volatiles	N/D	LOXH1	T07062	Leon et al. 2002
C6-volatiles	Fruit development	LoxC	T07408	Chen et al. 2004
JA	Wound responses	AtLOX2	CAB72152	Bell et al. 1995
N/D	N/D	Lox2:Hv:3	Q8GSM2	Bachmann et al. 2002
N/D	N/D	ZmLOX10	DQ335768	this paper
N/D	N/D	ZmLOX11	DQ335769	this paper
13-HOT	Induction of PR1	RCI-1	CAC01439	Zabbai et al. 2004
9-HOT, 9-HOD	Resistanse to nematodes	ZmLOX3	AAG61118	Gao and Kolomiets unpublished data
N/D	N/D	ZmLOX1	AAF76207	Kim et al. 2003
N/D	Tuber development	POTLX-1	AAB67858	Kolomiets et al. 2001
N/D	N/D	POTLX-3	AAB67865	Kolomiets et al. 2000
N/D	Resistanse to fungi	NaLOX1	AAF76207	Rance et al 1998
N/D	N/D	ZmLOX12	N/A	Nemchenko et al. unpublished data

Fig. 3 Maximum likelihood phylogenetic tree and reported biochemical and physiological functions of selected plant LOXs. Maximum likelihood tree was constructed by comparing ZmLOX10 and ZmLOX11 deduced proteins (in bold) with other plant LOXs function of most of which has been established either by transgenic suppression of expression or by overexpression strategies. Sequences were aligned using Muscle software (Edgar 2004b). A phylogeny was reconstructed using the neighbor-joining method implemented in the Phylip package using Kimura's correction for multiple substitutions Two well supported lineages of plant LOX proteins reflect accurately their regio-specificity and subcellular localization: cytoplasm-localized 9-LOXs and chloroplast-localized 13-LOXs.

Another maize LOX, ZmLOX12, encodes a 9-LOX (Nemchenko *et al.,* unpublished) and was equally distant from either of the two major groups.

Genomic structure and predicted promoter *cis*-acting elements

Using maize GSS and Maize Assembled Genomic Island (MAGI) databases we identified overlapping genomic sequences covering the entire full-length cDNAs of *ZmLOX10* and *ZmLOX11* and most of the promoter region for ZmLOX10. Genomic sequences with 98% or higher identity to the cDNAs were exclusively selected to assemble genomic contigs for each of the two genes. Estimated length genomic sequences (from start to stop codon) for *ZmLOX10* (DQ459365) and *ZmLOX11* (DQ459366) was 3360 bp and 3271 bp, respectively. Alignment of the cDNAs with corresponding genomic sequences to compare exon/intron

Both genes are comprised of four exons and three introns. The differences between *ZmLOX10* and *ZmLOX11*, though, were found in the length of the introns with the first intron of *ZmLOX10* being 4.3 fold longer than the first intron of *ZmLOX11* (481 bp and 112 bp, respectively). However, the other two introns of *ZmLOX11* were at least 1.5-fold longer than corresponding introns of *ZmLOX10*. In both genes exon-intron-exon boundaries followed the rule of consensus junction sequences GT/AG (Mount 1982).

Available GSS genomic sequences allowed us to reconstruct presumably the entire promoter region of the *ZmLOX10* gene containing 1,869 bp upstream

Table 1. Predicted cis-acting elements in the promoter of ZmLOX10 identified by PLACE program

Category	Function	sequence	copy number
Organ-specific	Chlorophyll-specific	TATTCT	2
	Chloroplast-specific, GATA-box	GATA	4
	Mesophyll-specific	YACT	24
Dehydration stress, ABA response	ABRE (ABA early responsive element)	ACGTG	7
	ABA-responsive element	ACGTSSSC	1
	ACGT element	ACTG	12
	DRE1	RCCGAC	2
	DRE2	ACCGAC	1
	MYB-site water stress	WAACCA	6
	MYC-binding site (dehydration)	CANNTG	29
Cold stress	LTRE	CCGAC	3
Light response	I-box	GATAAG	4
Defense related	GCC-box, in pathogen responsive genes	GCCGCC	4
	GT-1 motif pathogen-induced	GAAAAA	1
	WRKY71 binding (SA-mediated)	TGAC	9
	W-box (wounding)	TGACY	14
	Infected roots	CTCTT	4
	AGC-box, binding of ERF1	AGCCGCC	1
	ASF-1 SA/Auxin responsive	TGACG	1

of start codon. By using the PLACE Signal Scan Search software (http://www.dna.affrc.go.jp/PLACE/; (Higo et al. 1999;Prestridge 1991) we detected multiple putative *cis*-acting elements in the *ZmLOX10* promoter. Since the promoter sequence for *ZmLOX11* was not available we were not able to compare it to the promoter of *ZmLOX10*. Table 1 shows the categories of the regulatory sequence elements present in the promoter of *ZmLOX10* (Table 1). This data was used as a guideline in our further expression studies.

Biochemical properties of recombinant ZmLOX10

For further characterization of enzymatic properties, *ZmLOX10* coding region was amplified by PCR (described in Materials and Methods) and was placed under control of an IPTG-inducible promoter of an *E. coli* expression vector. Crude extract of bacteria containing the ZmLOX10 overexpression construct exhibited LOX activity. Similar to 13-LOXs from peanut (Tsitsigiannis et al. 2005), ZmLOX10 was most active at neutral to slightly alkaline pH conditions with maximal LOX activity observed at pH 8.0 (data not shown), which is within the range of physiological pH values during photosynthesis (Schaffrath et al. 2000). Therefore, regio-specificity was also determined at pH 8.0 using linoleic acid as a substrate. Since 96% of ZmLOX10-catalyzed products were 13*S* hydroperoxides of linoleic acid, we concluded that ZmLOX10 possessed clear 13-LOX regio-specificity. The preferred products of ZmLOX10 enzymatic

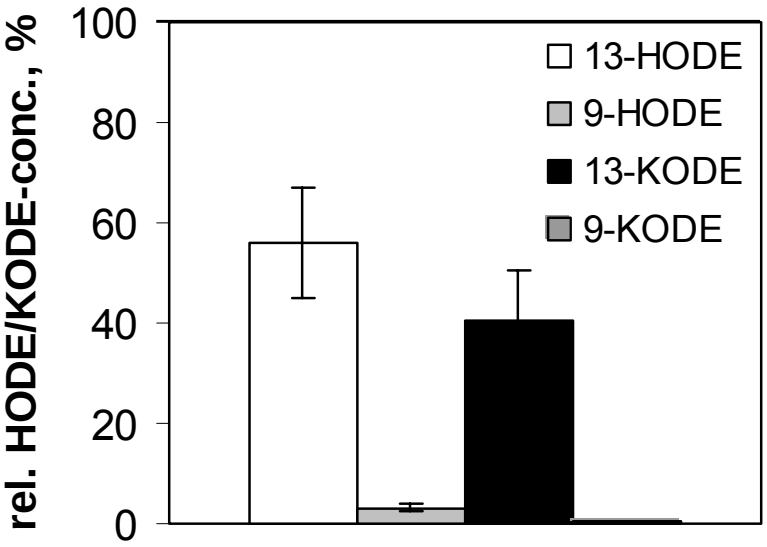


Fig. 4 Analysis of recombinant ZmLOX10 protein regio-specificity using linoleic acid as a substrate. Analysis of LOX- activity reflected in conversion of linoleic acid by ZmLOX10 into correspondent 13-HODE, (9*Z*,11*E*,13*S*)-13-hydroxy-octadeca-9,11-dienoic acid; 9-HODE, (9*S*, 10*E*, 12*Z*)-9-hydroxy-10,12-octadecadienoic acid; 13-KODE, (9*Z*,11*E*)-13-oxo-octadeca-9,11-dienoic acid, and 9-KODE, ketodiene (9*S*, 10*E*, 12*Z*, 15*Z*)-9-hydroxy-10, 12, 15-octadecadienoic acid.

reaction detected by HPLC were 13-H(P)ODE ((9*Z*,11*E*,13*S*)-13-hydro(pero)xy-octadeca-9,11-dienoic acid) and 13-KODE ((9*Z*,11*E*)-13-Oxo-octadeca-9,11-dienoic acid) (Fig. 4). The fact that the hydro(pero)xides were predominantly *S*-enantiomers suggests their enzymatic origin rather than being a result of autocatalysis (Mueller et al. 2006). Amongst 13-LOX products 13-H(P)ODE was preferentially produced (70%) as compared to 13-KODE (30%), whereas only minor quantities of 9-LOX products were produced as a racemic mixture of *S*- and *R*-enantiomers (data not shown). The negative control did not show any detectable LOX activity. These data suggest that ZmLOX10 is a linoleate 13-LOX which is most active at the pH conditions closest to those during photosynthesis.

Organ-specific and circadian clock-regulated expression of *ZmLOX10* and *ZmLOX11*

To rationalize the *in vivo* function of *ZmLOX10* and *ZmLOX11* we analyzed expression of both genes by the Northern blot technique. Comparison of cDNA sequences of *ZmLOX10* and *ZmLOX11* showed very high overall nucleotide identity in the protein coding sequence, therefore we generated gene-specific probes based on 3'-UTR portions that share only limited homology. Nevertheless, the gene-specificity of generated probes was tested by dot blot

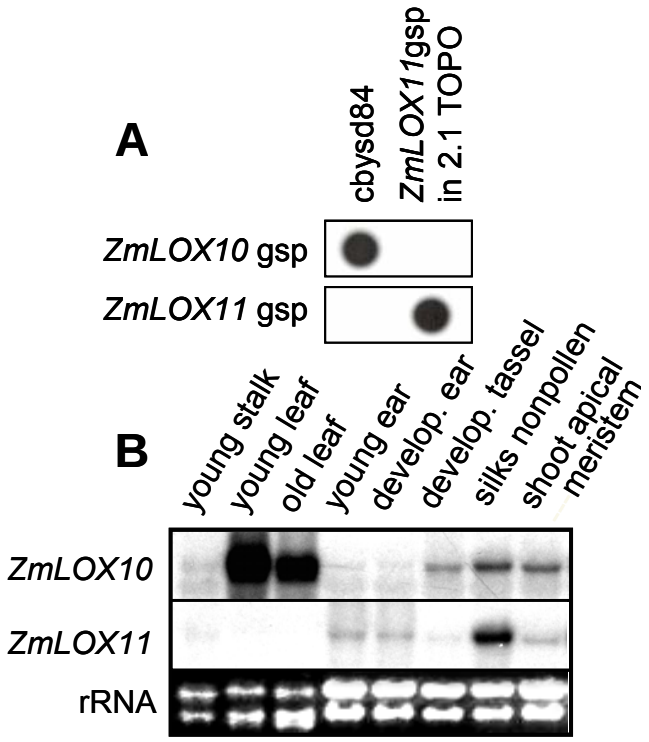


Fig. 5 Gene-specificity assay and organ-specific expression of *ZmLOX10* and *ZmLOX11* genes. (A) Dot blot assay. Gene-specific probes (gsp) for *ZmLOX10* and *ZmLOX11*, generated based on equivalent 3'-UTRs portions of both of the genes were hybridized to 100 ng of dotted recombinant DNA constructs representing respectively cDNA (cbysd84) with full length clone (1.8 kb) of *ZmLOX10*, and 3'-utr portion of ZmLOX11 cloned into pCR 2.1 TOPO. This blot illustrates that both probes hybridized only to the cDNA fragments of the corresponding genes indicating the probes used in this study are indeed gene-specific. (B) Northern blot analysis of organ and tissue-specific expression of *ZmLOX10* and *ZmLOX11.* Ten ug of total RNA were extracted from the following organs of maize plants at different stages of development: young stalk, young leaf and shoot apical meristem were harvested from seedlings at V2 developmental stage; and old leaf, ear, tassel and non-pollinated silks were harvested from mature plants at R1 stage. Duplicate blots were hybridized to ^{32}P-labeled *ZmLOX10* and *ZmLOX11* gene-specific probes (described in panel A). Equal loading of RNA was confirmed by visualizing ethidium bromide (EtBr) staining of ribosomal RNA (rRNA).

assay. No cross-hybridization of the two probes to each other or to their corresponding full length cDNAs was observed (Fig. 5A).

Analysis of organ- and tissue-specific expression showed that transcript levels of *ZmLOX10* were most abundant in both young (V2 developmental stage) and older (R1 stage) leaves (Fig. 5B). *ZmLOX10* is also expressed at relatively low levels in all rapidly growing tissues studied including developing tassel, silks and shoot apical meristem. In contrast to *ZmLOX10*, expression of *ZmLOX11* was the highest in silks and silk-bearing organs, ears. Moreover, expression of *ZmLOX11* in silks is much higher than that of *ZmLOX10*, while its transcripts are just at detectable levels in tassel and shoot apical meristem. Such differential distribution of transcripts for these two highly related genes in diverse organs suggests their dissimilar role during plant and organ development. However, we cannot exclude a possibility of partial functional overlap for both LOXs in silks.

