GENOMIC ANALYSIS OF 12-OXO-PHYTODIENOIC ACID REDUCTASE GENES OF *ZEA MAYS*

A Thesis

by

JINGLAN ZHANG

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

MASTER OF SCIENCE

December 2004

Major Subject: Plant Pathology

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Approved as to style and content by:

Michael Kolomiets (Chair of Committee)	Clint Magill (Member)
Javier Betran (Member)	Dennis Gross (Head of Department)

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ABSTRACT

Genomic Analysis of 12-Oxo-Phytodienoic Acid Reductase Genes of *Zea mays*.

(December 2004)

Jinglan Zhang, B.S., Wuhan University

Chair of Advisory Committee: Dr. Michael Kolomiets

The 12-oxo-phytodienoic acid reductases (OPRs) are enzymes of the octadecanoid pathway which converts linolenic acid to a phytohormone, jasmonic acid. Bioinformatics analysis of ESTs and genomic sequences from available private and public databases revealed that the maize genome encodes eight different *OPR* genes. This number of maize *OPR* genes has been independently confirmed by Southern blot analysis and by mapping of individual *OPR* genes to maize chromosomes using oat maize chromosome addition lines. Survey of massively parallel signature sequencing (MPSS) assays revealed that transcripts of each *OPR* gene accumulate differentially in diverse organs of maize plants. This data suggested that individual *OPR* genes may have a distinct function in development. Similarly, RNA blot analysis revealed that distinct *OPR* genes are differentially regulated in response to stress hormones, wounding or pathogen infection. ZmOPR1 and ZmOPR2 appear to have important functions in defense responses to pathogens because they are transiently induced by salicylic acid (SA), chitooligosaccharides and by infection with *Cochliobolus carbonum*, *Bipolaris maydis* and *Fusarium verticillioides* and not by wounding. In contrast to these two genes, *ZmOPR6* and *ZmOPR7/8* are highly induced by wounding and treatments

with wound-associated signaling molecules jasmonic acid, ethylene and abscisic acid. *ZmOPR6* and *ZmOPR7/8* are not induced by SA treatments or pathogen infections suggesting their specific involvement in wound-induced defense responses. Possible functions of specific *OPR* genes are discussed.

DEDICATION

To my wife, Hui Mei and my parents, Nianxiu Zhang and Liuhua Xu.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Michael Kolomiets, for giving me considerable advice in the direction of my research. I would also like to thank the collaborators of my research project, Dr. Carl Simmons, Dr. Nasser Yalpani and Dr. Virginia Crane for their generosity with their time and expertise. The members of the Kolomiets' Lab, past and present, have all contributed to my graduate work, either by engaging in helpful discussions or by giving me the assistance I needed to finish my research. Finally, special thanks to my friends and family for all their support over the years.

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INTRODUCTION

Maize is an ergonomically important crop consumed by humans and cattle. Both breeders and scientists have been seeking different means to improve the economic value of this crop. With the emergence of modern biology technique applied in modern agriculture, scientists are trying to understand how plant conducts adaptive responses to environmental signals at the molecular level. It may help scientists and farmers manipulate the plants to produce crops with broad spectrum resistance and high production. In another hand, maize has been used a model by biologist to study genetics for a long time. More recently, accumulated maize gene sequence data available to public makes biologists able to study this plant with genomics tools. A conservative estimation predicts maize has 59,000 genes while some private industrial parities can provide two million expressed sequence tags (ESTs) and more than 25,000 full-length cDNAs to the public **(**Timmermans et al., 2004; Martienssen et al., 2004**)** under licensed agreement**.** Also, available genome survey sequence (GSS) database covers 95% of the genic region of maize genome. With these resources, it is practical to identify almost any gene in maize. My interest of this research project to identify one of maize gene families, 12 oxo-phytodienoic acid reductase (OPR) gene family , that is associated with maize development control and resistance response. The goals of this project includes: 1) Identification of the entire maize OPR gene family; 2) Characterization of the expression profiling of maize OPR gene family members in different developmental stages and in

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This thesis follows the style of Plant Physiology.

response to stresses; 3)Generating the maize *opr1* and *opr2* mutants for functional studies of their role in defense responses.

In the past, much of our knowledge about the jasmonate biosynthesis and signaling pathway, also known as octadecanoid pathway, came from studies of dicotyledonous plants in which most genes encoding enzymes of this pathway have been characterized. The jasmonic acid pathway starts from the release of α-linolenic acid (α-LeA) from membranes by a phospholipase. This step is followed by addition of molecular oxygen at carbon atom 13 of α-LeA by a 13-lipoxygenase (13-LOX), leading to the formation of a fatty acid hydroperoxide 13*S*-hydroperoxy-(9*Z*,11*E*,15)-octadecatrienoic acid (13(*S*)-HPOT) (Shaller, 2000 and references therein). After sequential oxidization and cyclization by allene oxide synthase (AOS) and cyclase (AOC), the first two committed steps of this pathway, 13(*S*)-hydroperoxide gives rise to the jasmonic acid (JA) precursor, 12-oxo-phytodienoic acid (OPDA) (Stenzel et al., 1988). The initial steps of JA biosynthesis pathway including LOX, AOS and AOC reactions occur in chloroplasts determinated by the study of the subcellular localization of these enzymes (Schaller et al., 1998; Maucher et al., 2000; Ziegler et al., 2000; Froehlich et al., 2001). The following step in the pathway involves the reduction of the cyclopentenone ring of OPDA by OPDA reductase (OPR) which reduces the 10,11 double bond of OPDA and gives rise to 3- three rounds of β-oxidation, OPC is converted to the final product of this pathway, oxo-2-(2')(z)-pentenyl)-cyclopentane-1 octanoic acid (OPC) (Vick and Zimmerman., 1984). After jasmonic acid (JA). β-oxidation is believed to occur in peroxisomes where the enzymes for β-oxidation are located (Turner et al., 2002).

Jasmonates such as JA, OPDA and related cyclopentanones were shown to act as plant growth regulators in various developmental processes such as root elongation, senescence, anther dehiscence, pollen maturation, and tuber formation and they are also potent modulators of defense responses against insects and pathogens (Parthier, 1991; Creelman and Mullet., 1997; Turner et al., 2002). Jasmonic acid occurs constitutively at a basal level (Stenzel et al., 1998). Levels of JA increase dramatically in response to wounding and contribute to resistance against herbivores by activating defense genes such as proteinase inhibitors (Farmer and Ryan 1990). Studies using Arabidopsis and tomato mutants defective either in JA accumulation or perception demonstrated that these mutants are highly susceptible to insect predators (McConn et al., 1997). The role of JA as a major signaling molecule in defense responses to necrotrophic pathogens is well supported by numerous experiments (for reviews, see Farmer, 1994, and 2003). However, a recent study indicated that exogenously applied JA decreases disease severity evoked by a variety of fungal pathogens with a wide range of life styles including those having a biotrophic phase in their pathogenesis (Thaler et al., 2004). Besides the well known role of JA in defense signaling, OPDA, the natural precursor of JA and substrate of OPR, may have its own signaling role that is distinct from that of JA, because OPDA and JA can induce overlapping but distinct subsets of jasmonate-inducible genes (Stintzi et al., 2001).

Plant OPRs are usually encoded by a multigene family. For example, Arabidopsis and tomato genomes contain three *OPR* genes each (Biesgen and Weiler, 1999; Stintzi and Browse, 2000; Strassner et al., 1999, 2002), whereas rice appear to have 13 *OPR* genes (Agrawal et al., 2003). Recently, six members of one *OPR* gene subgrounp were cloned

and characterized suggesting that pea genome encodes more than six *OPR* genes (Ishiga et al., 2002; Matsui et al., 2004).

