Plant Physiol. (1994) 105: 1097–1105

Molecular Analysis of Two cDNA Clones Encoding Acidic Class I Chitinase in Maize[1]

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The cloning and analysis of two different cDNA clones encoding putative maize (*Zea mays* L.) chitinases obtained by polymerase chain reaction (PCR) and cDNA library screening is described. The cDNA library was made from poly(A)$^+$ RNA from leaves challenged with mercuric chloride for 2 d. The two clones, pCh2 and pCh11, appear to encode class I chitinase isoforms with cysteine-rich domains (not found in pCh11 due to the incomplete sequence) and proline-/glycine-rich or proline-rich hinge domains, respectively. The pCh11 clone resembles a previously reported maize seed chitinase; however, the deduced proteins were found to have acidic isoelectric points. Analysis of all monocot chitinase sequences available to date shows that not all class I chitinases possess the basic isoelectric points usually found in dicotyledonous plants and that monocot class II chitinases do not necessarily exhibit acidic isoelectric points. Based on sequence analysis, the pCh2 protein is apparently synthesized as a precursor polypeptide with a signal peptide. Although these two clones belong to class I chitinases, they share only about 70% amino acid homology in the catalytic domain region. Southern blot analysis showed that pCh2 may be encoded by a small gene family, whereas pCh11 was single copy. Northern blot analysis demonstrated that these genes are differentially regulated by mercuric chloride treatment. Mercuric chloride treatment caused rapid induction of pCh2 from 6 to 48 h, whereas pCh11 responded only slightly to the same treatment. During seed germination, embryos constitutively expressed both chitinase genes and the phytohormone abscisic acid had no effect on the expression. The fungus *Aspergillus flavus* was able to induce both genes to comparable levels in aleurone layers and embryos but not in endosperm tissue. Maize callus grown on the same plate with *A. flavus* for 1 week showed induction of the transcripts corresponding to pCh2 but not to pCh11. These studies indicate that the different chitinase isoforms in maize might have different functions in the plant, since they show differential expression patterns under different conditions.

Simultaneous induction of chitinase and β-1,3-glucanase has long been implicated in defense reactions of plants against potential pathogens (see review by Bol et al., 1990). Chitinase genes respond to an attack by potential pathogens, elicitor treatments, and stress conditions, including exposure of the plant to the stress hormone ethylene (Mauch et al., 1984; Boller, 1985, 1988). Presumably, chitinase and β-1,3-glucanase are involved in the breakdown of β-1,3-glucan and chitin, which are found to be major components of the cell walls of many fungi (Wessels and Sietsma, 1981). Since there is no obvious natural substrate for chitinase in plants, the induction of chitinase activity has been suggested to play a crucial role in defense against fungal pathogens. It has been shown that both chitinase and β-1,3-glucanase activities increase coordinately in tomato when inoculated with *Fusarium solani*, and that both hydrolytic enzymes act synergistically in the degradation of fungal cell walls (Benhamou et al., 1990). Likewise, chitinase and β-1,3-glucanase from pea pods act synergistically in the degradation of isolated fungal cell walls and cause swelling and disruption of hyphal tips in vitro (Mauch et al., 1988). In addition, physiological concentrations of chitinase and β-1,3-glucanase effectively inhibit growth of many potentially pathogenic fungi (Schlumbaum et al., 1986; Mauch et al., 1988). Thus, chitinase and β-1,3-glucanase appear to be part of the inducible defense response of higher plants resulting from plant-pathogen interactions and the hypersensitive response. Numerous chitinase genes have therefore been isolated and characterized from various plant species.

The primary structures and expression of various chitinases have been determined in a variety of dicot and monocot plants (Swegle et al., 1989; Shinshi et al., 1990; Huang et al., 1991; Leah et al., 1991; Zhu and Lamb 1991; Huynh et al., 1992; Sela-Buurlage et al., 1993). These studies show that all stress-induced expression of chitinase activity is controlled by transcription rather than by enzyme activation, modification, or translation (Hedrick et al., 1988). On the basis of amino acid sequence data and subcellular localization, three chitinase classes have been proposed (Shinshi et al., 1990). Class I chitinases are basic isoforms and may contain a Cys-rich domain (also called the hevein domain), a hinge domain (Pro-, Gly-, and Arg-rich), and a major catalytic domain structure. Class I chitinases have been identified in tobacco (Shinshi et al., 1990), potato (Gaynor, 1988), bean (Broglie et al., 1986), *Arabidopsis* (Samac et al., 1990), and rice (Huang et al., 1991; Nishizawa and Hibi, 1991; Zhu and Lamb, 1991). Class II chitinases are acidic, lack the N-terminal Cys-rich domain and hinge domain, and have a high sequence similarity to class I chitinases within the catalytic domain. This class includes two chitinases from tobacco (Linthorst et al., 1990a; Payne et al., 1990), a petunia chitinase (Linthorst et al., 1990b), and a chitinase from barley (Leah et al., 1991). Class III chitinases share no homology to the class I or class

[1] This work was supported by funds from the Illinois Agricultural Experiment Station and U.S. Department of Agriculture grant AG-90-34215-5031.