Because the promoter region of *ZmLOX10* harbors light responsive cis-acting elements such as I-box (Table 1) we hypothesized that expression of *ZmLOX10* and/or *ZmLOX11* may be regulated by light/dark conditions or circadian clock. In order to test this hypothesis we subjected V2 stage seedlings to different light treatments and harvested leaf tissues at 4-hour intervals over two days. Samples harvested within each treatment were analyzed for steady-state mRNA levels of *ZmLOX10* and *ZmLOX11* using northern blot assay (Fig. 6). Plants grown at 12 h light/12 h dark photoperiod showed cyclic time-

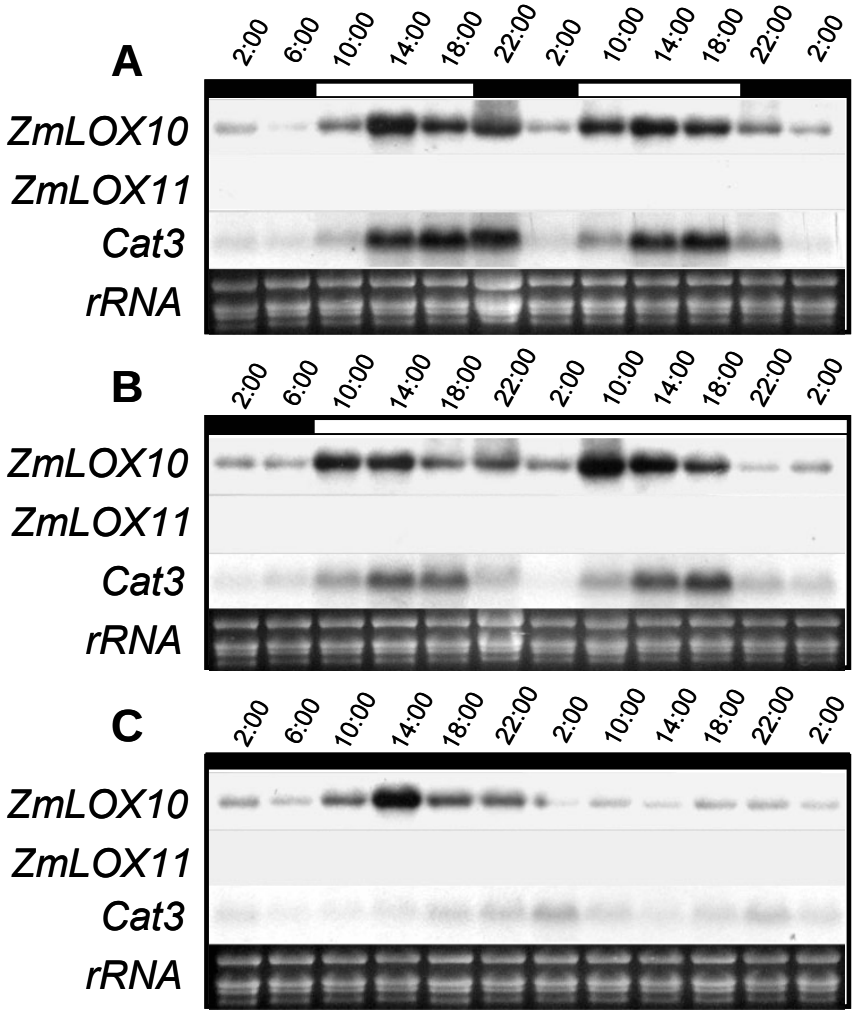


Fig. 6 Temporal variation in *ZmLOX10, ZmLOX11* and *Cat3* transcript levels in the leaves of maize seedlings grown under different light conditions. (A) at the 12 h light/12 h dark photoperiod or, (B) transferred to continuous light or, (C) constant darkness. Within each treatment leaves were harvested every 4 h for 48 h. Total RNA was extracted and 10 μg was used for northern blot assay. Equal loading of RNA was visualized by EtBr staining and exposure to UV-light revealing amounts of rRNA. Periods of light and darkness, as well as the time of sample harvesting is indicated correspondently by white and black bars and the time in h (on 24-hour clock) above the northern blot figure.

dependent fluctuation of transcript levels with the maximum at 14:00 (6 h after onset of light) and minimum at 2:00 (6 h after offset of light). The plants maintained the same pattern of expression for the rest of the experiment duration even after being transferred to constant light. When the plants were transferred into constant darkness, *ZmLOX10* exhibited similar cyclic expression pattern with the similar amplitude but only during the first 24 h. However, in contrast to constant light conditions, no detectable fluctuation of *ZmLOX10* transcripts was observed during the next 24 h of constant darkness. This pattern is typically observed for other well characterized plant circadian-regulated genes such as maize catalase 3 (Polidoros and Scandalios 1998). Fig. 6 illustrates that accumulation of *ZmLOX10* transcripts followed closely a pattern observed for *Cat3* expression except that maximum of *ZmLOX10* expression preceded the highest levels of *Cat3* transcripts by at least four hours. This tendency was maintained through the time course of both light/dark and constant light experiments (Fig. 6A and 6B). However, in contrast to *ZmLOX10*, expression of *Cat3* was significantly reduced under conditions of constant dark. Closer examination of the available promoter region of *ZmLOX10* revealed presence of a promoter element, TTAATATCT, so called “site 2” (starting at position 725 bp upstream from the start codon), that may confirm circadian rhythmicity to a number of Arabidopsis cycling genes (Harmer et al. 2000). These data suggests that the expression of *ZmLOX10* in maize leaves is regulated by circadian clock. No *ZmLOX11* mRNA was detected throughout the

entire time course under all the dark/light conditions used in this experiment (Fig. 6).

Wound-, cold- and hormone-regulated expression of *ZmLOX10* and *ZmLOX11*

In order to study potential involvement of *ZmLOX10* and *ZmLOX11* in stress-induced defense responses we examined mRNA accumulation levels in response to various defense-related hormones such as JA, salicylic acid (SA), abscisic acid (ABA), and ethylene (Fig. 7). As was the case with organ-specific expression, these genes were differentially expressed following most of the treatments applied. In leaves, exogenous JA application transiently induced steady-state levels of *ZmLOX10* transcript starting 3 h and reaching maximum at 12 h after treatment (Fig. 7A). Similarly, wounding, known to induce endogenous accumulation of JA (Pena-Cortes et al. 1991), had the same effect on *ZmLOX10* mRNA accumulation with maximum at 12 h (Fig. 7B). Available evidence suggests that JA and ethylene usually act synergistically in defense pathways most likely via the involvement of the ERF1 transcription factor (Berrocal-Lobo et al. 2002;Lorenzo et al. 2003;Penninckx et al. 1996;Penninckx et al. 1998). In our study, however, mRNA levels of *ZmLOX10* declined rapidly after treatment with ethylene and were minimal at 12 h post treatment but later started to increase and reached maximum at 48 h (Fig. 7A). In control untreated plants there was no secondary phase of mRNA accumulation detected (data not

shown). Interestingly, at the time point of minimal levels of *ZmLOX10* transcripts after ethylene treatment this gene was induced by JA to the highest levels, suggesting a certain level of antagonism between these two hormones in regulating *ZmLOX10* expression. *ZmLOX10* was also inducible by SA with mRNA accumulation reaching the maximal levels at 6 h after treatment but lower compared to those induced by JA. SA is a required signaling molecule in the resistance mechanisms against biotrophic pathogens and the development of SAR (McDowell and Dangl 2000;Ryals et al. 1995). In sharp contrast with *ZmLOX10*, *ZmLOX11* did not show any mRNA accumulation in response to JA, SA, ethylene or wounding. However, transcript levels of both *ZmLOX10* and *ZmLOX11* were induced strongly by ABA as early as 3 h post application and stayed high throughout the course of the experiment or decreased by 48 h, respectively (Fig. 7C).

Interestingly, ABA, the key regulator of osmotic stress responses, was the only phytohormone tested, that was competent to induce *ZmLOX11* transcripts in leaves. Therefore, we hypothesized that *ZmLOX11* may be inducible under osmotic stress-related conditions such as cold and/or drought stress responses. To examine this possibility we measured accumulation of *ZmLOX10* and *ZmLOX11* in response to low temperature. In this experiment, after exposing the young seedlings to chilling stress by shifting the plants from 22°C to 4 °C, *ZmLOX10* transcripts were induced rapidly and reached maximum at 3 h but

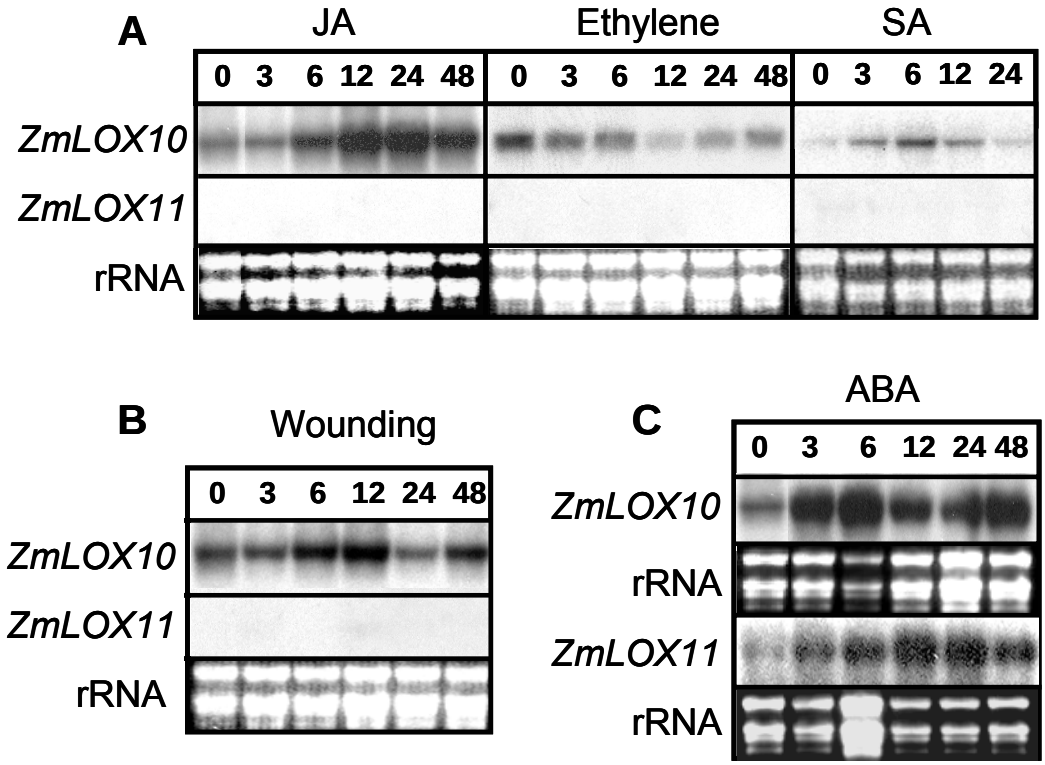


Fig. 7 RNA blot analysis of wound responsiveness and hormonal regulation of *ZmLOX10* and *ZmLOX11* expression. Analysis of transcript levels of *ZmLOX10* and *ZmLOX11* in V2 leaves in response to treatment with 200 μM JA, ethylene, 2.5 mM SA (A), 100 μM ABA (C) and wounding (B). Equal loading of RNA was visualized by EtBr staining and exposure to UV-light revealing amounts of rRNA. Numbers on top indicate hours after treatment.

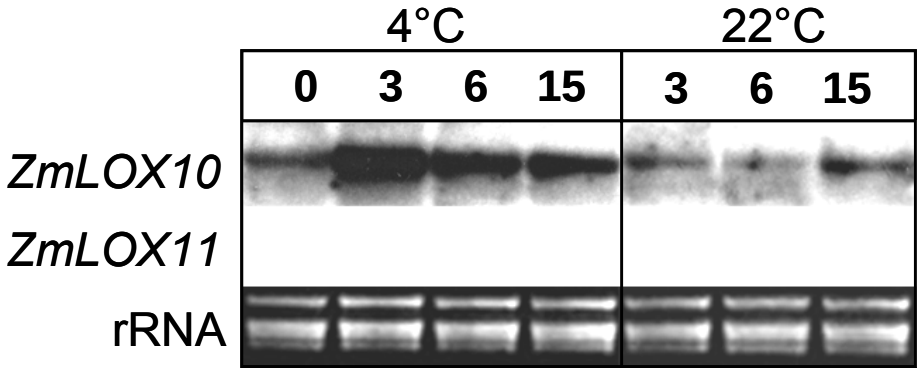


Fig. 8 Nothern blot analysis of *ZmLOX10* and *ZmLOX11* transcript accumulation in maize seedlings subjected to cold treatment (4 °C) and grown at room temperature (22 °C) control plants. Time points at which leaf tissue was harvested is indicated in the boxes above the figure in hr after the treatment was applied. After extraction 10μg of total RNA was used for RNA blot assay. Equal loading is visualized by exposure of EtBr-stained rRNA to UV-light.

declined thereafter (Fig. 8). However, no induction of *ZmLOX11* transcripts was observed in response to chilling stress.