All plant OPRs are classified into two groups depending on their substrate specificity. Members in subgrounp OPRI preferentially catalyze the reduction of *cis*-(-) OPDA over cis-(+) OPDA and is not required for JA biosynthesis. Members of the OPRII subgrounp are required for JA biosynthetic pathway and preferentially catalyze the cis-(+) OPDA over cis-(-) OPDA to form the natural precursor of JA, 3-oxo-2-(2')(z)-pentenyl)-cyclopentane-1 octanoic acid (OPC 8:0) (Schaller et al., 1998). Substrate specificities of several more plant OPR isoforms have been reported recently. In both Arabidopsis and tomato, AtOPR3 and LeOPR3 respectively, are classified into subgrounp OPRII (Stintzi and Browse, 2000; Strassner et al, 2002). AtOPR1, AtOPR2, PsOPR1-6 and OsOPR1 are OPRI enzymes (Schaller et al., 1998; Matsui et al., 2004; Sobajima et al., 2003). The biological significance to plants of having multiple OPRs is not clearly understood. To date, the biochemical and physiological role of only one plant OPR isoform, Arabidopsis AtOPR3 has been relatively well established. This OPR enzyme was shown in vivo to be required for JA biosynthesis and male fertility (Stintzi and Browse, 2000). A great challenge is to identify the biologically active products that are catalyzed by OPRI enzymes as well as their physiological role in plant adaptive response. It is possible that individual OPR family members have distinct functions based on their regulation of gene expression in response to environmental cues, substrate specificity, subcellular and tissue distribution. For example, wounding only stimulated the expression of LeOPR3, the only OPR involved in the JA biosynthesis but did not

affect expression of other OPR genes (Strassner et al., 2002). In contrast to these findings, Arabidopsis OPR1 and OPRR2, two enzymes that belonging to the OPRI subgrounp, were shown to be wound inducible (Biesgen and Weiler, 1999). *OsOPR1* is the only *OPR* gene from a monocot species that was characterized molecularly so far (Agrawal et al., 2003). This gene was rapidly and transiently up-regulated by a variety of environmental cues including JA, SA, Eth and H_2O_2 (Agrawal et al., 2003).

In maize, OPR activity was first reported and characterized about two decades ago by Vick and Zimmerman (1986). However, the constitution of the entire maize OPR family and the physiological function of any corn *OPR* gene remains unknown. In this study, we report on the identification, cloning, and sequencing of eight *OPR* genes in maize. The putative genomic sequences of these genes were assembled by a homology search of maize GSS database which allowed us to identify genomic structures and some putative *cis*-elements in these genes. That the maize genome contains eight OPR genes was independently confirmed by Southern blot analysis and by mapping each *OPR* gene to a particular chromosome using a PCR approach and oat-maize chromosome addition lines (Ananiev et al., 1997). RNA blot analysis and MPSS expression profiling data suggested that individual OPR genes are expressed differentially in diverse tissues of unchallenged maize plants and in response to stress-related signals or pathogen infection. The data suggests that OPR genes may have a diverse and highly specialized function in plant development and in defense responses to wound- and pathogens.

MATERIALS AND METHODS

Genomic DNA Isolation and Southern Blot Analysis

One gram of freshly harvested leaves from two-week old seedlings was used for DNA isolation. Ground leaf tissue was suspended in a mixture of 3 ml Urea Extraction Buffer (5.5 M Urea, 0.25 M NaCl, 0.04 M Tris, 0.016 M EDTA, 0.8% Sarcosine, pH (change throughout) 7.0,) and 3 ml of Phenol: CH_3Cl_3 (1:1 v/v, PH 8.0) at room temperature for 15 minutes. After phase separation by centrifugation at 5,000g for 15 minutes, DNA was precipitated from the supernatant with an addition of 250 µl 3 M NaOAc (PH 5.0) and 3ml isopropanol. DNA pellet was dissolved in Tris-EDTA buffer (PH 8.0). RNA was removed with RNase treatment at 37°C overnight. For genomic Southern blot analysis 10 µg genomic DNA from several maize inbred lines was digested with EcoRI, BamHI, HindIII, XbaI and XhoI. Digested DNA was separated in a 0.8% agarose gel by electrophoresis. The DNA was transferred onto a nylon membrane (Magna Nylon Transfer Membrane, Osmonics Inc., Minnetonka, MN) with 0.025M phosphate transfer buffer and was cross-linked to the membrane by UV exposure. The blots were hybridized in ULTRAhyb hybridization buffer (Ambion, Austin, TX) at 42°C overnight with *ZmLOX* gene specific fragments of individual LOX generated by PCR. Washes were performed with low strength buffer (2× SSC and 0.1% SDS) at 42°C for 2 times 5 min, followed by an additional wash with high strength buffer (0.2M SSC and 0.1% SDS) at 42°C two

times for 15 min. The blots were exposed to the X-ray film (Kodak, Rochester, NY) in cassettes at -80°C for 72 h.

Localization of Maize OPR Genes to Individual Chromosomes Using Oat Maize Chromosome Addition (OMA) Lines

Detailed information for generating OMA plants was described previously (Riera-Lizarazu et al., 1996). In our study, Oat Maize Chromosome Addition (OMA) line genomic DNA containing individual maize chromosome DNA was used as template for PCR. Several pairs of PCR primers for each *OPR* were designed using the DNAStar PrimerSelect program. Gene specific forward (F) and reverse (R) primers used in this study were,

OPR1F 5'-AAGTGACCTGCGGTGTTGCATCACATC-3',

OPR1R 5'-TTCACTTCACACCAGTACATGA-3',

OPR2F 5'-AAGAAGATGGCAAGAATGAGG-3',

OPR2R 5'-TAGATTTATTTCACTTCACACC-3',

OPR3F 5'-GCCAACCCAGACCTGCCTAAAAGGTT-3',

OPR3R 5'-GCAACACACCGGTATACTCAACTAG-3',

OPR4F 5'-CTTTTGGCTGCAGCATCATCATCG-3',

OPR4R 5'-CCTGGTCGGGCGTCCACACTCC-3',

OPR5F 5'-GCTGAGGCCACGGGGGTTTCAG-3',

OPR5R 5'-CGGCAGCGGTTCTCCAGACT-3',

OPR6F 5'-AGCAGGCTTTGATGGAGTGGA-3',

OPR6R 5'-TTGGCAAAACGCATCGGAAGG-3',

OPR7F 5'-CGGCTGTTCATCGCTAATCCCGA-3',

OPR7R 5'-CAATCGCGGCATTACCCAGATGT-3,

OPR8F 5'-CCGCCTCAACGCTCTCCAGGAG-3,

OPR8R 5'-GACTGCTGATTGCTGTGCAAAT-3.

PCR conditions were adjusted for each primer pair to amplify one LOX gene specifically. The PCR contained 1×PCR buffer for the *Taq* polymerase (Thermopol Buffer, New England BioLabs, Beverly, MA) , 2.5 mM of each dNTP, 10% (m:v) sucrose, 1 unit of enzyme, 20 ng of OMA genomic DNA and 0.4 μM forward and reverse oligonucleotide primers. Higher annealing temperature or the PCR additive DMSO (5%) was added if necessary. PCR products were loaded in a 1.0% agarose gel and separated by electrophoresis. Maize parent genomic DNA, oat parent genomic DNA from which the OMA addition lines were generated (Riera-Lizarazu et al., 1996), and B73 genomic DNA were used as controls for the PCR in this study.

Plant Material and Hormonal Treatments

Maize plants were grown in 7 cm pots in Strong-Lite (circleR) potting soil (Universal Mix, Pine Bluff, AZ) in a greenhouse at 22ºC to 30ºC under a 16 hour daylength, 50% average relative humidity, 560-620 µE of light from both sun and halogen lamps. For all the hormonal treatments and organ-specific expression studies except for the ear and the

silk tissues, we have used two-week-old plants of the maize inbred line B73 at the developmental stage V3. Tassel 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 RNA extraction and Northern blotting (see Fig. 1). For JA treatments, the seedlings were cut at the soil level and were incubated with the cut end placed in 100 mL of either 0.1% ethanol, 0.01 % Tween-20 (control) or 200 μM JA (Sigma, St. Louis). Treatment with 10 $\mu L\ L^{-1}$ ethylene was conducted in hermetically sealed, 5.6 L dessicators. The entire seedlings (two seedlings per replicate, three replicates) were harvested at different times of treatment, frozen immediately in liquid N_2, and stored at -80ºC.

Pathogens, Inoculum Production, and Inoculation

For inoculation with *B. maydis* we used maize lines provided by the Maize Genetics Cooperative, University of Illinois, Urbana/Champaign, stock reference 1526 (in B73 genetic background) bearing the recessive *rhm1* gene for resistance to *B. maydis* or 1650 (a heterozygous, susceptible near-isogenic line derived from the outcross of 1526 with B73). *B. maydis* isolate TX001 (incompatible on the 1526 line and compatible on the 1650 line), was obtained from field sources at Pioneer Hi-Bred, and was maintained on potato dextrose agar (PDA) medium (Difco). The fungi were grown for two weeks at 22°C for sporangia production, and the sporangia were harvested by washing the plates with 0.02% Tween-20. Sporangial suspensions were diluted to approximately 10,000 sporangia per ml. Inoculations were performed by spraying spore suspensions as an

aerosol on leaves of V3 stage plants. Inoculated seedlings were immediately covered with plastic tents in order to increase humidity and incubated in a greenhouse under the growth conditions described above. Samples were taken after 3, 6, 12, 24 and 48 h, frozen in liquid N_2, and stored at -80°C.

For inoculation with *Cochliobolus carbonum,* we used two-week old seedlings of near-isogenic lines Pr or Pr1 witch are either susceptible or resistant to *C. carbonum* race 1 Tox2+, respectively (Multani et al., 1998). For the study of gene expression in response to *Cochliobolus* carbonum race 1 we used near-isogenic strains of *Cochliobolus carbonum* race 1 that did (Tox2+) or did not (Tox2+) produce its pathogenecity factor, HC-toxin. 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. Upon inoculation, plants were immediately covered with a plastic cover and were incubated for 3, 6, 12, 18, 24, 48 and 72 h in the greenhouse. After each incubation time, infected leaves were collected and frozen immediately in liquid N_2 and stored at -80°C until used for RNA extractions.

Massively Parallel Signature Sequencing and Expressed Sequence Tag mRNA Profiling

The maize OPR transcript expression levels were assayed by the Massively Parallel Signature Sequencing (MPSS) technique of Lynx Technologies (Brenner et al., 2000), and

separately by EST distribution. The MPSS profiling involves deep transcript sampling on microbeads, with a 17 base sequence read (Tag) beginning with 'GATC'. A total of 203 MPSS libraries covering diverse tissues, developmental stages, and treatments were assayed. However, for this study we focused on B73 genotype samples belonging to 12 distinct non-overlapping tissues and two treatments. Limiting the data to B73 avoided SNP effects on Tag sequences. Tag to gene associations were done by direct sequence matching. Most genes were represented by Tags corresponding to the plus strand and proximal to the polyA tail. The EST distribution analysis involved standard EST sequences obtained from 265 diverse cDNA libraries, from public and private sources. The 'UniCorn 4.1' EST assembly set (Mark Whitsitt, Pioneer Hi-Bred, personal communication) assisted in EST gene assignment. EST distributions were not limited to B73 because identification is largely immune to SNPs.

Northern Blot Analysis

Corn leaves were harvested and were immediately frozen in liquid nitrogen for RNA extraction. Total RNA was extracted using TRI reagent (Molecular Research Center Inc, Cincinnati, OH) according to manufacturer protocols. 10 µg of total RNA was loaded and subjected to electrophoresis in 1.4% (w/v) formaldehydagarose gels for separation and transferred onto a MagnaGraph nylon membrane (Micron Separations Inc., Westboro, MA). Equal loading of RNA samples and uniform transfer onto a nylon membrane was confirmed by visualizing RNA stained with ethidium bromide under UV light. The probe

was amplified by PCR from genomic DNA or an EST clone representing the *OPR* gene. Membranes were hybridized with a ^{32}P-labeled *OPR* fragment using UltraHyb solution (Ambion, Austin, TX) under hybridization conditions described in the manufacturer's protocol. Membranes were washed in 1X SSC, 0.1% SDS at room temperature for 15 min, in 0.1X SSC, 0.1% SDS at room temperature for 30 min, followed by a final wash in 0.1X 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) by using intensifying screens for 2 to 7 days. Blots presented are representative examples of at least two independent experiments.

RESULTS

Identification of the Maize *OPR* Gene Family Members

Identification of the entire maize OPR family was initiated by BLAST search (Altschul et al., 1997) of extensive DuPont/Pioneer and public expressed sequence tag (EST) databases representing more than 265 different cDNA libraries (see Materials and Methods for detailed description). 257 EST clones were found encoding maize OPR-like sequences and they were grouped into eight contigs and singletons. Full insert sequencing was carried out for 12 full length clones that represented each group. Sequence analysis of these sequenced cDNA clones revealed that the maize genome contains at least eight genes (Table 1). We designated these genes as *ZmOPR1, 2, 3, 4, 5, 6, 7* and *8* in an order that reflects their level of sequence similarity. All *OPR* clones but the clones representing *ZmOPR4* were full length containing short 5' UTR and 3'UTR sequences and entire open reading frames as well as a poly-A tail. EST clones representing *ZmOPR4* gene sequenced did not appear to have any poly-A tail. Three *OPR*-like gene sequences were previously reported in the NCBI database including two full-length cDNA sequences, AY108052, AY106849, and one partial mRNA sequence AY106967.

Southern blot analysis was performed to identify the copy number for each *OPR* gene. To avoid potential problems associated with crosshybridization of probes derived from different genes, we PCR-amplified the 3' portions of the OPR cDNA clones and used them as gene-specific probes. Unfortunately, it was impossible to generate gene-specific probes to distinguish *OPR1* and *OPR2* or *OPR7* and *OPR8*, because these two pairs of

Table 1. Percentage of the identity of maize OPRs at the amino acid level.

	ZmOPR2	***ZmOPR3***	***ZmOPR4***	***ZmOPR5***	***ZmOPR6***	***ZmOPR7***	***ZmOPR8***
ZmOPR1	96.5	78.3	69.2	66.9	64.3	50.6	55.8
ZmOPR2		77.2	67.9	65.6	63.5	50.9	55.9
ZmOPR3			66.8	63.7	62.1	51.1	55.8
ZmOPR4				64.0	64.8	52.1	55.0
ZmOPR5					66.9	52.4	56.2
ZmOPR6						50.6	55.2
ZmOPR7							95.2

duplicated genes are highly similar throughout their entire cDNA sequences. Therefore, as expected we detected at least two bands hybridizing to *OPR 2* or *OPR7* probes. However, it is clear from the blot hybridized to the *OPR2* gene-specific probe that the weaker second band appear to represent *OPR1* rather than an additional copy of *OPR2*. Southern analysis (Fig. 1) indicated that *OPR3*, *OPR4*, *OPR5* and *OPR6* gene-specific probes hybridized to a single band and therefore, these genes are likely encoded by a single copy.

Mapping of *OPR* Genes to Maize Chromosomes

Due to the high sequence identity of maize maize *OPR* genes to each other (Table 1), for instance, *ZmOPR1* and *ZmOPR2* cDFNAs sharing more than 96% identity, the question arose whether these highly homologous *OPR*s represent different genes or different alleles. Obviously, Southern blot analysis alone was not sufficient to discriminate amongst the highly similar sequences. Therefore we used recently developed OMA lines (Ananiew et

al., 1997; Okagaki et al., 2001) to map each *OPR* gene to maize chromosomes. Chromosome location of gene sequences may help us determine whether two similar *OPR*