ZmLOX10 but not *ZmLOX11* is pathogen inducible

Induction of *ZmLOX10* transcripts in response to JA and SA suggests that this gene may play an important role in maize defense reactions against pathogens. Therefore, we elucidated whether expression of this gene can be altered differentially during compatible or incompatible plant-pathogen interactions. For this experiment, two-week old seedlings of the Pr inbred line were infected with two strains of well characterized necrotizing fungal agent of Northern corn leaf spot *Cochliobolus carbonum* race 1. Pr plants are susceptible to the HC-toxin producing wild type strain (Tox2+) and resistant to the mutant strain (Tox2-) that is abolished in HC-toxin production (Multani et al. 1998). Fig. 9 illustrates that *ZmLOX10* transcripts were induced to higher levels reaching maximum at 24 h post-inoculation during incompatible interaction with *C. carbonum* Tox2- strain. The compatible interaction with the HC-toxin producing strain resulted in only a slight increase in mRNA accumulation comparable to the mock inoculated controls. It is possible that low but detectable increases in the transcript levels in the mock- and Tox2+ strain treated plants could be due to fluctuations of gene expression driven by circadian rhythm. Alternatively, this induction can be the result of close proximity of the mock- and Tox2+ strain-inoculated plants to the

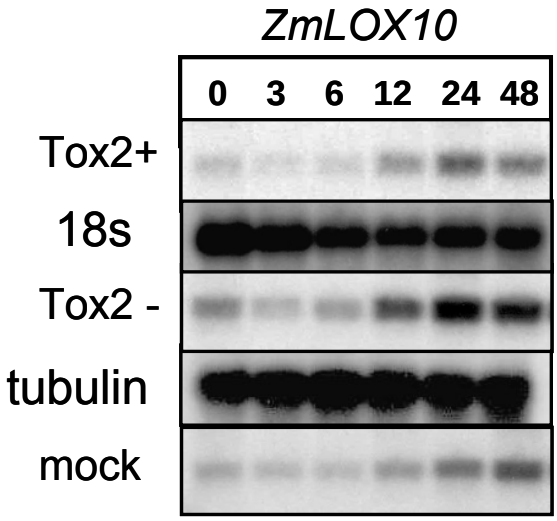


Fig. 9 Northern blot analysis of *ZmLOX10* expression in response to infection with *Cochliobolus carbonum*. Leaves of Pr inbred line inoculated with fugal pathogen that causes Nothern corn leaf spot - *Cochliobolus carbonum* race 1: wild type strain (Tox2+) and mutant strain non-producer of HC-toxin (Tox2-). Conidial suspension of *C. carbonum* race 1 either Tox2+ and Tox2- were sprayed on maize seedlings at V2 developmental stage. Equal loading of total RNA was tested by hybridizing ^{32}P-labeled cDNA encoding either 18S rRNA or tubulin, correspondingly.

plants inoculated with the avirulent strain that might have produced certain volatile defense signals such as hexenal or methyl jasmonate capable of inducing *ZmLOX10* expression. No induction of mRNA was observed for *ZmLOX11* in response to infection by either pathogen tested (data not shown). These data suggest that *ZmLOX10* but not *ZmLOX11* may be specifically involved in defense mechanisms against necrotrophic fungal pathogens.

Discussion

The biological significance of oxylipin metabolism initiated by 13-LOXs in general and jasmonates in particular in any monocot species is little understood. To gain more insights into the role of maize 13-LOXs and their metabolites, in this study we report on cloning and characterization of two novel maize LOXs, *ZmLOX10* and *ZmLOX11*. High degree of identity between these two genes at both cDNA (93%) and protein (90%) sequence levels reflected by their extremely close clustering on the phylogenetic tree and their localization to different chromosomes suggest that these genes evolved as a result of a relatively recent segmental duplication event. Both predicted proteins have a recognizable N-terminal chloroplast localization signal. In fact, ZmLOX10 is localized to chloroplasts as it has been shown by recent comparative proteomics study of maize bundle sheath and mesophyll chloroplasts (Majeran et al. 2005). This study identified and sequenced two LOX proteins that predominantly

accumulated in the chloroplasts of mesophyll cells. One of these proteins (TC298873, formerly TC234252 in (Majeran et al. 2005) is 100% identical to ZmLOX10 at the amino acid level. Because both predicted proteins appear to be localized to plastids and cluster together with other *type-2* 13-LOXs (Feussner and Wasternack 2002) we concluded that ZmLOX10 and ZmLOX11 can be classified as *type 2* 13-LOXs. This conclusion was further supported by the fact the recombinant ZmLOX10 protein is indeed a linoleate 13-LOX. On the grounds of their extremely high sequence homology, it is very likely that ZmLOX11 possesses the same regio-specificity as ZmLOX10. Similarly to our case, Tsitsigiannis et al. (2005) reported that another highly similar pair of 13-LOXs from peanut, PnLOX2 and PnLOX3, had identical biochemical properties as it is the case for the three plastidic 13-LOXs from barley (Bachmann 2002).

To understand the physiological function of ZmLOX10 and ZmLOX11, it is important to address the key question: what are the final oxylipin products of the pathway(s) initiated by these LOX isoforms. The comparison of ZmLOX10 and ZmLOX11 to the well studied LOXs from other plant species indicated that the barley *lox2:Hv:3* gene is the closest monocot relative. *Lox2:Hv:3* is also expressed predominantly in leaves and is proposed to be involved in channeling of linolenic acid substrate into the HPL-mediated production of C6-volatiles (Bachmann 2002;Vörös et al. 1998). Another close monocot homolog of these genes, the rice RCI-1 does not to contribute to JA biosynthesis (Zabbai et al. 2004). Moreover, antisense suppression of tobacco *NaLOX2* (Halitschke and

Baldwin 2003) and potato *LOXH1* (Leon et al. 2002), the two most closely related dicot homologs of *ZmLOX10* and *ZmLOX11*, were devoid in the production of C6-volatiles rather than in JA. Therefore, we hypothesize that the most likely biochemical function of ZmLOX10 and ZmLOX11 is in the HPL-mediated production of C6-aldehydes and alcohols and not in the biosynthesis of JA. It is well established that JA is produced from linolenic acid via sequential enzymatic action of a 13-LOX, an allene oxide synthase, an allene oxide cyclase, a 12-oxo-phytodienoic acid reductase (OPR) and three consecutive β–oxidation steps (Feussner and Wasternack 2002). The transcripts of the genes coding for the JA-producing enzymes including 13-LOXs are usually consecutively induced in response to JA treatment with each of the genes downstream in the biosynthetic pathway being induced at a later time point (Sasaki et al. 2001). Recently, we cloned and characterized the maize OPR gene family consisting of eight genes, only two of which, *ZmOPR7* and *ZmOPR8*, most likely encode the JA-producing enzymes (Zhang et al. 2005a). In support of our hypothesis that ZmLOX10 is not involved in JA biosynthesis, accumulation of *ZmOPR7* and/or *ZmOPR8* transcripts was increased and peaked at least 3 and 6 hours prior to *ZmLOX10* expression in V2 leaves in response to JA treatment and wounding, respectively. Since OPRs are three enzymatic steps downstream of 13-LOXs, it may be unlikely that ZmLOX10 supplies 13-hydroperoxy linolenic acid for JA production. This role is most likely confined to three other 13-LOXs found in maize some of which are induced by

JA at much earlier time points (Kolomiets et al., unpublished data). However, the possibility that ZmLOX10 and/or ZmLOX11 may be responsible for production of JA can not be ruled out completely, as AtLOX2 that exhibited the highest homology to ZmLOX10 amongst Arabidopsis LOXs, participates in the wound-induced JA synthesis (Bell et al. 1995).

Analysis of organ- and tissue-specific mRNA accumulation showed that despite their extremely high level of sequence similarity *ZmLOX10* and *ZmLOX11* are expressed differentially in diverse organs of unchallenged plants. While *ZmLOX10* is expressed predominantly in leaves, transcripts of *ZmLOX11* accumulated abundantly in silks only, suggesting that this gene is silk-specific. In agreement with this finding only ZmLOX10 protein but not ZmLOX11 accumulate to high levels in mesophyll chloroplasts (Majeran et al. 2005). In our experiments with silks we did not detect any change in *ZmLOX11* transcript levels in silks at different times after pollination (data not shown). Thus, the biological significance of silk-specific expression of *ZmLOX11* is currently not clear.

Our study showed that *ZmLOX10* transcript levels increased transiently by wounding, chilling and treatments with JA, SA and ABA, while *ZmLOX11* was only induced by ABA, thus supporting our hypothesis that physiological function of *ZmLOX11* maybe different from that of *ZmLOX10*. SA is a key signaling molecule required in the development of systemic acquired resistance (SAR) (Ryals et al. 1995) and it also mediates defense reactions against biotrophic

pathogens (Glazebrook 2005). Conversely, JA inhibits SA-induced expression of SAR (Traw et al. 2003), and is a key component of resistance mechanisms against necrotrophic pathogens and insects (reviewed in (Dong 1998). Induction of *ZmLOX10* by both JA and SA may seem rather surprising since numerous published reports indicated that JA and SA act antagonistically in pathogen- and especially in insect-induced defense reactions (Cipollini et al. 2004;Feys and Parker 2000;Pena-Cortes et al. 1993). However, a recent Arabidopsis microarray study showed that in response to JA, SA and ethylene treatments 55 out of 705 differentially expressed genes were co-induced by SA and JA suggesting a more complex interaction between SA- and JA-mediated regulation of gene expression (Schenk et al. 2000). Similar to *ZmLOX10*, a barley chloroplast-targeted 13-LOX is induced by both methyl jasmonate and SA (Weichert et al. 1999). Interestingly, in their study, SA treatment of barley leaves resulted in preferential channeling of 13-LOX primary products 13-fatty acid hydroperoxides into reductase-mediated production of 13-hydroxyoctadecatri(di)enoic acids (HOT(D)), thus blocking biosynthesis of JA. Because of observed stronger induction of *ZmLOX10* by JA compared to SA, it is tempting to speculate that this gene and its products maybe primarily involved in defense responses to insects and necrotrophic pathogens.

Several 9- and 13-LOXs from Arabidopsis and other dicot plants participate in plant defense responses against herbivorous insects and diverse pathogens (Bell et al. 1995;Halitschke and Baldwin 2003;Montillet et al.

2005;Rance et al. 1998b;Royo et al. 1999). Here we studied *ZmLOX10* and *ZmLOX11* involvement in defense mechanisms in the plant-pathogen system where compatible as well as incompatible interactions can be compared using a single maize genotype but different near-isogenic strains of *C. carbonum*. In Pr plants inoculated with the avirulent strain of *C. carbonum* (incompatible interaction) expression levels of *ZmLOX10* were notably higher when compared to mock treatment and treatment with the virulent strain Tox2+ (compatible interaction). Interestingly, accumulation of the highest levels of *ZmLOX10* preceded visual appearance of hypersensitive response (HR) -like necrotic lesions suggesting its involvement in the HR-associated cell death. Similarly to *ZmLOX10* a 13-LOX homolog from rice is induced only during incompatible interaction of rice seedlings with rice blast fungus *Magnaporthe grisea* (Peng et al. 1994). Expression of *ZmLOX11* was neither detected nor induced in all variants at least by northern blot analysis (data not shown), thus suggesting no role for this duplicated gene in defenses against *C. carbonum*.

ABA is the only hormonal signal that induces both *ZmLOX10* and *ZmLOX11*. This hormone was demonstrated to be the main signaling molecule that orchestrates plant responses to osmotic stress such as those associated with drought or chilling stresses (Zeevaart and Creelman 1988). To test the possibility of functional overlap between *ZmLOX10* and *ZmLOX11* in leaves during the stress caused by the change in plant osmotic status we tested accumulation of the transcripts of these genes in response to low temperature.

ZmLOX10 but not *ZmLOX11* mRNA levels increased rapidly in response to chilling treatment, thus excluding the possibility of subfunctionalization of *ZmLOX10* and *ZmLOX11* in chilling stress responses. Nevertheless, induction of *ZmLOX10* expression during chilling stress suggests a role, although as yet unclear, of this LOX isoform in maize response to chilling and possibly other osmotic stresses.

A very important finding from our study is that *ZmLOX10* is regulated by circadian clock. To the best of our knowledge, this study is the first reported case of a plant LOX gene regulated transcriptionally by the circadian clock. Moreover, the period of the increased accumulation of transcripts (10:00 – 18:00) coincided with the period of most active photosynthesis. Interestingly, ZmLOX10 mRNA exhibited oscillation with 24 h-periodicity similar to the typical rhythm described for Arabidopsis genes encoding seven photosystem I and three photosystem II proteins that also peaked at around midday (Harmer et al. 2000). Besides, our data showed that enzymatic activity of ZmLOX10 protein is the highest at pH 8.0, which is also within the range of physiological pH-values during photosynthesis (Schaffrath et al. 2000). Based on our current surprisingly limited knowledge of diurnal regulation of lipid metabolism in plants, we propose three alternative hypotheses to explain the possible physiological significance of clock-controlled *ZmLOX10* expression. Firstly, *ZmLOX10* may fulfill a role proposed for 13-LOXs in the complex membrane lipid peroxidation during photosynthesis (Feussner et al. 1998). Localization of ZmLOX10 in close

proximity to chloroplast membranes mediated through the PLAT domain identified in this study may aid in such a process. With regard to this proposed role of *ZmLOX10* in photooxidative stress it is interesting that two circadian-regulated rat LOXs, 12-LOX and 5-LOX, also showed their maximum activity during the day time and have been suggested to play key roles in a number of neurobiological processes including generation of ROS and cell death (Li et al. 1997). These clock-controlled rat LOXs were also shown to have a role in calcium intracellular release (Reynaud et al. 1994) and modulation of ion-channels activities typically observed during neurotransmission processes (Piomelli et al. 1987). Having this data in mind our second hypothesis is that clock-dependent ZmLOX10-derived oxylipins might be involved in the modulation of ion-channels and calcium homeostasis, and thereby regulate stomatal conductance during the period of active photosynthesis.