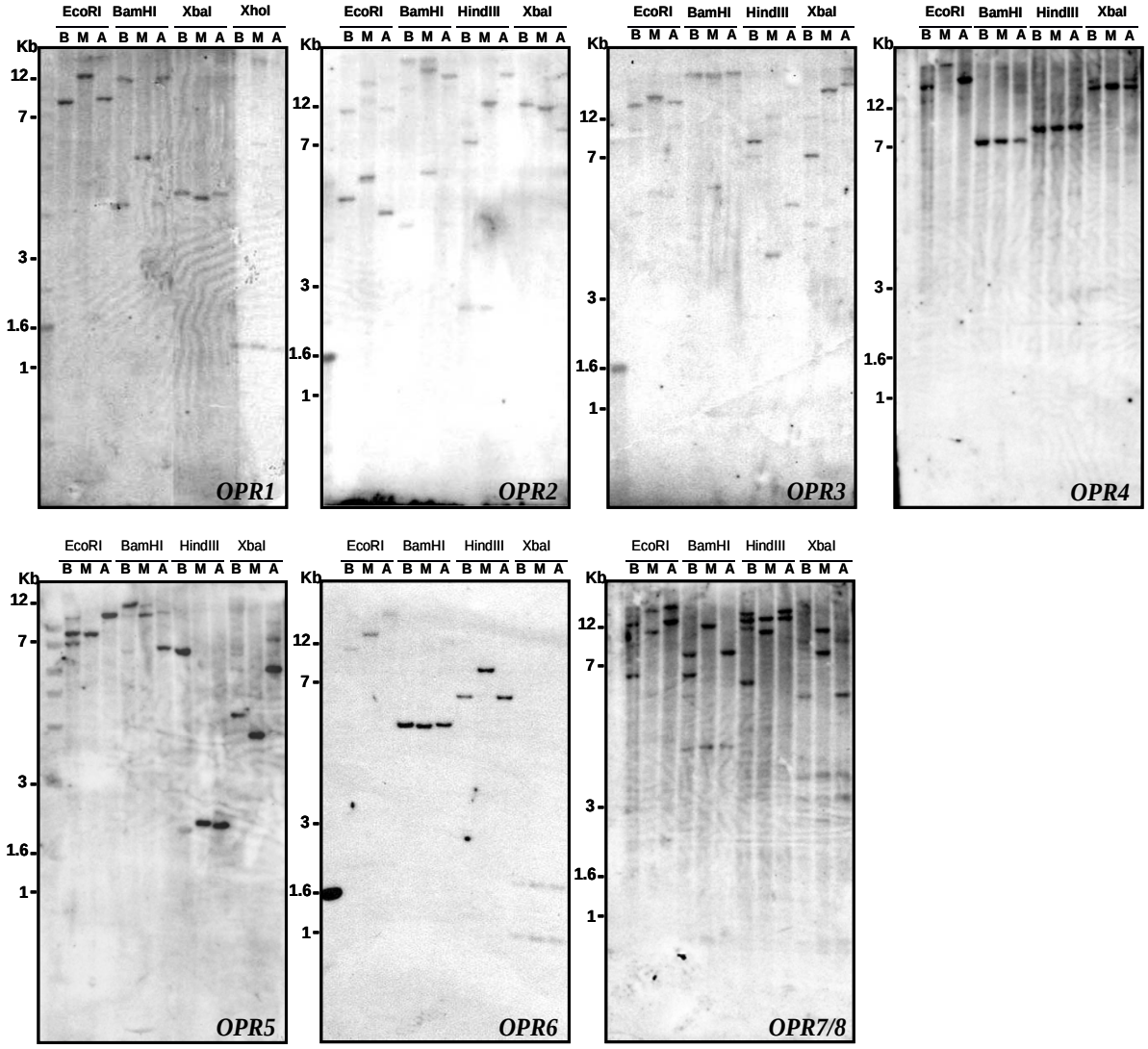

Figure 1. Southern blot analysis of maize genomic DNA with *OPR* probes. 10µg genomic DNA of maize B73 (B), MO17 (M) and A632 (A) inbred lines were isolated, digested with *Eco*RI , *BamH*I , *Hind*III, *Xba*I, and *Xho*I, separated by electrophoresis, blotted onto nylon membranes and hybridized with *OPR* gene specific probes. *OPR7* and *OPR8* were crosshybridized to a probe derived from 3' end of *OPR7* cDNA. DNA size markers in kilobases (kb) are indicated on the left.

sequences represent different loci or alleles of a single locus. Oat-maize chromosome addition lines were produced by crossing maize and oat resulted in preferential elimination of maize chromosomes (Ananiew et al., 1997). The recovered oat-maizehybrid contains a complete oat chromosome set and a single maize chromosome. OMA materials were ideal to identify on which maize chromosome a particular *OPR* gene is located by a PCR approach. We designed several pairs of gene-specific PCR primers for each maize *OPR* gene and used OMA genomic DNA as a template to perform PCR. To avoid detection of duplicated sequences or amplification of oat *OPR*-like sequences, different primer pairs or PCR conditions were tested (see Materials and Methods). Fig. 2 illustrates the localization of *OPR* genes to maize chromosomes. These results were confirmed independently by using at least two different gene-specific primer pairs for each *OPR* gene. *OPR1-7* are located on chromosome 9, 8, 6, 8, 2, 3, and 1, respectively. Interestingly, although *OPR1* and *OPR2* cDNAs share more than 96% sequence identity at the nucleotide level, they are located on chromosomes 9 and 8, respectively, meaning they represent two separate loci in the maize genome.

The Genomic Structure of Maize *OPR* Genes and Their Deduced Amino Acid Sequences

To determine the genomic organization of *OPR* genes, we identified the Survey Sequences (GSS) (available in www.plantgdb.org/cgi-bin/PlantGDBblast) that are related

to the *OPR* cDNA sequences. Only were those genomic sequences with 98% or above sequence identity to the overlapping region of cDNA sequences selected to assemble the

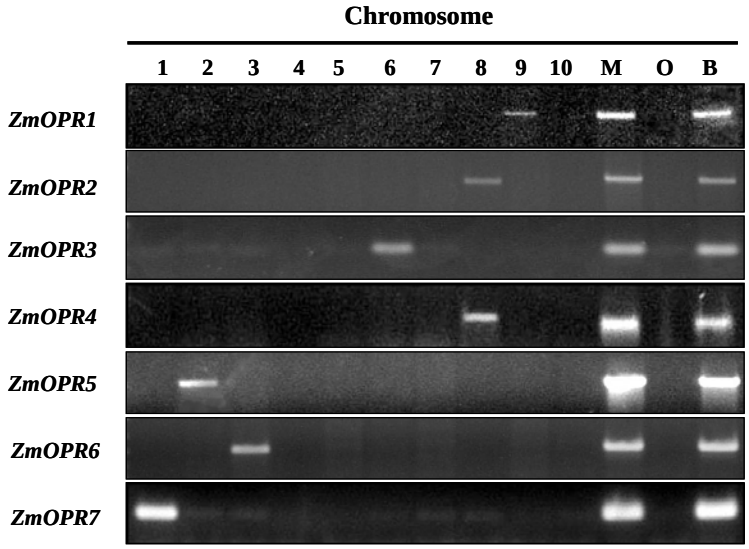

Figure 2. Localization of maize *ZmOPR*s to maize chromosomes by using oat-maize chromosome addition lines. PCR products obtained by using OPR gene specific primers of genomic DNA of ten oat-maize chromosome addition lines (1 to 10) , maize parent (M), oat parent (O) and B73 (B) inbred lines were separated by electrophoresis in an agarose gel. Bands represent the PCR products amplified by maize *OPR* gene specific primers and indicate the chromosome on which individual *OPR* genes are located.

genomic contigs for each *OPR* gene. It should be noted that we were able to identify the genomic sequences covering the full length of cDNA for *OPR2, 4, 6, 7* and *8* (Fig. 3) or a portion of full length maize *OPR* genes for *OPR1*, *3*, and *5* from GSS database. Some maize *OPR* transcription start points were tentatively predicted by using a computational tool (available at http://www.fruitfly.org/seq_tools/promoter.html.).

The predicted distances from the transcription starting point to the TATA box were conserved among the maize *OPR* gene family members, ranging from base -28 to base -32 (data not shown). By comparing the cDNA sequences and the genomic sequences, we were able to identify the intron/exon composition in all of maize *OPR* genes. There are 1, 1, 1, 0, 4, 3, 4 and 4 intron(s) in *OPR1* to *8* respectively (Fig. 3). All exon-inron-exon borders follow the GT/AG splicing rule (Mount, 1982).

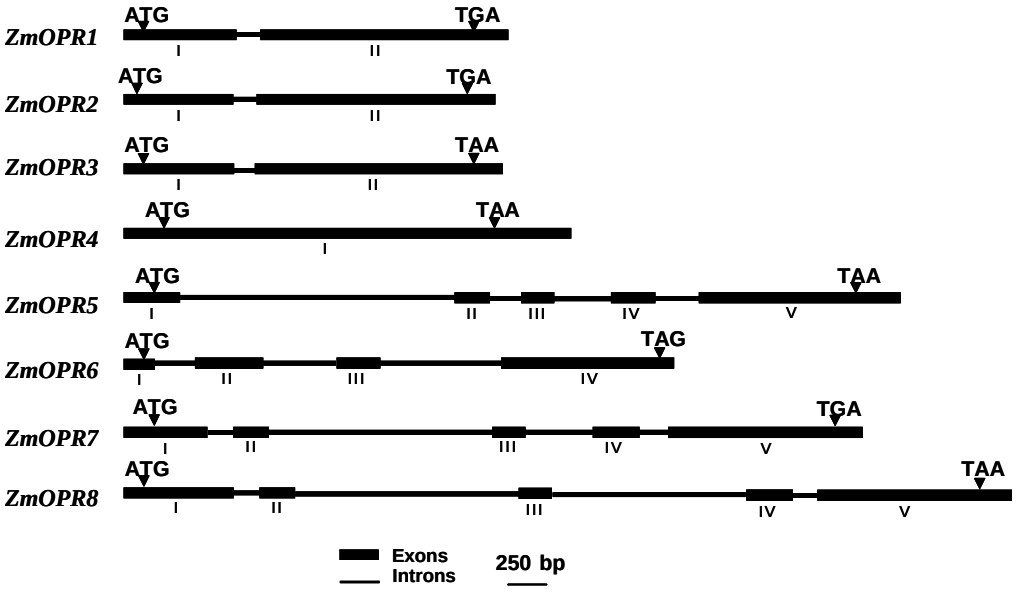

Figure 3. Predicted genomic structures of maize *OPR* genes. Exons are represented as the black boxes. Introns are presented as black lines. Translation start codon and termination codon are indicated as ▼.

The Prediction of Putative *cis* Elements in the Promoters of *OPR* Genes

With the predicted genomic sequences of all *OPR*s, we identified putative promoter sequence of *OPR1* and *OPR2* by a computational program Softberry-TSSP (available at

\ www.softberry.com). Two *cis*-elements , TG**ACGT**TC and TG**ACGT**GC, were found in *OPR1*, located from –218 to –211, and from –257 to –250 respectively (data not shown). Both are typical *As-1*-like element sequences with a palindrome core sequence **ACGT** that can be recognized by TGA transcription factors. The TGA transcription factors interact directly with NPR1, a positive regulator of SA-mediated signal transduction pathway (Johnson et al., 2003). The presence of an *As-1*-like elements in the *OPR1* promoter is consistent with results of Northern Blot analysis and reverse transcription-PCR (the figure on page 23) demonstrating that Zm*OPR1* is induced in maize seedlings treated with SA. Duplicated *As-1*-half elements –TGACG- were also found in the promoter of Zm*OPR2*, located at -259, -136, and -124.

Phylogenetic Analysis of OPR Proteins

Fig. 4 shows the phylogenetic tree for the amino acid sequences of OPR family members of different organisms in plant and fungus. Six maize OPRs, ZmOPR1, ZmOPR 2, ZmOPR 3, ZmOPR 4, ZmOPR 5, and ZmOPR 6 are classified to OPRII subgroup including AtOPR1, AtOPR2, LeOPR1, and OsOPR1 which are not required for JA biosynthesis (see above). ZmOPR7 and ZmOPR8 are classied into OPRII subgroup with AtOPR3 and LeOPR3, so they are most likely to involve in JA biosynthesis in maize. OPRx was identified from our original EST analysis, but we can not amplify this sequence when a maize genomic DNA template was used in PCR (data not shown). Therefore, we suspected this sequence may come from a contaminant in the EST library which was

prepared from *Colletotrichum graminicola* infected tissues. We performed PCR by using *Colletotrichum graminicola* genomic DNA as template and there was still no PCR products. We excluded the possibility that this is a *Colletotrichum graminicola* gene based on the phylogenetic analysis.

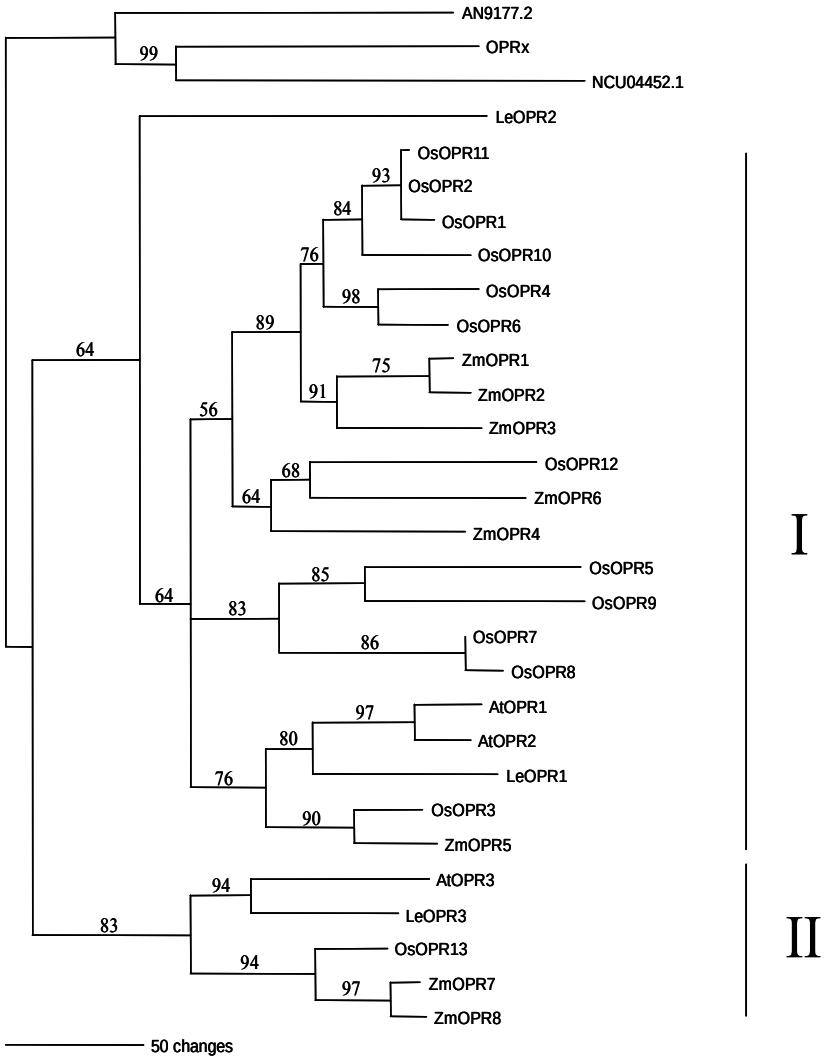

Figure 4. Phylogenetic analysis of plant and fungal OPR deduced amino acid sequences from maize (Zm), Arabidopsis (At), rice (Os), tomato (Le), and *Aspergillus nidulans* (An), *Neurospora crassa* (Nc). The phylogenetic tree was built by using the neighbor-joining program of PAUP4.0 phylogenetic analysis software package. (The amino acid sequence of ZmOPR8 was not included in this analysis)

RNA Profiling of Organ-Specific Expression of *ZmOPR* Genes

The maize OPR transcript expression levels in diverse maize organs were assayed by the Massively Parallel Signature Sequencing (MPSS) technique of Lynx Technologies (Brenner et al., 2000), and separately by EST distribution in diverse maize tissues. The MPSS profiling involves deep transcript sampling on microbeads, with a 17 base sequence read (tag) beginning with 'GATC'. A total of 203 MPSS libraries were assayed covering diverse tissues, developmental stages, and treatments (see Materials and Methods).

However, for this study we focused on B73 genotype samples as all cDNA and genomic sequences were derived from this inbred line. We deployed MPSS tech for transcription profiling of *OPR* genes its advantage that highly homologous sequences can be discriminated by this technology based on the 17-base tag signature. MPSS also provides high sampling sensitivity ensuring that *OPR* genes expressed at very low levels are detectable. Fig.5 demonstrates the transcript levels of *OPR* genes measured in parts per million (ppm) in selected maize organs including vegetative (Fig. 5A), reproductive (Fig. 5B) organs and kernel (Fig. 5C). *OPR* genes appear to be transcribed more actively in leaves at later developmental stage (R1) while they are expressed at a higher level earlier in root at the V2 stage (Fig. 5A). In kernel and reproductive organs, most *OPR* genes are expressed at a relatively low level in different developmental stages (Fig. 5B and C). OPR6 has the most abundant transcripts in the kernel and reproductive organs. The putative JA biosynthesis OPR isoform in maize ZmOPR7 is the predominant OPR

member expressed in shoot apical meristem from stages V2 to V4 while it remains inactive in root apical meristem. It has been shown that jasmonic acid inhibits the cell division in root apical meristem. Thus, OPR7 or jasmonic acid may have a different role in the development of shoot apical meristem. It appears that the regulation of OPR genes is fine tuned in a specific spatial and timely manner to control the plant development.