Another alternative explanation of circadian regulation of *ZmLOX10* is that this isozyme may be involved in the production of oxylipins with signaling activities which in turn may help maize plants to better adjust to low night temperatures and, therefore contribute to chilling tolerance. Recent study of genome-wide expression of circadian regulated Arabidopsis genes revealed that several enzymes involved in lipid modification were under the clock control and were proposed to have a role in plants adaptation to chilling temperatures during night (Harmer et al. 2000). The fact that ZmLOX10 was induced early following the chilling treatment indirectly points towards this hypothesis. This idea is also

supported by the presence of multiple ABA-, cold- and dehydration responsive cis-acting elements such as ERD1, ABRE and DRE (Zhang et al. 2005b) in the *ZmLOX10* promoter region. It is interesting that the gene encoding Arabidopsis transcription factor DREB1a/CBF3 was itself clock-controlled and peaked at around subjective midday, similar to *ZmLOX10* (Harmer et al. 2000). Involvement of ZmLOX10 in chilling adaptation may be further supported by the fact that amounts of LOX substrates linoleic and linolenic acids fluctuate in a light-dependent manner and are increased upon low temperature treatment (Rikin et al. 1993). Regardless of which of the proposed hypotheses is correct, diurnal regulation of ZmLOX10 is intriguing and warrants further examination.

In summary, based on mapping of *ZmLOX10* and *ZmLOX11* to different chromosomes and on extremely high sequence identity to each other we speculate that this gene pair arose from relatively recent segmental gene duplication event. Recent comparative study of two types of gene duplications, tandem (linked) and segmental (unlinked) duplications, in Arabidopsis suggested that (1) the average age of segmentally duplicated pairs is twice as high as that of tandemly duplicated genes, and (2) perhaps, because of this difference in time of gene divergence, a significantly larger proportion of segmentally duplicated genes show a less similar expression pattern compared to tandemly duplicated genes (Haberer et al. 2004). Remarkably dissimilar expression pattern of *ZmLOX10* and *ZmLOX11* in all but ABA treatments fit this observation. This study represents an example of evolutionary employment of

duplicated genes that have distinct physiological functions despite their high overall homology. In order to determine conclusively the physiological function of the pathogen-inducible 13-LOX, *ZmLOX10*, we identified *Mu*-transposable element insertional mutants for this gene. Currently, we are in the process of introgressing these mutant alleles into the appropriate genetic backgrounds differing in their levels of resistance to diverse pathogens and pests.

CHAPTER III

MUTATION IN A MAIZE 9-LIPOXYGENASE *ZMLOX12* RESULTS IN DECREASED COLONIZATION OF MESOCOTYLS BY *FUSARIUM VERTICILLIOIDES*

Introduction

Fusarium verticillioides (synonym, *Fusarium moniliforme* Sheldon; teleomorph, *Gibberella moniliformis* [synonym, *Gibberella fujikuroi* mating population A]), is an ascomycete fungal pathogen causing ear and stalk rots of maize (corn, *Zea mays* L.) producing large yield losses in many parts of the world (Desjardins 2003;Rheeder et al. 2002). *F. verticillioides* most frequently can be isolated from kernels where it can reduce grain quality by producing fumonisins - mycotoxins carcinogenic to humans and animals (Proctor et al. 2006;Rheeder et al. 2002). Invasion through the silks is the most common among the possible routs of kernel infection, but as a result only a minor portion of infected kernels become symptomatic (Munkvold 1997;Oren et al. 2003). Another well established pathway for infection is through the underground organs where inoculum inside or on the surface of the seed spreads to the roots and through the mesocotyl to stalk, and later to the ears (Munkvold 1997;Oren et al. 2003). In addition, ability

of *F. verticillioides* to successfully survive in the soil on the plant debris makes that debris an additional source of inoculum (Rheeder and Marasas 1998). Systemic infection by *F. verticillioides* especially on early stages of development may result in blight of seedlings and root and stalk rots (Nelson 1992). Interestingly, it was observed that in some inbred lines transmission of the fungus from the seedling mesocotyl and crown to stalk was a limiting step of infection (Munkvold 1997). Conveniently, etiolated maize mesocotyls were proven to be an effective tool to investigate maize-pathogen interactions (Heim 1983).

The infection process of maize seedling tissues by *F. verticillioides* is influenced by a number of environmental and molecular factors. Among the latter, lipids were described as critical components for many physiological reactions in *Fusarium* spp. and other phytopathogenic fungi (Yu and Keller 2005). A subclass of lipid molecules called oxylipins was reported to regulate fungal development, reproduction and pathogenicity (Calvo et al. 2001;Kunz et al. 2006). Oxylipins are oxygenated fatty acids produced by the enzymatic or nonenzymatic incorporation of oxygen into the fatty acid carbon chain (Feussner and Wasternack 2002;Shah 2005). In ascomycete fungi, hydroperoxides of polyunsaturated fatty acids including palmitoleic (16:1), oleic (18:1) and linoleic (18:2) acids produced in mixtures, and collectively called psi-factor, were shown to regulate both asexual and sexual spore development (Calvo et al. 2001;Champe and el-Zayat 1989;Champe et al. 1987). In *Aspegillus spp*.,

linoleic acid and its derivatives are conserved signal molecules modulating mycotoxin biosynthesis, spore production, survival and other aspects of fungal differentiation processes (Brodhagen and Keller 2006).

Because some plant-derived oxylipins are structurally similar to fungal psi factors, they were implicated as possible signals mimicking action of endogenous fungal psi-factors (Brodhagen and Keller 2006). Indeed, purified linoleic acid and hydroperoxy linoleic acids derived from corn seed promoted sporogenic activities in several *Aspergillus* species (Calvo et al. 1999a). Plant oxylipins are strongly implicated as signaling molecules that modulate reproductive development, germination, senescence, programmed cell death, tolerance to cold, drought and salt stresses, plant-to-plant and plant-to-pest communication, and resistance to diverse pathogens (Feussner and Wasternak 2002; Shah 2005). Most plant oxylipins identified to date are formed via lipoxygenase (LOX) enzymatic activity. LOX catalyzes oxygenation of free polyunsaturated fatty acids, linoleic (18:2) and linolenic (18:3), either at carbon position 9 and 13 in the fatty acid backbone, thereby resulting in the formation of distinct 9- and 13-hydroperoxides, respectively. Thus, based on the position of carbons in fatty acid chain targeted for oxygenation, LOXs are referred to as 9- or 13-LOXs (Feussner and Wasternack 2002;Liavonchanka and Feussner 2006;Shah 2005). Much attention was devoted to the 13-LOX-initiated pathways of which the chloroplast-localized allene oxide synthase (AOS) pathway and hydroperoxide lyase (HPL) branches of the pathway are the best characterized.

AOS-mediated or so called octadecanoid pathway produces jasmonate compounds that function as signals in transduction pathways that regulate expression of defense-related and developmental genes (Turner et al. 2002). The HPL-derived oxylipins C_6 green leafy volatiles (GLVs) have been shown to possess both anti-microbial (Prost et al. 2005) and defense signaling activities (Bate et al. 1998).

Although, 13-LOXs are often found to confer resistance to insects and pathogens (Howe and Schilmiller 2002;Porta and Rocha-Sosa 2002), physiological function of 9-LOXs and their metabolites in dicots and even more so in monocots remains elusive. Accumulating largely circumstantial evidence suggests that specific 9-LOXs may function in the regulation of vegetative growth. The only definitive proof of such a function was shown for a tuber-specific potato 9-LOX *POTLX-1,* where antisense suppression resulted in decreased radial growth and malformation of tubers (Kolomiets et al. 2001). However, 9-LOXs are most often implicated in plant defense responses associated with resistance to diverse biotic and abiotic stress (Blee 1998;Howe and Schilmiller 2002). Role of 9-LOXs in resistance to fungal pathogens was conclusively demonstrated in antisense knockout experiments in tobacco (Rance et al. 1998b), where deficiency in the *LOX1* transcripts resulted in loss of hypersensitive response (HR)-mediated resistance towards *Phytophthora parasitica*. The closest homolog to this tobacco gene, *POTLX-3*, is also induced by pathogens during incompatible interactions with maximal levels of expression

preceding the appearance of HR-type necrotic lesions (Kolomiets et al. 2000). These results are in agreement with the recent findings that 9-LOX-catalyzed lipid peroxidation is the primary driving force behind cell death processes in tobacco leaves treated with the elicitor cryptogein and an avirulent strain of *Pseudomonas syringae* pv *syringae* (Montillet et al. 2005;Rusterucci et al. 1999). Similarly, in multiple other reports 9-LOXs were induced either exclusively or more rapidly and to higher levels during incompatible interactions with avirulent pathogen strains compared to infections with virulent strains (Bohland et al. 1997;Gobel et al. 2001;Hamberg et al. 2003) further suggesting the active role of 9-LOXs in plant-host resistance mechanisms. In contrast to these studies, Wilson et al. (2001) showed that two different 9-LOX gene in maize (*ZmLOX2*, formely chssh76 and *ZmLOX3*, formely cssap92) were induced by *F. verticillioides* and *Aspergillus* spp., suggesting the involvement in maize-pathogen interaction. Corroborating this idea are the pharmacological studies showing that low levels of linoleic acid and, even more so, its immediate 9-LOX product, 9S-HPODE, stimulated spore production and mycotoxin production in Aspergillus spp. In vitro (Burow et al. 1997;Calvo et al. 1999b). These findings prompted a hypothesis (Brodhagen and Keller 2006;Wilson et al. 2001) that 9-LOX activity resulted in the production of oxylipins, that were able to mimic sporogenesis-inducing psi factors and, therefore, contributed to host susceptibility. However, until now, no direct support for this hypothesis has been obtained.

As a first approach to test this hypothesis, seven maize 9-LOX genes were identified (Kolomiets, unpublished data). Here we report the identification, cloning, molecular and biochemical characterization of a novel cytoplasmic 9-LOX form maize, designated *ZmLOX12*. This monocot-specific gene was found to be inducible by JA, wounding and the mycotoxigenic fungus *F. verticillioides,* indicating its possible involvement in maize defense responses against pathogens. To elucidate the function of this 9-LOX in maize interaction with *F. verticillioides* we generated near isogenic mutant lines in which expression of *ZmLOX12* was abolished by *Mutator (Mu)*-transposible element inserted in the coding sequence of the gene. Phenotypical analysis of *lox12* mutants and wild type plants revealed that the mutants were more resistant to *F. verticillioides* colonization of mesocotyls and, therefore, supported production of fewer conidia on this organ. This finding is a strong evidence that biosynthesis of host plant 9-LOX derived oxylipins can be manipulated by fungal pathogens which results in plant susceptibility and stimulation of fungal developmental processes.

Materials and methods

Plant material and treatments

Maize plants were grown in a growth chamber in 7cm pots in Strong-Lite® potting soil (Universal Mix, Pine Bluff, AK), temperature was maintained from

22ºC to 30ºC under a 16 h day length, 50% average relative humidity, 560 to 620 µE of light. For all of the organ-specific expression studies, wounding and hormonal treatments, 2-week-old maize (*Zea mays L.*) seedlings of inbred lines B73, VA46 and R168 at the V2 developmental stage were used. For wounding experiments, the second fully expanded leaves of B73 seedlings at V3 stage were wounded by crushing the lamina with a hemostat. For hormonal treatments, the seedlings were cut at the soil level and were incubated with the cut end placed in 100 mL of either 200 µM JA, 2.5 mM SA, 100 µM ABA and 0.01% Tween-20 as a control (Sigma, St. Louis, MO). Treatment with 10 µL/L ethylene was conducted in hermetically-sealed 5.6 L dessicators.

Isolation of genomic DNA and Southern blot analysis

Freshly harvested leaves from maize line B73, sorghum (*Sorghum bicolor*. cv. B1), rice (*Oryza sativa* cv. Cutter), proso (*Panicum miliaceum* L.), barley (*Hordeum vulgare* cv. Legacy), wheat (*Triticum aestivum*), soybean (*Glycine max* (L.) Merr.) and tobacco (*Nicotiana benthamiana*) were used for extraction of genomic DNA as described (Zhang J. *et al.*, 2005). For Southern blot assay genomic DNA (10 µg) from plant species listed above was cut with the restriction enzyme *Xba*I. Digested DNA was separated on a 0.8% agarose electrophoresis gel, transferred (0.025 M phosphate transfer buffer) and then cross-linked to the nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc.,

Minnetonka, MN) by UV Stratalinker^{TM} 2400. The blots were hybridized overnight at 42 °C with a ^{32}P-labeled *ZmLOX12*-specific probe. Original EST p0006.camah52 clone 2689 bp long containing almost full length cDNA sequence lacking only 24 bp after the start codon was used as a template for labeling to produce this probe. The membrane was hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX). Washes were performed: first with low stringency buffer (2×SSC and 0.1% SDS) at 42 °C twice for 5 min, followed by an additional wash with high stringency buffer (0.2 M SSC and 0.1% SDS) twice at 42 °C for 15 min. The blots were exposed to X-ray film (Kodak, Rochester, NY) in cassettes at -80 °C for 72 h unless otherwise indicated.