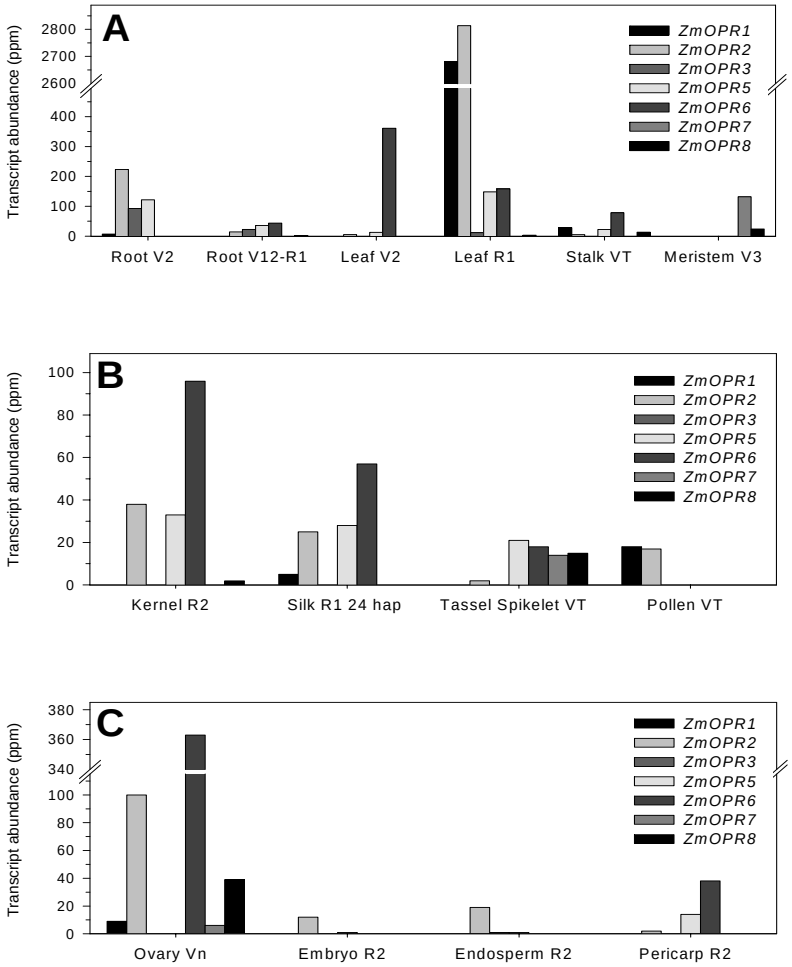

Figure 5. The expression levels of *OPR* genes measured in parts per million (ppm) in selected maize organs of 209 cDNA libraries representing total RNA expressed in specific tissues at different developmental stages. A. Vegetative organs. B. Kernel. C. Reproductive organs. *OPR4* transcript was not demonstrated because a practical Tag is not available for it in MPSS.

OPR1/2 Are Dramatically Induced by Fungal Elicitor Treatment, Pathogen Infection and Salicylic Acid

Both EST analysis and MPSS data suggest the OPR1/2 are more actively expressed than other OPR genes in unchallenged tissues or treatments. Fig. 6A demonstrates the induction of *OPR1/2* upon fungal elicitor (Chito-oligosaccharide) treatments and *Fusarium verticillioide* spore infection in silk cell suspension culture. To further our understanding of the expression of *OPR1/2* in response to stress, we treated two-week old plant with stress related signaling molecules including jasmonic acid, salicylic acid,

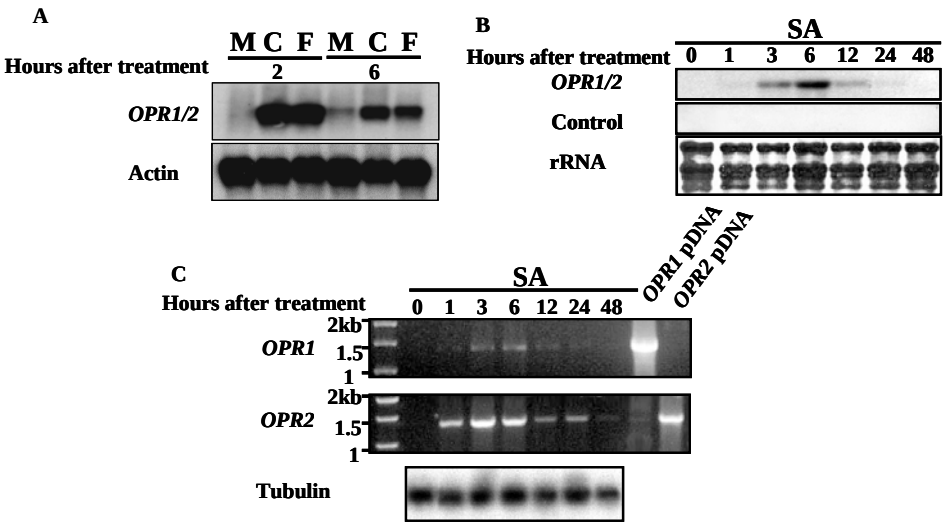

Figure 6. *OPR1/2* mRNA accumulates upon fungal elicitor treatment, pathogen infection and SA treatment. A. Corn suspension cell cultures treated with C-Chito-oligosaccharides(100 ug/ml); B. F-*Fusarium verticillioides* spores(10^6 spores/ml); M-Mock – inoculated. Leaves were treated with SA and RNA was isolated for B. Northern blot analysis; C. reverse transcription PCR. *OPR1* and *OPR2* plasmid DNA were used in PCR to assure the PCR was performed in a gene specific manner.

ethylene and abscisic acid. We found *OPR1/2* transcripts only accumulated in response to SA treatment suggesting *OPR1/2* may be controlled by a salicylic acid signaling pathway. We performed reverse transcription PCR to see if both *OPR1* and *OPR2* are inducible by SA because it is not plausible to generate a gene specific probe for *OPR1* and *OPR2* in Northern blot analysis due to their high sequence identity. In agreement with our finding in the promoters of *OPR1* and *OPR2* both of which contain SA-responsive cis elements, *OPR1* and *OPR2* are both indeed inducible by SA (Fig.6 B).

Expression of *OPR1/2* in Response to Pathogen Challenge

Because *OPR1/2* are induced strongly by fungal elicitors and pathogen in cell culture and by stress related signal SA, we used diverse pathogens to study the dynamics of *ZmOPR1/2* expression during plant-pathogen interactions. It appears that *OPR1/2* are induced much more strongly in a corn resistant line against *F. verticillioides* (Fig. 7A).

Also, OPR1/2 transcripts were accumulated in an earlier time in the resistant line to *Bipolaris maydis* than in susceptible line (Fig. 7B). However, when we tested *OPR1/2*expression upon a necrotrophic pathogen, *Cochliobolus carbonum,* we found that the strain could significantly induce *OPR1/2* expression but the avirulent strain failed to induce *OPR1/2* expression. It suggested OPR1/2 might contribute to the compatibility in this plant-pathogen interaction (Fig. 7C).