Bioinformatics analysis

Homology search using ZmLOX12 translated sequences was done using the BLAST search that is publicly available at the National Center for Biotechnology Information (NCBI) web-site (www.ncbi.nlm.nih.gov/BLAST/). Percentage identity of ZmLOX12 with other plant LOXs was evaluated using ClustalW software available at the European Molecular Biology Laboratory, the European Bioinformatics Institute (EMBL-EBI) (www.ebi.ac.uk/clustalw/). Subcellular localization was predicted based on the identification of signal peptide sequences by two different programs ProtComp (www.softberry.com/ berry.phtml), Proteome Analyst - Subcellular Specialization Server 2.5

(http://pasub.cs.ualberta.ca:8080/pa/Subcellular). Presence of conserved domains in the deduced amino acid sequences was determined by NCBI Conserved Domain Search (www.ncbi.nlm.nih.gov/Structure/cdd/cdd. shtml) and Pfam software (www.sanger.ac.uk/Software/Pfam/). To determine the genomic structure of *ZmLOX12,* a homology-based blast search was performed by utilizing the databases of the maize Genome Survey Sequences (GSS) (available at www.ncbi.nlm.nih.gov/BLAST/, www.plantgdb.org/ PlantGDB-cgi/blast/PlantGDBblast and (http://tigrblast.tigr.org/tgi_maize/index. cgi) as well as Maize Assembled Genomic Island databases (MAGI) (available at www.plantgenomics.iastate.edu/ maize/).

Construction of overexpression vector and analysis of ZmLOX12 biochemical properties

The entire open reading frame was PCR amplified from root-specific cDNA using primers with adopted in-frame *EcoRI*-sites 5'-CTT-TGA-ATT-CAT-GCA-AAT-GCC-CTT-CTG-TC-3' and 5'-CGA-GAA-TTC-CAA-TTG-TCA-TAT-GGA-GAC-GCT-G-3'). Following separation and elution from gel, the purified fragment harboring the *ZmLOX12* coding sequence was ligated into the pCR2.1 TOPO® vector (Invitrogen, Carlsbad, CA). After restriction digestion with *Eco*RI, the coding region of *ZmLOX12* was placed under the control of isopropyl thio-*β*-D-galactopyranoside (IPTG)-inducible promoter of an *E. coli* expression vector

pET28a containing Amp+ and Kan+ resistance genes as selection markers (EMD Biosciences, Inc., San Diego, CA) using T4 DNA Ligase (Roche Applied Science, Indianapolis, IN). After ligation *ZmLOX12*/pET28a expression construct was heat-shock transformed into BL21 (DE3) strain of *E. coli* chemically competent cells (EMD Biosciences, Inc., San Diego, CA). Two independent *E. coli* BL21 (DE3) cultures containing the expression constructs were grown overnight at 37 °C in 20 ml LB medium containing 50 µg/mL ampicillin and 25 µg/mL kanamycin. When the cell culture reached absorbtion value A600 = 0.7, 1 mM IPTG was added to induce expression of the constructs at 15 °C for 48 h. Cells collected from 200 mL of culture were resuspended in 30 mL of lysis buffer (50 mM Tris-HCL, pH 7.5) that contained 10% (v/v) glycerol, 0.5 M NaCl and 0.1% Tween-20. Cells then were disrupted using a sonifier tip with a frequency of 5 pulses per 30s. To remove cellular debris, samples were centrifuged at 12.000×g for 15 min. The supernatant was divided into 1 mL aliquots and stored at -20 °C (Feussner *et al.*, 1998). Oxygenation of linoleic acid was carried out by incubating the crude extract of bacteria containing *ZmLOX10* overexpression construct with the substrate (120 µM final concentration) diluted in 1 mL of 0.1 M sodium phosphate buffer (pH 5.7-7.0) or in 0.1 M Tris buffer (pH 7.7–8.6), respectively, for 20 min at room temperature. The reaction was stopped by addition of 100 µL of glacial acid. After centrifugation, the organic phase was vacuumed. Remaining lipids were resuspended in 0.1 mL of HPLC solvent. Detection of LOX products by HPLC

was carried out on an Agilent (Waldbronn, Germany) 1100 HPLC system, where absorbances were recorded simultaneously at 234 nm and 210 nm. The enantiomer composition of the hydroxyl fatty acids was analyzed as described by Feussner and Kühn (1995).

Infection of plants with *Fusarium verticillioides*

Two-week-old seedlings grown in dark for etiolated mesocotyls were used to study the gene expression of *ZmLOX12* in two genetic backgrounds during *F. verticillioides* infection. For all studies related to *F. verticillioides* infection, we used strain 7600 (M3125; Fungal Genetics Stock Center, Kansas City, KS), which was stored at −80 °C in 20% glycerin. For conidia production, the fungus was grown at 28 C on potato dextrose agar medium (PDA; B&D, Sparks, MD) as described by Shim and Woloshuk (1999). Suspensions containing 10^6 conidia/ mL in 0.1% Tween-20 were prepared by collecting conidia in 5 mL of sterile water from 3-week-old V8 agar cultures and then filtering through four layers of miracloth. Control plants were inoculated with sterile water containing 0.1% Tween-20. Plant mesocotyls were first slightly wounded using the syringe needle at three separate sites (3 cm apart from each other) on each mesocotyl. *F. verticillioides* conidial suspension (10 µL), was applied to each wound site on horizontally placed mesocotyls. Following inoculation, plants were immediately placed under a plastic cover to create moisture environment conducive for

conidial germination. Inoculated and control plants were incubated for selected times under indicated conditions. After each incubation period, infected and control mesocotyls were collected and frozen immediately in liquid N_2 and stored at -80 °C until used for total RNA or DNA extractions.

RNA extraction and northern and dot blot analysis

Total RNA from corn tissues was extracted by using TRI reagent (Molecular Research Center Inc., Cincinnati, OH) according to the protocol provided by the manufacturer. After extraction, 10 µg of total RNA from each experiment was separated using electrophoresis in 1.5% (w/v) formaldehyde agarose gels in 1×MOPS buffer and then transferred onto a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA). To confirm equal loading of RNA samples into the agarose gel and uniform transfer onto the nylon membrane for all experiments, rRNA was stained with ethidium bromide and was visualized by UV light. Northern blot membranes were hybridized with ^{32}P-labeled gene-specific probes using UltraHyb hybridization solution (Ambion, Austin, TX) under conditions described in the manufacturer's protocol. Membranes were washed in 1 x SSC, 0.1% SDS at room temperature for 15 min, in 0.1 x SSC, 0.1% SDS at room temperature for 30 min, followed by a final wash in 0.1 x SSC, 0.1% SDS at 42 °C for 30 min. For autoradiography, RNA blots were exposed to a BioMax X-ray film (Kodak, Rochester, NY) for 2 to 7 days at –80 ºC. Blots

presented in this manuscript are representative examples of at least two independent experiments.

Generation of *lox12* mutants

The reverse genetics resource [Trait Utility System for Corn (TUSC)] generated at Pioneer Hi-Bred Intl. that represents a library of Mutator (Mu)-transposon-mutagenized F_2 was used to identify *lox12* mutants (Meeley 1995). Genomic DNA extracted from individual plants in this library was used to identify insertions in *ZmLOX12* by PCR-based screening as described by Bomblies et al (2000) with minor modifications. For this screening procedure we used a combination of Mu-Terminal Inverted Repeat (Mu-TIR)-specific primer (5'-AGA-GAA-GCC-AAC-GCC-AWC-GCC-TCY-A-3') with either of the Zm*LOX12*-specific primers: 61683 5'-ATG-GAG-ATG-TCC-TCG-GCC-ATC-TAC-TC-3'; lox12utrR1 5'- CCA-AAC-CAA-TCA-TCG-CAA-TAA-AAC-ATA-T-3'. Initial screening of the mutant library identified one PCR-positive individual. Fragments flanking both sides of the insertion were PCR-amplified, cloned into the pCR®2.1 TOPO® vector (Invitrogen, Carlsbad, CA), and sequenced to identify the location of *Mu*-insertions. Sequence analysis revealed that a Mu-element was inserted 23 bp ustream of the stop codon site. This mutant allele was designated *lox12-1*. In addition to the mutation in *ZmLOX12,* it is highly probable that the *lox12* mutant individual contained a number of unrelated *Mu*-elements elsewhere in the

genome. Therefore, the original *lox12-1* mutant allele was back-crossed three times (BC_3) to selected inbred lines to reduce the probability of these irrelevant mutations. Resultant BC_3 individuals were self-pollinated to generate a BC_3F_2 population of near-isogenic individuals in which homozygote mutants (*lox12-1/lox12-1*) and wild types (*LOX12/LOX12*) as well as heterozygotes at the *ZmLOX12* locus were identified by PCR and confirmed by Southern blot assays as described.

RT-PCR confirmation of *lox12-1* being a true null allele

Total RNA was extracted from mesocotyls of *lox12-1* mutant and wild type seedlings. Concentration was quantified using ND-1000 Spectrophotometer (Technologies Inc., Centerville, DE). After removing contaminating DNA using DNA-*free*™ (Ambion Inc., Austin, TX), RNA was equalized and 5 µg was used for construction of cDNA using First-Strand cDNA Synthesis kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) following the manufacturer's protocol. Total of 500 ng of each cDNA sample served as a template in PCR assay using *ZmLOX12*-specific primers: 61683 5'-ATG-GAG-ATG-TCC-TCG-GCC-ATC-TAC-TC-3'; lox12utrR1 5'- CCA-AAC-CAA-TCA-TCG-CAA-TAA-AAC-ATA-T-3'.

F. verticillioides conidiation assays

Mesocotyls of *lox12-1* and wild type seedlings in the B73 background were inoculated as described above. After twelve days post inoculation 1cm-long sections spanning the site of inoculation of infected and control mesocotyls were collected into separate microfuge tubes containing 200 µL of 0.05% Triton in sterile dH_2O for liberation of conidia. After vortexing for 10 sec, the spore suspension was immediately counted using hemocytometer. The experiments were repeated at least three times and consistent results were obtained. The data were expressed as the means of 5 mesocotyl sections/ treatment.

Results

Isolation and sequence analysis of *ZmLOX12* gene

Among a total of 767 EST clones encoding for maize LOX genes identified in 265 diverse cDNA libraries, only two clones (P0105.camah52r and P0126.cnldn76r) represented *ZmLOX12*. This suggested that *ZmLOX12* was expressed at a low level in maize. The sequence from the longest EST clone (P0105.camah52) revealed that it represents almost the entire coding sequence of the gene, missing only 24 bp of the 5'-coding sequence of the gene. This portion together with a portion of 5'-UTR sequence, which was cloned by a 5'-

RACE-like technique from a root cDNA library. The combined full-length cDNA sequence contained a complete open reading frame of 2550 bp and was named *ZmLOX12* (GeneBank accession number: DQ335770) in the order that reflects its level of homology to *ZmLOX1* as compared to the rest of the twelve maize LOX genes identified (Kolomiets, unpublished data). By using PCR with gene-specific primers and template genomic DNA from oat-maize chromosome addition lines we determined that *ZmLOX12* was located on chromosome 3, (data not shown). The deduced amino acid sequence encodes a predicted 850 amino acid peptide with the estimated molecular mass of 96 kDa and pI of 8.1. Deduced amino acid sequence of ZmLOX12 exhibited the highest homology (57%) to the translated rice cDNA AK064795 (Bachmann et al. 2002), only between 39% and 45% identity to the rest of maize LOXs, and lower than 40% to any other reported LOX from other dicot and monocot species. Such low sequence identity to other plant LOXs indicated uniqueness of ZmLOX12 and warranted the further biochemical characterization.

We used Southern blot analysis to determine whether a gene closely related to *ZmLOX12* is present in other monocot or dicot species, especially those with limited genome sequences available. The entire insert from the EST clone *P0105.camah52r* was used as a probe, and restriction fragments strongly hybridizing with the probe were found in sorghum and rice (Fig. 10). In the maize B73 inbred line only a single band was identified, suggesting that a single copy of *ZmLOX12* is present in the maize genome. However, in sorghum, the

ZmLOX12 probe hybridized to two bands with similar intensity, suggesting the presence of two *ZmLOX12*-like sequences. In addition, the presence of a sequence with less homology as evidenced by lower intensity of hybridization signal to *ZmLOX12* was also detected in the proso genome (Fig. 10). Unlike the monocot species tested, there were no hybridization signals detectable in genomic DNA from the dicots soybean, tobacco or Arabidopsis (data not shown), suggesting that this gene is monocot-specific.

For further analysis of *ZmLOX12* gene architecture, full length cDNA was aligned with corresponding genomic sequence derived from the available maize genome sequence survey (GSS) database. This analysis revealed that the *ZmLOX12* gene is comprised of six exons and five introns (Fig, part A, p74). In all cases exon-intron-exon boundaries followed the rule of consensus junction sequences GT/AG (Mount 1982).

Plant lipoxygenases were found in cytosol and various cell organells including vacuoles, chloroplasts, lipid bodies, and microsomal membranes (Liavonchanka and Feussner 2006). Analysis of predicted subcellular localization of ZmLOX12 using the publicly available programs, ProtComp and Proteome Analyst - Subcell Specialization Server 2.5, independently indicated targeting of this protein to the cytosol. Deduced amino acid sequences of ZmLOX12, like other known plant LOXs, harbored all five canonical residues involved in iron binding and enzyme catalytic activity (Prigge et al. 1996): His-532, His-541, His-699, Asn-703 and Ile-850. Similar to other LOXs, ZmLOX12

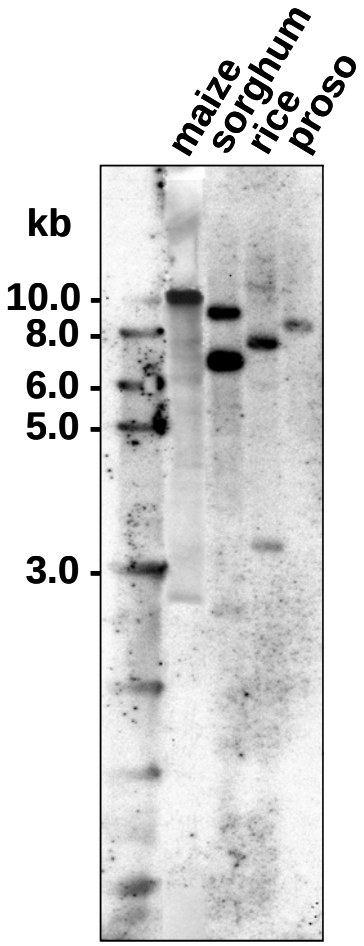


Fig. 10 Southern blot analysis reveals presence of genes homologous to *ZmLOX12* in other monocot species. Ten µg of genomic DNA extracted from maize line B73 (*Zea mays L.*), sorghum (*Sorghum bicolor*. cv. B1), rice (*Oryza sativa* cv. Cutter), proso (*Panicum miliaceum* L) was digested with *Xba*I, separated by electrophoresis, blotted onto nylon membranes and hybridized to the original EST clone p0006.camah52 which is 2689 bp long and contains almost full length cDNA sequence lacking only 24 bp after the start codon. DNA size markers in kilobases (kb) are indicated on the left.

possessed motifs required for substrate (AWRSDEEFAREMLAG) and oxygen binding (ASALHAAVNFGQY) (Santino et al. 2005) in positions 355-369 and 695-707, respectively. Unusually, instead of the common conventional motif Thr/Val reported to be indicative of all plant 9-LOXs known to-date (Sloane et al., 1991; Liavonchanka and Feussner, 2006), ZmLOX12 contains Thr/Met (566/567), which distinguishes it as a novel LOX. Analysis of the domain architecture using the Pfam program showed that the protein was comprised of two major domains: the lipoxygenase domain typical for all LOXs and the PLAT domain known to mediate membrane attachment via other protein binding partners (Marchler-Bauer et al. 2005).

Biochemical properties of recombinant *ZmLOX12*

It was impossible to predict whether ZmLOX12 is a 9- or 13-LOX based solely on sequence data because it shares only limited homology to the LOX proteins with well-established regio-specificity. Therefore enzymatic properties of ZmLOX12 were examined by overexpressing the recombinant protein in *E. coli* under the control of an IPTG-inducible promoter. ZmLOX12 was highly active at neutral to slightly acidic pH, with maximal LOX activity observed at pH 6.0 (data not shown). Therefore, regio-specificity was also determined at pH 6.0 using linoleic acid as a substrate. Since 98.1% of ZmLOX12-catalyzed products were 9*S* hydroperoxides, it was concluded that ZmLOX12 possessed a clear 9-LOX

regio-specificity. With linoleic acid as a substrate, the preferred products of ZmLOX12 enzymatic reaction detected by HPLC were 9-KOD, ketodiene (9S, 10E, 12Z)-9-hydroxy-10, 12- octadecadienoic acid and 9-HOT, (9S, 10E, 12Z, 15Z)-9-hydroxy-10, 12, 15- octadecadienoic acid (Fig. 11). The fact that resultant oxygenated fatty acids were predominantly *S*-enantiomers suggested that they were not the result of autocatalysis, but rather a product of enzymatic conversion (Mueller *et al*., 2006). Amongst 9-LOX products 9-HOD was preferentially produced (92%) as compared to 9-KOD (6.25%), whereas only minor quantities of 13-LOX products were produced as a racemic mixture of *S*- and *R*-enantiomers (data not shown). The negative control did not show detectable LOX activity. These data suggest that ZmLOX12 is a linoleate 9-LOX that is most active at the pH conditions natural for cytosol.

Organ-specific expression and regulation by wounding and defense-related hormones

To determine the function of ZmLOX12, we analyzed accumulation of specific transcripts in various maize organs and tissues. Since, ZmLOX12 mRNA was not detected by northern blotting in stalk, leaves, silks, ears, tassel or shoot apical meristem (data not shown), further analysis of organ-specific expression focused on underground organs (root, mesocotyl, crown and coleoptile) using the maize inbred lines B73, VA46 and R168 (Fig. 12A). Expression varied

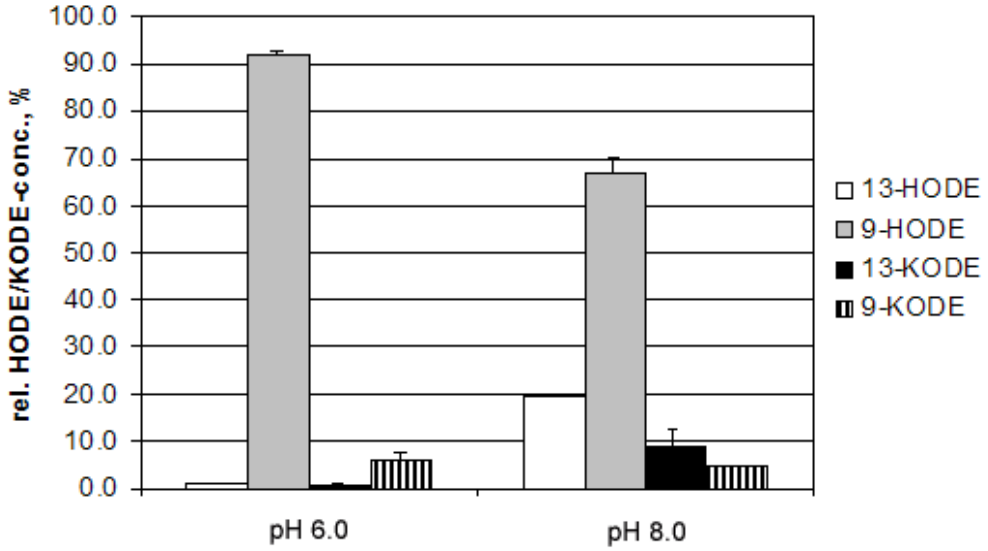


Fig. 11 Regio-specificity analysis of recombinant ZmLOX12 protein with linoleic acid used as a substrate. Analysis of LOX regio-specificity at pH-values 6.0 and 8.0 is reflected in conversion of linoleic acid by recombinant ZmLOX12 into correspondent 13-HODE, (9Z,11E,13S)-13-hydroxy-octadeca-9,11-dienoic acid; 9-HODE, (9S, 10E, 12Z)-9-hydroxy-10,12-octadecadienoic acid; 13-KODE, (9Z, 11E)-13-oxo-octadeca-9,11-dienoic acid; and 9-KODE, ketodiene (9S, 10E, 12Z, 15Z)-9-hydroxy-10, 12, 15-octadecadienoic acid.

depending on inbred line. Unlike other lines, VA46 showed highest levels of *ZmLOX12* transcripts in all tested underground organs, whereas roots of R168 had the highest levels of *ZmLOX12* mRNA. The highest expression of this gene in B73 line was in the coleoptile, however overall mRNA levels were lower in all organs tested compared to other two inbred lines.

Possibility of *ZmLOX12* contribution to stress-induced defense responses by measuring *ZmLOX12*-specific mRNA levels in responses to the defense-related hormones JA, salicylic acid (SA), abscisic acid (ABA), and ethylene we examined. *ZmLOX12* transcripts in leaves increased to substantial levels only in response to wounding and treatment with JA (Fig. 12B), but not in response to SA, ABA or ethylene (data not shown). Wounding is known to increase endogenous levels of JA, thus it was not surprising to note slightly earlier induction of *ZmLOX12* transcripts by JA compared to wounding.

ZmLOX12 is induced in *Fusarium verticillioides*-infected mesocotyls

Maize mesocotyl is usually located below the ground and often becomes a target of invasion by seed- and soil-borne pathogens. Induction of *ZmLOX12* transcript accumulation was observed at 3 and 6 hours post inoculation in VA46 and R168, respectively (Fig. 13). In mesocotyls of VA46, *ZmLOX12* mRNA accumulated earlier, and to higher levels compared to those of R168. This

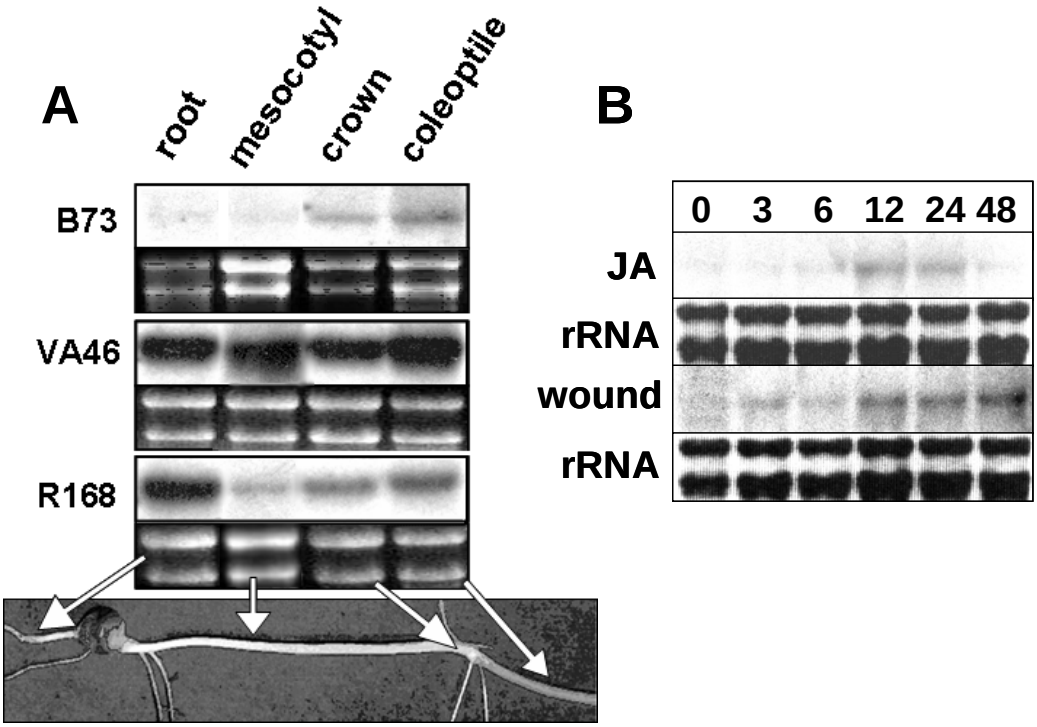


Fig. 12 Organ-specific expression of *ZmLOX12* gene. Northern blot analysis of organ and tissue-specific accumulation of *ZmLOX12* mRNA. Ten µg of total RNA were extracted from the following organs of maize plants: root, mesocotyl, crown and coleoptile. The tissues were harvested from seedlings at V2 developmental stage. The blots were hybridized to ^{32}P-labeled *ZmLOX12* probe described in Figure 10. Equal loading of RNA was confirmed by visualizing ethidium bromide (EtBr) staining of ribosomal RNA (rRNA).

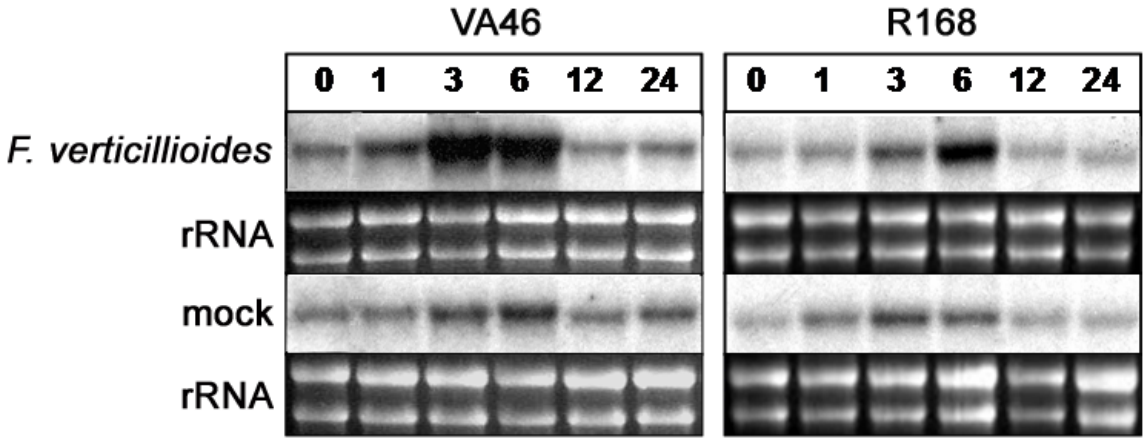


Fig. 13 *ZmLOX12* transcript levels are induced by infection of mesocotyls with *Fusarium verticillioides*. Seedlings of two different maize inbred lines VA46 and R168, which are resistant and susceptible to Fusarium ear rot, respectfully, were grown in the dark to obtain elongated mesocotyls. Mesocotyls were inoculated with 10^6 conidia/mL in 0.01% Tween-20 water (*F. verticillioides*) and sterile water with 0.01% Tween-20 (mock)-inoculated.

suggested that maize *ZmLOX12* is inducible by fungal pathogens and that its expression may affect the outcome of *F. verticillioides*–maize interaction. Although maize silks were long established to be a major pathway and potential limiting factor of *F. vericillioides* infection of kernels, in this study inoculation of silks with spores did not induce detectable levels of *ZmLOX12* transcripts (data not shown).