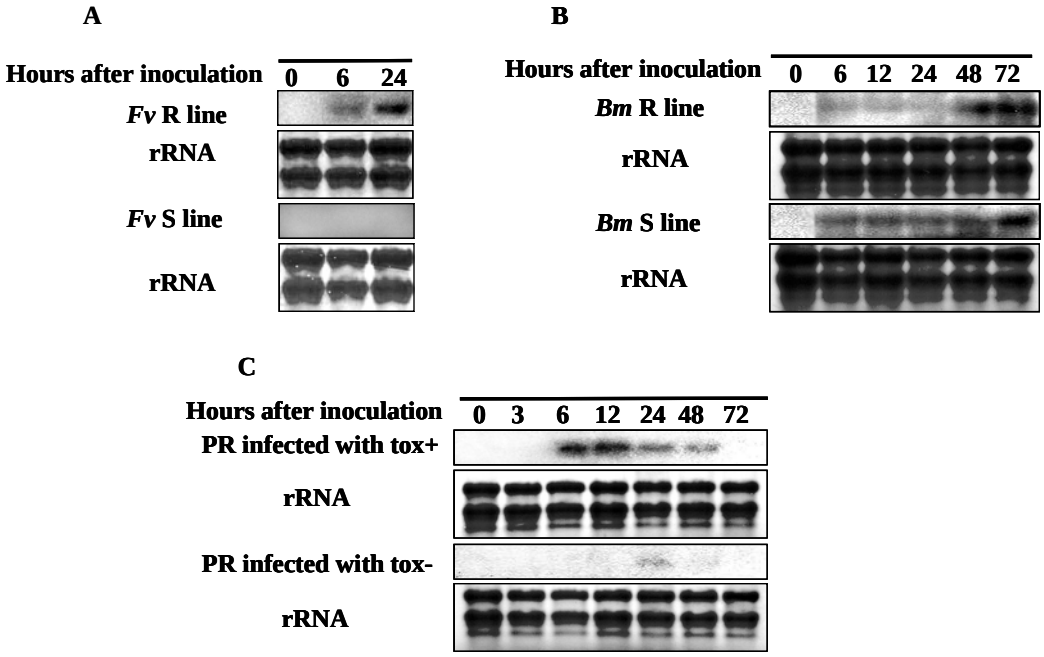

Figure 7. OPR1/2 gene expression in response various pathogens. A. *OPR1/2* gene expression in response to infection with *Fusarium verticillioides.* Two corn lines that are either susceptible(S) or resistant (R) to *Fusarium verticillioides* were infected with conidial suspension and total RNA was extrated from the infected silks from different time points upon infection. B. RNA gel-blot analysis of *ZmOPR1/2* gene expression in response to infection with *Bipolaris maydis*. Two corn lines that are either susceptible (S) or resistant (R) to *B. maydis*(Bm) were infected with conidial suspension and total RNA was extracted from leaves at different time points upon infection. RNA gel blots were hybridized to a 32P-labeled cDNA encoding 18S rRNA as a RNA loading control (only a representative blot from Rhm1/rhm1 experiment is shown). C. Conidial suspension of *Cochliobolus carbonum* race 1 either producing Hc-toxin (tox +) or deficient in toxin production (tox-) were sprayed on maize seedlings at the developmental stage V3. 10 ug of total RNA were loaded in each well and transferred to nylon membrane and hybridized to a cDNA fragment of *OPR1*. Equal loading of RNA was tested by staining to an rRNA with methylene blue.

ZmOPR6 and *7/8* mRNAs Accumulate upon Treatment with Defensive Signals and Wounding

Zm*OPR6* and *7/8* are rapidly induced within 1 hour after jasmonic acid, ethylene, and abscisic acid treatments (Fig. 8) while all these signals had no significant effect on expression of other *OPR* genes (data not shown). It should be noted that *OPR6* and *OPR7/8* were not responsive to SA treatment (data not shown) suggesting inductions of maize OPR individual genes are regulated in distinct pattern. Fig. 8C shows that only

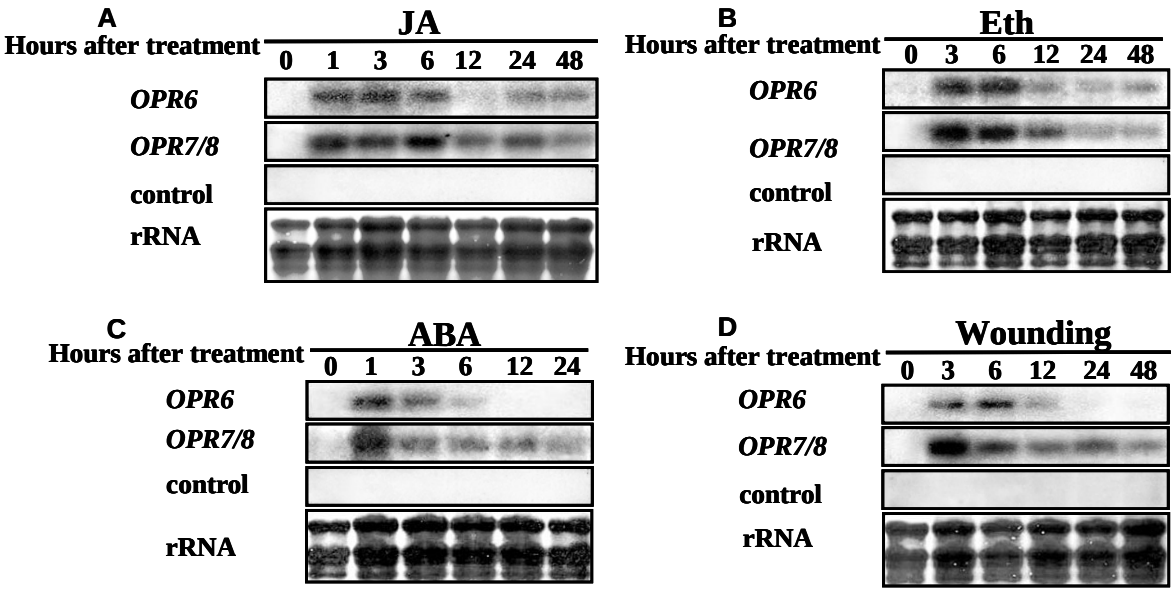

Figure 8. Activation of *OPR* by defense signals or wounding. Total RNA (10 µg lane -1) from B73 leaves treated with A.JA (200 µM), , B. Ethylene (10 µL L -1) or C. ABA and subjected to D. mechanical wounding was used for Northern blotting analysis with a probe derived from *OPR6* and OPR7/8 gene.

OPR6 and *OPR7/8* are induced by mechanical wounding which is in agreement that JA, ethylene and ABA are all believed to be wounding-related response signals.

Advance Genetically *opr2* Mutant Alleles into Genetic Backgrounds That Contrast in Their Levels of Resistance to Diverse Pathogens Using the *Mu*-element Insertion Materials

We have identified as described in introduction, an exonic and an intronic mutant allele of Fusarium-inducible *ZmOPR2* gene (Fig. 9). To identify unambiguously the function of this gene in resistance to diverse pathogens, we plan to generate near isogenic lines that are either resistant or susceptible to specific pathogens. This will be achieved by backcrossing the original allele to several lines that are resistant or susceptible to wheat streak mosaic virus, maize dwarf virus, rust fungus, and contamination with fumonisins and aflatoxins. The current genetic stage for this mutant is BC2. To perform the functional study of OPR2, we need to advance the mutant material to genetic stage BC3F4. This project is still undergoing.

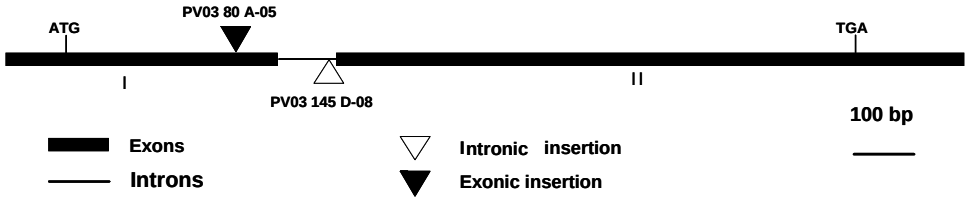

Figure 9. Schematic representation of *Mu* elements in *ZmOPR2* gene.

DISCUSSION

Phylogenetic Analysis

We showed and confirmed that it is very likely the entire maize gene family was identified in this study because of the depth of both EST and GSS databases. Agrawal et al., (2003) determined that rice had at least 13 OPRs (*OsOPR1-13*), a number much higher than that seen in any other plant species and the authors suggested a functionally diverse octadecanoid pathway in rice. Our data revealed maize has the second largest OPR gene family of all plant species that have been reported. Schaller et al. (1998) suggested that two subgroups of OPRs in plants with regard of their substrate preferentiality. Members of OPRII are able to effectively convert the natural 9*S*, 13*S*-OPDA to form the precursor of (+)-7-epi-JA and can also reduces three other OPDA steroisoforms. Proteins belonging to OPRI have enzyme activity more specific to 9*R*, 13*R*-OPDA and have moderate reduction activity with other iso-forms of OPDA (Schaller et al., 1998). Therefore, it is possible that OPRI subgrounp proteins may not be an enzyme of octadecanoid biosynthesis but in vivo have a different, yet unidentified substrate and give rise to a potential signaling molecule in adaptive responses. It was reported that tomato LeOPR1 reduced only 9*R*, 13*R*-OPDA, while LeOPR3 reduced both 9*R*, 13*R*-OPDA and 9S, 13S-OPDA which is the precursor of natural biologically active jasmonic acid (Strassener el., 2002). However, LeOPR2 did not accept any OPDA or α,β-unsaturated carbonyl as a substrate (Strassener el., 2002). In Arabidopsis, AtOPR3 was shown to effectively convert the natural JA precursor, 9*S*,13*S*-OPDA to the corresponding OPC-8:0 while AtOPR1 and AtOPR2 preferentially

catalyze the reduction of 9*R*,13*R*-OPDA (Schaller et al., 1998; Schaller et al., 2000). Based on the isomer preferences of previously characterized OPRs and our phylogenetic analysis, it is very likely the OPRs in group B belong to OPRI and those in group C are OPRII. Thus, we hypothesize that ZmOPR7 and ZmOPR8 are most likely to be the OPR isozymes involved in JA biosynthesis in maize. Group A contains a large number of OPRs and represents exclusively the monocot plants maize and rice. These OPRs may have additional functions in monocot plants. LeOPR2 is the only member of Group C and it doesn't have any OPR reduction activity (Strassener el. 2002).

Mapping of ZmOPR Genes to Chromosomes by Using Oat Maize Addition Lines

All of the maize *OPR* cDNA sequences in this study were derived from EST libraries. To determine if two similar *OPR* sequences represent two different loci or two alleles of a single locus, localization of these gene sequences on maize chromosomes was needed. For this purpose, OMA lines are ideal for mapping maize *OPR*s to their chromosomes by PCR due to the simplicity of this method. Though it can not reveal the precise location of a gene on a chromosome, we can obtain substantial evidence to determine that two highly homologues sequences are representing two different genes as long as they are localized to different chromosomes. In the OMA mapping study, we designed gene-specific primers based on the cDNA sequences of OPRs. All of the PCR products, when we used the OMA genomic DNA as templates, had the expected sizes corresponding to their genomic sequences assembled by GSS sequences. Notably, though ZmOPR1 and ZmOPR2 share

95% nucleotide sequence identity, they were localized to different chromosomes. This information will be especially valuable when we generate and identify specific mutants for ZmOPR1 and ZmOPR2.

Genomic Organization of OPR Genes

The genomic sequences of maize OPR genes were obtained from an available GSS database. Having full-length cDNA sequences for all maize OPR genes, we were able to assemble genomic sequences covering the entire length of cDNA by an extensive GSS database search. This approach allowed us to identify the intron/exon structure , putative promoters, transcription start points and cis- elements of these OPR genes. No promoter sequences could be identified for ZmOPR4 and ZmOPR5 due to the limit of the GSS database. The number and length of introns and exons of maize *OPR* genes are diverse suggesting this gene family may undergo different molecular evolution events. Typical binding elements for TGA-transcription factors were found in *ZmOPR1* and other Zm*OPR* promoters. It has been shown that TGA-transcription factors can interact with NPR1 which is a disease resistance protein stimulated by SA (Johnson et al., 2003). In the SA signaling pathway, NPR1 is activated by SA and it in turn activates the TGA-transcription factors which induce expression of numerous defense genes including *PR-1* in Arabidopsis (Johnson et al., 2003). Our northern blot and RT-PCR analysis also showed that *ZmOPR1* and *ZmOPR2* were inducible by SA treatment. Interestingly, It seems that the As-1 elements are only present in the OPRs in Group A, which exclusively

consists of monocot *OPR*s. Thus, we speculate that *OPR*s in Group A may have a regulatory mechanism that is different from those *OPR*s found in Group B or Group C, which includes OPRs involved in octadecanoid biosynthesis and are inducible by octadecanoids.

Survey of MPSS Data

MPSS provided us an in-depth approach to perform transcription profiling for highly related genes such as gene family members. The MPSS data shown in this study demonstrated that individual *OPR* genes have tissue and temporal specific expression pattern. For instance, *ZmOPR1* and *ZmOPR2* are expressed predominantly in leaves both of whose transcripts exceed 2,000 ppm in leaves. ZmOPR6 is mainly transcribed in ovary and leaf. Interestingly, the putative JA biosynthesis OPR isoform in maize ZmOPR7 is expressed at the highest among all OPRs in shoot apical meristem from V2 to V4 stage while it remains inactive in root apical meristem. Those observations suggest OPR7 or jasmonic acid play distinct role in shoot apical meristem but not in root apical meristem.

Hormone Regulation of Expression of ZmOPR Genes

Examination of environmental and hormonal stimuli that induce *OPR* expression should increase our understanding of the role this enzyme family has in general adaptive and stress responses. We, thus, used Northern blot analysis to determine *OPR* expression

patterns upon treatment with a variety of signaling molecules treatment or stress. Octadecanoid pathway genes were previously shown to be differentially regulated by wounding. In tomato, *LeOPR3* RNA was accumulated upon wounding in local leaves while *LeOPR1* and *LeOPR2* RNA were not affected (Strassner et al., 2002). Arabidopsis *AtOPR1* and/or *AtOPR2* were induced by wounding in local leaves as well as in systemic leaves at a moderate level (Biesgen and Weiler., 1999). Wounding also induced rice *OsOPR1* from as early as 30 minute in local leaves (Agrawal et al., 2003). In this study, we found wounding was able to dramatically induce expression of *ZmOPR6* and *ZmOPR7/8* within 3 hours which then declined after 6 hours. The expression of Zm*OPR1, 2, 3*, 4, and 5, however, were not affected by wounding treatment.

ZmOPR1 and *ZmOPR2* RNA levels did not increase in response to exogenous JA while the *ZmOPR3* RNA level was slightly increased. However, *Zm*OPR6 and *Zm*OPR7/8 RNA levels increased significantly in response to JA treatment. SA treatment was only able to increase *OPR1* and *OPR2* expression at a high level but failed to significantly induce other *OPR* genes. Plants apparently regulate these *OPR*s through different mechanisms. Although this regulation mechanism is not fully understood, it seems that it occurs through a cascade of proteins including transcription factors such as TGA-TF that is regulated by salicylic acid or another phytohormone. ABA and ethylene treatments were also able to increase *OPR6* and *OPR7/8* RNA levels but did not affect transcription modulation of other *OPR*s. It is well established that ABA is a required signal along with JA for the wound-induced defense responses (Pena-Cortes et al., 1989). Therefore, it was not surprising to find that transcripts of ABA and JA inducible OPR6, OPR7/8 are also up

regulated in response to mechanical wounding. Interestingly, the northern blot analysis demonstrated that *OPR6* and *OPR7*/*8* have induction patterns different from *OPR1*/*2*. *OPR6* and *7*/*8* appeared to be induced by wounding response related signals such as JA, ethylene and ABA. Since OPR7/8 are the closest homologs of Arabidopsis and tomato OPR3 that is required for JA biosynthesis, we hypothesize that OPR7/8 may be specifically involved in wound-induced defense responses to insects. On the other hand, OPR1 and/or OPR2 may be specifically involved in SA-dependent defense responses against pathogens.

Induction of OPR Transcript Accumulation in Response to Pathogens

Although the role of maize OPRs in plant/pathogen interaction is not understood, it is possible that oxylipins including jasmonates, or other products catalyzed by OPRs are factors that play a role in resistance or susceptibility to pathogens. ZmOPR1/2 appeared to be highly active in response to pathogens or elicitors. Our RNA blot analysis indicated that of all maize OPR genes, only OPR1 and/or OPR2 can be induced. They are more likely to be induced in resistant but remain unaffected in susceptible lines. Taken that *OPR1* and *OPR2* are highly inducible by SA but not JA, the yet unidentified OPR1/2 catalyzed products are more likely to be confined in SA signaling pathways that contribute to plant resistance against biotrohpic pathogens with the regard that SA is generally believed to primarily induce defense against biotrophic pathogens. Indeed, OPR1 and/or OPR2 are induced in mazie silks of an inbred line that is highly resistant to Fusarium ear

rot caused by F. verticillioides. Intriguingly, no significant induction of OPR1/2 RNA levels was observed in the silks of a susceptible line to *Fusarium* ear rot. Similarly, OPR1/2 are induced more rapidly in a resistant line to *Bipolar maydis* than their induction in susceptible line suggesting a potential contribution of OPR1/2 in defense response against this pathogen. Though the induction of OPR1/2 in compatible interaction between the maize and *Cochliobolus carbonum* does not contradict our hypothesis that OPR1/2 are primarily involved in SA-mediated defense response to biotrophic pathogens, we need to use strictly-lifestyle-defined pathogens to test OPR1/2 function in response. This project is underway.

SUMMARY

Eight maize *OPR* genes have been identified which may consist of the entire maize *OPR* gene family due to the thorough EST and GSS database search. Individual OPR genes are confirmed their presence in maize genome by Southern blot analysis and mapping them on chromosomes using OMA materials. Genomic structures of OPR genes demonstrated they have undergone different molecular evolution events. Putative cis-acting elements were found in some OPR genes suggesting they are regulated by certain phytohormone. The maize OPR genes appear to have distinct tissue expression pattern as MPSS data showed. OPR1 and OPR2 are mostly expressed in leaves. Other OPRs may have important role in specific organ development such as the putative JA biosynthesis OPR7/8. Northern blot analysis revealed that OPR1/2 have a very active role in defense responses against pathogens which are regulated by a SA-dependent signaling pathway. OPR6, OPR7 and OPR8 are involved in wounding defense response as they are inducible by JA, ethylene and ABA as well as mechanical wounding.

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