Generation of *lox12-1* mutant and wild type near isogenic lines

To determine the physiological function of the *ZmLOX12* gene, reverse genetic approach was used. *ZmLOX12*-specific and *Mutator* (*Mu*) Terminal Inverted Repeat-specific primers were used to screen 42,000 *Mu*-insertional individual plants with mutations throughout the maize genome available at Pioneer Hi-Bred Intl (Johnston, IA). As a result of this effort a single mutant allele (*lox12-1*) with *Mu*-insertion in the *ZmLOX12* gene was identified. Sequence analysis of the adjoining sequences on both sides of the insertion showed that *lox12-1* had a *Mu*-element inserted in exon VI, 23 bp upstream of stop codon (Fig. 14A). Total RNA extracted from mesocotyls of both wild type and homozygous mutant individuals was tested for accumulation of *ZmLOX12* transcripts to verify that *Mu*-element insertion in *lox12-1* led to suppression of expression *ZmLOX12*, and thus represented true null allele. RT-PCR assay using *ZmLOX12* gene-specific primers confirmed the absence of *ZmLOX12* message in the mesocotyls of

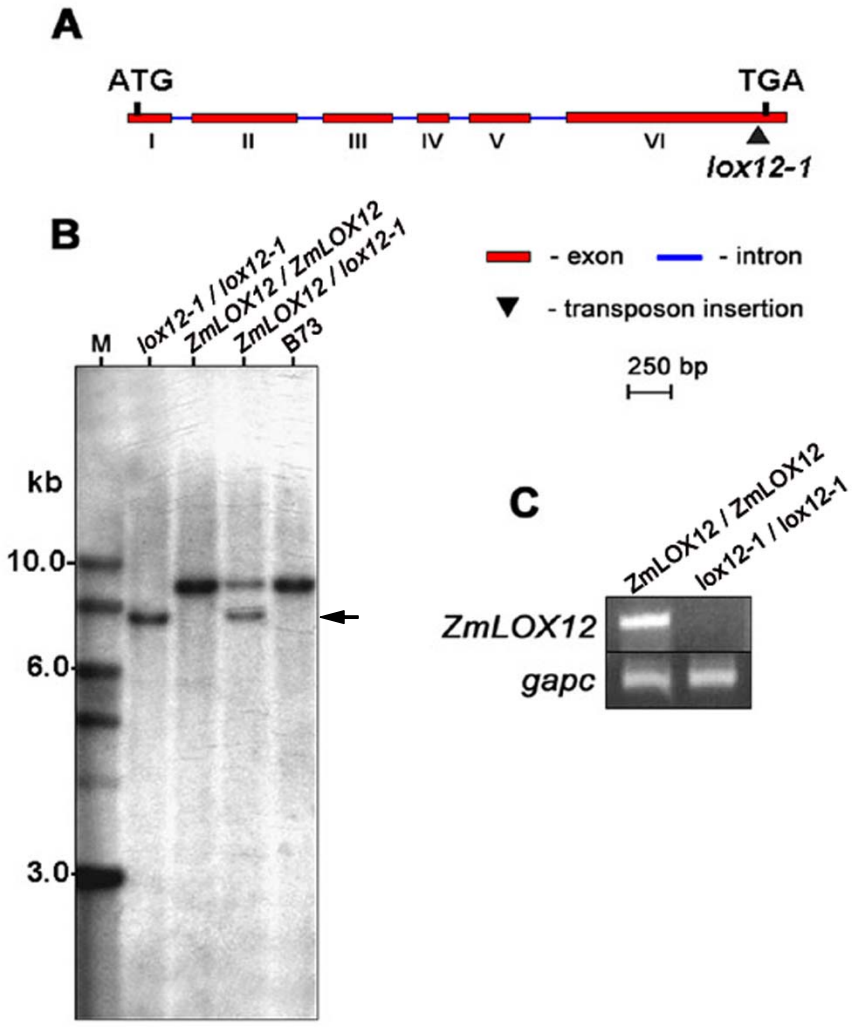


Fig.14 Identification of maize *lox12-1* mutants and verification of complete suppression of *ZmLOX12* expression by the *Mu*-element insertion in the gene. (A) Schematic representation of the *ZmLOX12* genomic DNA showing the *Mu*-element insertion site. (B) Southern blot analysis of genomic DNA extracted from individual plants of BC3F2 family segregating for *lox12-1* mutants and near isogenic wild types in B73 genetic background. Twenty μg of genomic DNA was digested with *XbaI* and hybridized with the *ZmLOX12* gene-specific probe. The arrow indicates the mutant band. M, molecular weight marker; diverse allelic combinations are indicated as follows: *lox12-1/lox12-1* (homozygous mutant), *ZmLOX12/lox12-1* (heterozygote), *ZmLOX12/ZmLOX12* (homozygous wild type) and B73 as a wild type control. (C) RT-PCR analysis of *ZmLOX12* transcript accumulation in mesocotyls of *lox12-1* mutants and WT plants. No *ZmLOX12*-specific steady state mRNA signal was detected in mesocotyls of *lox12-1* mutant seedlings, indicating that the *lox12-1* is a true null allele of *ZmLOX12*: WT - wild type; mut - homozygous mutant.

homozygous mutant *lox12-1* plants (Fig. 14C). Then *lox12-1* was out-crossed into appropriate genetic backgrounds to generate near isogenic lines and eliminate unrelated mutations. For this study, *lox12-1* was introgressed into B73 and CML176, two inbred lines that are susceptible and resistant to colonizationof mescotyls by *F. verticillioides,* respectively (Fig. 15A and Fig 16A). For both genetic backgrounds homozygous wild type and mutant plants at BC_3F_2 stage (Fig. 14B) were then used for analysis of the *ZmLOX12* gene function in host interactions with pathogens.

lox12-1 mutants are less susceptible to *F. verticillioides*

With the aim to determine if *ZmLOX12* is associated with susceptible responses of maize to *F. verticillioides*, we inoculated mesocotyls of *lox12-1* mutant and wild type seedlings in B73 and CML176 genetic backgrounds with 30uL of 10^6/mL conidia suspension. Disease development following *F. verticillioides* infection in inoculated and control plants was monitored for 12 day. Twelve days post inoculation, the mutant seedlings had noticeably less mycelial growth on their mesocotyls compared to the ild type plants, especially in the B73 line (Fig. 15A). Control mutant and WT mesocotyls were free of visible fungal colonization (Fig. 15B). Because *F. verticillioides* infection in B73 lines containing mutated and native copies of *ZmLOX12* gene resulted in rather striking difference between the two, we examined conidia production in both

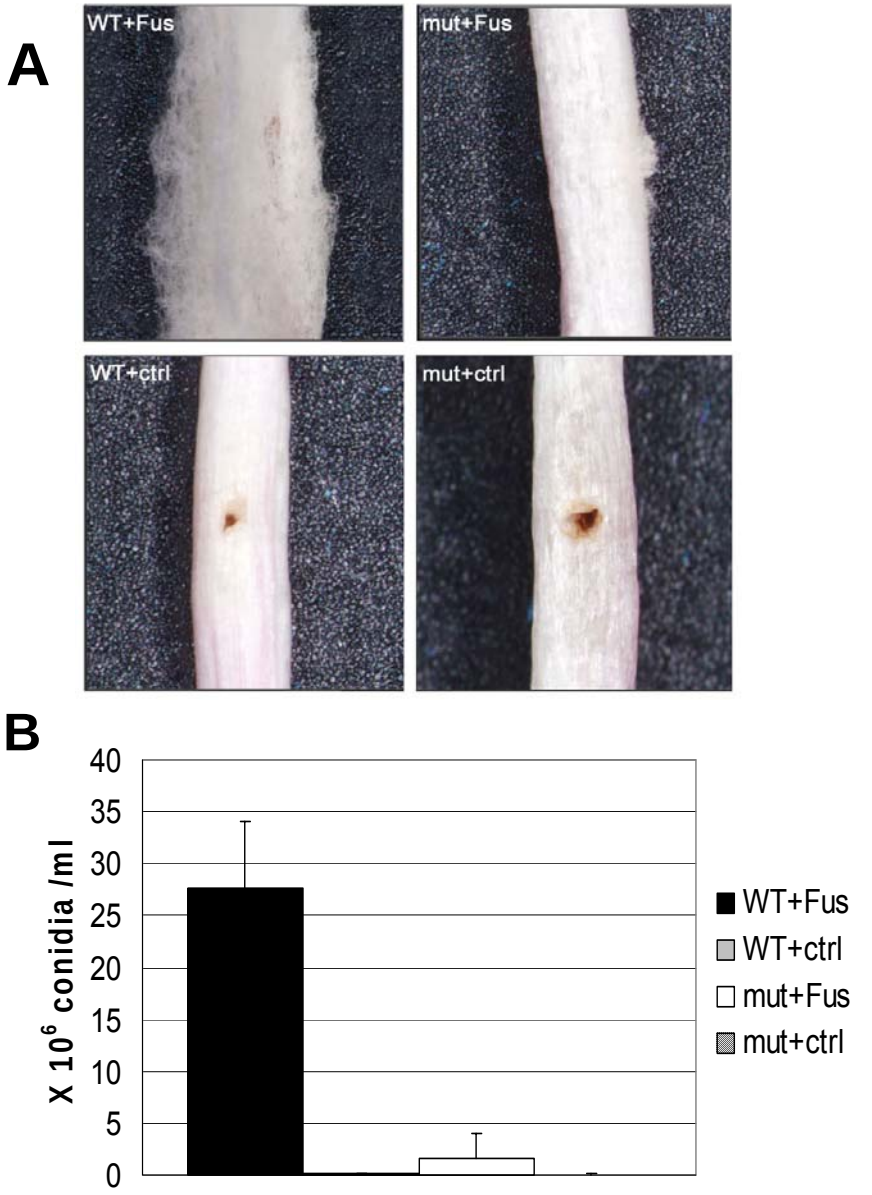


Fig. 15 *Fusarium verticillioides* mycelial growth (A) and production of conidia (B) are prominently reduced in mesocotyls after 12 days post inoculation of *lox12-1* mutants compared to near isogenic wild type in B73 genetic background. (A) Mesocotyls of WT and mutant plants were inoculated with 10^6 conidia/mL of *F. verticillioides* strain 7600 (WT+Fus) and (mut+Fus), and control (WT+ctrl) and (mut+ctrl), correspondingly. (B) Number of conidia produced on infected mesocotyls of maize *lox12-1* mutants and near-isogenic wild types 12 days after inoculation.

near isogenic lines. Twelve days post inoculation, seedlings with the wild type copy of *ZmLOX12* supported production of at least 9 times more conidia on their mesocotyls in comparison to *lox12-1* mutants (Fig. 15B). Despite surface sterilization, non-inoculated mesocotyls of wild type and mutant plants alike supported a small amount of conidia of which was, however, irrelevant when compared to inoculated plants.

The mesocotyls of CML176 line appeared resistant to *F. verticillioides* infection because response to the fungus was manifested by appearance of necrotic lesions characteristic of HR. Therefore, disease development on mesocotyls of mutant and near-isogenic wild type plants in the CML176 genetic background was monitored by measuring lesion areas (Fig. 16B). Interestingly, it was observed that all plants in CML176 background, both mutant and wild type, independent of treatment, accumulated large amounts of anthocyanins in root and mesocotyls reflected in their reddish to pinkish coloration (Fig. 16A). Production of higher levels of anthocyanins in CML176 background compared to B73 line also coincided with lower rate of fungal growth on mesocotyls, supporting the indicative role of anthocyanins in resistant plants.

Microscopic observations revealed that *F. verticillioides* infection resulted in the formation of larger lesions around the site of inoculation in WT plants compared to *lox12-1* mutants (Fig. 16B). Taken together, these data strongly support the hypothesis that ZmLOX12 and the products of its enzymatic activity may contribute to maize susceptibility to seed-borne Fusarium seedling rots at

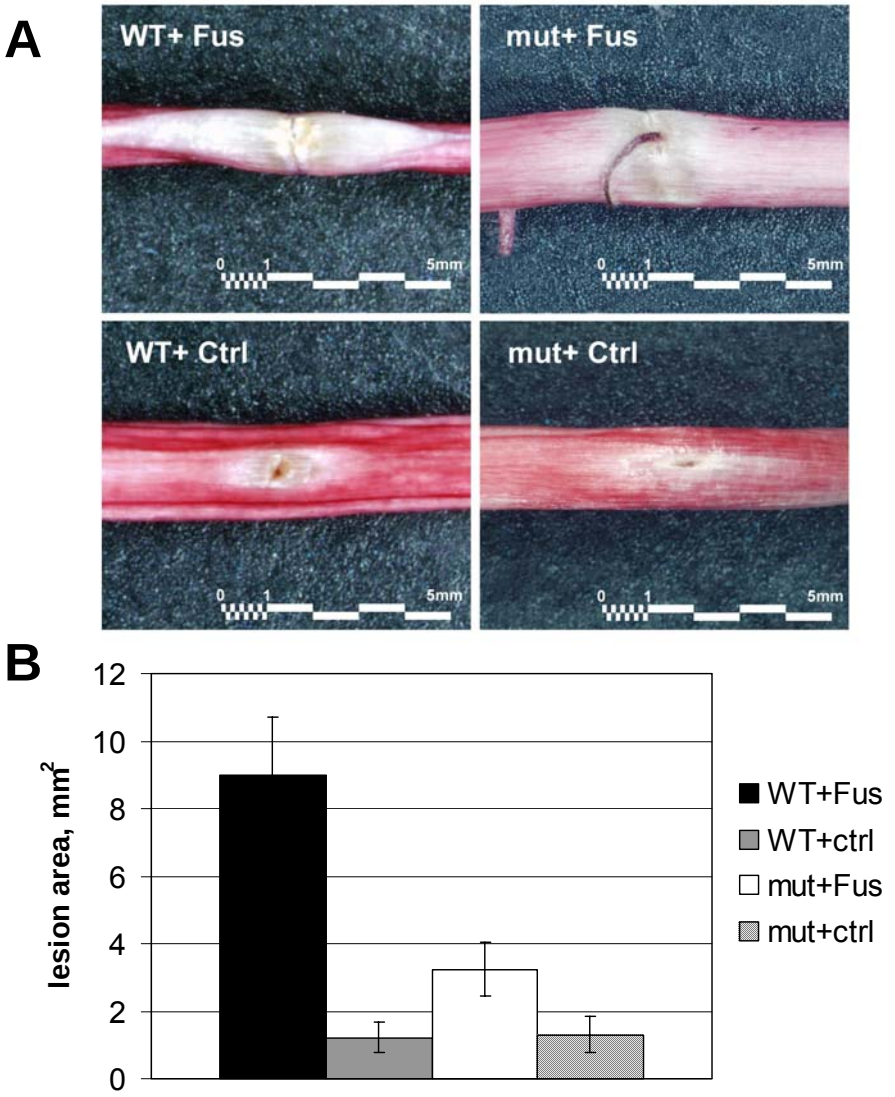


Fig. 16 Mutation in the *ZmLOX12* gene in CML176 genetic background results in reduced lesion formation on maize mesocotyls during the infection with *Fusarium verticillioides.* Mesocotyls of *lox12-1* mutants and near isogenic wild types at in the genetic background of CML176 inbred line (at the BC4F3 genetic stage) were inoculated with 10^6 conidia/mL of *F. verticillioides* strain 7600 (WT+Fus) and (mut+Fus), and mock treated (WT+ctrl) and (mut+ctrl), correspondingly. Twelve days after treatment inoculation sites were phographed using microscope and lesion areas were calculated using ImageJ program (http://rsb.info.nih.gov/ij/; (Abramoff 2004).

early stages of plant development, by positively regulating funal developmental processes such as mycelial growth and production of asexual spores.

Discussion

One measure to effectively reduce contamination of corn by *F. verticillioides,* would be to reduce infection incidence and fungal growth by targeting the vital regulators of fungal development. Diverse metabolic substances of lipid nature, especially, products of fatty acid biosynthesis and degradation, called oxylipins, were reported to possess such regulatory function (Kock et al. 2003;Noverr et al. 2003). Lipoxygenases (LOXs), widely distributed among eukaryotes, are the key enzymes involved in the biosynthesis of an array of oxylipins with distinct functions. The biological importance of 13-LOXs and their derivatives, mainly AOS-originated jasmonates and green leafy volatiles produced through the HPL branch of the LOX pathway, were reported to be required for effective defense responses to pests and pathogens. On the other hand, 9-LOX and their products have only recently gained attention as potential defense compounds. However, their significance was convincingly recognized only in solanaceous plants through manipulation of specific 9-LOXs using antisense or overexpression techniques (Rance et al. 1998b). These studies demonstrated involvement of 9-LOXs in the establishment of resistance in plant-microbe interactions. However, despite progress in understanding the role of specific 9-

LOXs in dicots, the function of these enzymes in monocot species has not been elucidated. Here we report on cloning and analysis of molecular and biochemical properties of the maize 9-LOX, ZmLOX12, and provide conclusive evidence of a role of this particular 9-LOX in maize susceptibility *to F. verticillioides*.

Low sequence homology of ZmLOX12 to other maize LOXs was suggested that this LOX isoform is unique among the 9-LOXs. This observation of ZmLOX12 was supported by sequencing analysis that revealed the presence of the T/M motif instead of the conventional T/V known to be indicative for all previously described 9-LOXs. Moreover, Southern blot analysis demonstrated that genes homologous to *ZmLOX12* were also acquired in other monocot but not dicot species, thus suggesting that *ZmLOX12* is a unique monocot-specific LOX.

The absence of a recognizable chloroplast signaling peptide and overall sequence identity to other 9-LOX suggested that the ZmLOX12 predicted protein is a *type 2*-9-LOX. This assumption was further supported by the analysis of regio-specificity of recombinant ZmLOX12 protein, which clearly indicates that this protein is a linoleate 9-LOX. Depending on the inbred line *ZmLOX12*-specific transcript accumulation was solely localized to underground organs including seminal roots, mesocotyl, crown and coleoptile, whereas no expression was detected in unchallenged aboveground organs. It is interesting

that the closest dicot LOX, potato 9-LOX, *POTLX-1*, is also preferentially expressed in tubers, and other underground organs (Kolomiets et al. 2001).

Root, mesocotyl, crown and stalk were proposed to be the major targets of for systemic infection of maize by *F. verticillioides* that usually causes rotting of these organs in maize seedlings (Munkvold 1997). Mesocotyl, defined as the intermediate tissue between the root and hypocotyl is strategically important for early seedling development. It is usually located below the ground and serves to position the photosynthesizing organs above the ground level and to supply them with nutrients from endosperm and minerals and water from the roots. In this study we examined the possibility of *ZmLOX12* involvement in maize-*F. verticillioides* interactions, where underground organs (mesocotyl) of two inbred line known to accumulate more (R168) and less (VA46) fumonisin in kernels were inoculated with *F. verticillioides*. Our observation, that early induction of *ZmLOX12* (3 to 6 hr post inoculation) in response to *F. verticillioides* occurred in both inbred lines, suggest active role of this gene at the early stages of the plant-fungus interaction. In comparison to early induction of *ZmLOX12* expression in mesocotyls, the previously reported maize 9-LOX gene (Wilson et al. 2001) was induced only 2 days after inoculation of kernels, suggesting spatial and temporal sub-functionalization for these genes in maize-*Fusarium* interaction.

To elucidate the role of *ZmLOX12* in *F. verticillioides* pathogenesis in maize mesocotyls, a *Mu*-insertional knockout mutant allele for this gene was identified. Disruption of expression of this gene resulted in decreased

colonization of mesocotyls by *F. verticillioides* in both genetic backgrounds, B73 and CML176. Supporting the main hypothesis, this study represents the first evidence that underground organ-specific expression of monocot 9-LOX is a susceptibility factor to seed-borne pathogen *F. verticillioides*. Taken together these data further provides strong evidence in support of the hypothesis that 9-LOXs may be induced to the benefit of the phytopathogenic fungi by producing compounds that promote their growth and conidia production.

Similar to 13-LOXs, 9-LOXs and their downstream products were also proposed to have both anti-microbial and signaling activity in plant defense responses against pathogens. However, signaling activity of 9-LOXs and their derivatives was demonstrated only in few reports (Gobel et al. 2002). Therefore, one possible explanation of *ZmLOX12*-mediated susceptibility is that oxylipins, derived from this 9-LOX may be converted into signaling molecules capable of activation of mycelial growth and conidiation. Plant 9-LOXs and their products were previously reported to affect vital metabolic processes in fungi. For example, Wilson et al. (2001) observed accumulation of high levels of primary 9-LOX products - fatty acid hydroperoxides 9*S*-HPOT(D)E in response to *Aspergillus flavus* infection that coincided with elevated sporogenesis and increased in aflatoxin accumulation. In addition, importance of oxylipins as key regulators of sporogenesis was shown in mutant strains of *A. flavus* (Tsitsigiannis and Keller 2006) and *Fusarium sporotrichioides* (McDonald et al. 2004) where genes involved in fungal oxylipin formation were depleted.

Collectively, these data prompted a hypothesis that plant-derived products of 9-LOXs, due to their structural resemblance to fungal psi-factors, may mimic the fungal regulatory molecules and have an effect on mycelial growth and production of conidia (Brodhagen and Keller 2006;Wilson et al. 2001). In the light of this hypothesis, *ZmLOX12* may act as a negative regulator of plant effecter genes that directly limit fugal growth and production of conidia.

Alternatively, increase in the production of 9-LOX-derived oxylipins in response to fungal infection can lead to down-regulation of the 13-LOX pathways. Taking into account, that 13-LOX products were reported to inhibit fungal growth and conidia formation (Brodhagen and Keller 2006;Wilson et al. 2001), this possibility seems promising. Inhibition of enzymatic activity of specific LOXs by downstream products of LOX pathway was described in several reports (Corey et al. 1987;Grechkin 2002). In addition, evidence exists suggesting that fungi are modulate plant metabolism. For instance, presence of substances that are able to inhibit plant LOXs was found in the ascomycete fungi *A. oryzae*, *A. niger* and *Streptomyces* spp. (Rao et al. 2002). These known fungal inhibitors of plant LOXs were shown to be produced through polyketide synthase pathway (Rao et al. 2002) which is an analog of plant fatty acid biosynthesis machinery (Pasanen et al. 1996). Finally, selective 13-LOX-inhibitory activity was described for *A. flavus* (Tsitsigiannis et al. 2005).

Although this study focused on the role of *ZmLOX12* in maize-*F. verticillioides* interaction in the mesocotyl, its fuction may not be strictly limited to

responses in underground organs. We showed transient inducibilty of *ZmLOX12* mRNA accumulation in leaves by wounding and exogenous application of JA, suggesting a possibility of defense-related function for this gene in leaves in response to pests or pathogens. The first seems more plausible, especially with regard to chewing insects, since, wounding is a known stimulus for endogenous production of JA (Feussner and Wasternack 2002).

Although, the precise physiological function of ZmLOX12 in plant development and the mechanism by which it contributes to maize susceptibility still remains to be elucidated. The employment of the *lox12-1* mutant and correspondent near isogenic wild type maize lines will be a valuable tool tounderstand the molecular and biochemical basis underprinting the increased resistance of *lox12* mutants to *F. verticillioides* colonization. This finding may contribute to the improvement of strategies to control maize seed-borne diseases caused by *F. verticillioides* by limiting at least systemic spread of the pathogen on early stages of plant development.

CHAPTER IV

GENERAL CONCLUSION

The major goal for this project was to clone and functionally characterize 3 maize LOX genes ZmLOX10, ZmLOX11 and ZmLOX12. We found that ZmLOX10 and ZmLOX11 are 13-LOXs. *ZmLOX10* and *ZmLOX11* were both categorized as *type-1*-13-LOX as they encode a chloroplast-targeting signal peptide and share 90% identity to each other. ZmLOX12 is clearly a *type-2*-9-LOX. These phylogenetic data was supported by analysis of the biochemical properties of ZmLOX10 and ZmLOX12, which had pH-optima of 8.0 and 6.0, respectively.

Using northern blot assays, preferential sites of transcript accumulation for all three genes were established. The highest levels of *ZmLOX12* steady state mRNA were detected in underground organs. Interestingly, expression of *ZmLOX10* was high in maize leaves, whereas, ZmLOX11 was highly expressed exclusively in silks. In addition, these two closely-related genes were mapped to different chromosomes, proving that they are true distinct duplicated genes. All studied LOXs were inducible in response to treatment with defense-related hormones in leaves. *ZmLOX10* transcripts were increased in response to JA, wounding, ABA in leaves, while *ZmLOX11* could only be induced by ABA. Surprisingly, *ZmLOX12*, the expression of which was not detected in

unchallenged aboveground organs, was induced by JA and wounding. *ZmLOX10* has a light responsive element in the promoter sequence and was shown to be regulated by circadian clock, with the highest accumulation of transcripts coinciding with the highest photosynthetic activity. Thus, *ZmLOX10* appears to be the first evidence of a circadian clock-regulated plant LOX. Expression of neither *ZmLOX11* nor *ZmLOX12* was clock-controlled in leaves. Taking into account inducibility of *ZmLOX10* by lower tremperatures, it is tempting to speculate that this gene functions most actively during the day to prepare plants for colder night temperatures. Supporting the hypothesis that maize LOXs function in orchestrated plant defenses against pathogens, ZmLOX10 and ZmLOX12 were induced by avirulent strain of *C. carbonum* and virulent strain of *F. verticillioides* in mesocotyls, respectively. Taken together our data suggests that *ZmLOX10* is likely to be involved defense mechanisms against pests and necrotizing pathogens. On the contrary, role of *ZmLOX12* in maize interaction with *F. verticillioides*, was established in this study conducted on mesocotyls of mutant and WT plants, where Mu-insertional mutation caused *lox12-1* plants to be more resistant to fungal growth incidence and supported production of fewer conidia. These data is the first direct evidence in support of the hypothesis that specific 9-LOX in monocots contribute to increased plant susceptibility.

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