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II enzymes, but are homologous to the acidic chitinases of cucumber (Bernasconi et al., 1987; Metraux et al., 1989), *Arabidopsis* (Samac et al., 1990), and tobacco (Lawton et al., 1992). However, there is an exception to the proposed classification system: an acidic chitinase has been isolated from bean that has a Cys-rich domain (Margis-Pinheiro et al., 1991). This classification system was based on sequence information solely from tobacco and other dicotyledonous plants before any detailed studies of chitinases from monocots were available.

The antifungal activity of chitinase makes this protein an attractive candidate for overexpression to produce disease-resistant agriculturally important crop plants, as demonstrated by Broglie et al. (1991) with tobacco plants. Although the structure, expression, subcellular localization, and antifungal activity of a number of plant chitinases have been studied, the pathogen specificity, biochemical properties, and roles of chitinases in plant defense remain to be further investigated. Previously, Huynh et al. (1992) cloned and determined the antifungal properties of two chitinases from maize (*Zea mays* L.) seeds and presented the amino acid sequence of a third chitinase, CHITD. In this paper, we report the isolation of two cDNA clones of putative class I chitinase isoforms from maize seedlings that had been treated with mercuric chloride. Although both are apparently class I chitinases, pCh2 belongs to a larger gene family than does pCh11, and their regulation patterns were dramatically different in response to mercuric chloride and to challenge by the fungus *Aspergillus flavus*.

MATERIALS AND METHODS

Plant Materials and Treatments

Maize (*Zea mays* L.) plants were grown in trays in a growth chamber with a 16-h photoperiod with 28°C days and 18°C nights. Seven-day-old seedlings of the maize inbred line Va26 were sprayed with a solution containing either 1 mg/mL ethephon, 1 mg/mL salicylic acid, or 0.2% mercuric chloride. Pots were then covered with plastic bags and seedlings were harvested between 6 and 48 h after the chemical treatment. Control plants did not receive any chemical treatment but were covered with plastic bags. Harvested tissues were frozen in liquid N_2 and stored at −70°C.

To obtain germinating maize embryos, the kernels were allowed to imbibe for 2 h in water either with or without 100 μM ABA and were then placed on filter paper saturated with the same solution in glass Petri dishes. Embryos were excised from the kernels after imbibition and after 1, 2, or 3 d and were immediately frozen in liquid N_2 and stored at −70°C.

Field-grown plants of the maize F_1 hybrid B73 × Mo17 were used for analysis of gene expression patterns in developing kernels. Ears were inoculated with *Aspergillus flavus* 20 to 24 d after midsilking by using a modified pinboard inoculation technique (Calvert et al., 1978). In the center of the pin array was a 16-gauge hypodermic needle through which 5 mL of a spore suspension, consisting of 2×10^5 conidia/mL, was injected through the husk. The inoculator was aligned with the ear axis and the pins were pushed through the husk and into the kernels. The inoculum was a mixture of four isolates prepared from lyophilized cultures of *A. flavus* (Northern Regional Research Laboratory [Peoria, IL] isolates 6536, 6539, and 6540 and a 1988 isolate from Illinois). The damaged controls were treated the same as inoculated ears except that no inoculum was included in the inoculator. Kernels were harvested at 9 to 23 d after inoculation and manually separated into aleurone plus pericarp, endosperm (minus most of the aleurone), and embryos. All tissues were immediately frozen in liquid nitrogen and stored at −70°C.

For in vitro studies, regenerable callus cultures were initiated from immature embryos of Mo17 × LB31 and LB31 × Mo17, maintained in the dark at 28°C on D medium (Duncan et al., 1985), and subcultured at 14-d intervals. Callus pieces (about 4 mm in diameter) were placed near the edge of a 10-cm Petri dish and *A. flavus* spores were then inoculated in the center. Tissue was harvested after 1 week of incubation, before the fungus had grown over the callus, and frozen in liquid N_2. Calli were then stored at −70°C before RNA isolation.

Nucleic Acid Isolation and Gel Blot Analysis

Total RNA was isolated from frozen tissue as previously described by using a guanidine-HCl method (Belanger and Kriz, 1989). For northern blot analysis, 10 μg of total RNA was subjected to electrophoresis in formaldehyde-agarose gels and transferred to nylon membranes (Magnagraph, Micron Separations, Inc., Westborough, MA). Isolation of maize genomic DNA from leaves of 7-d-old seedlings and Southern blot analysis were performed as described (Belanger and Kriz, 1989). For both RNA and DNA blots, the transferred nucleic acids were UV linked to the membrane by using a Stratalinker 1800 apparatus (Stratagene). The blots were hybridized overnight at 42°C using 50% formamide, 5× SSC, 1× Denhardt's solution, 20 mM sodium phosphate (pH 6.8), 0.1% SDS, and 5% dextran sulfate. The filters were washed with 2× SSPE (360 mM NaCl, 20 mM sodium phosphate, 50 mM EDTA, pH 7.4) and 0.5% SDS once at room temperature and once at 68°C for 15 min each time. Then the filters were washed for another 15 min at 68°C with 0.2× SSPE and 0.1% SDS. The Tm for hybridization of the pCh11 clone was calculated according to the following equation: $Tm = 81.5°C - 16.6(\log[Na^+]) + 0.41(\%\ G + C) - 0.63(\%\ \text{formamide})$ (Sambrook et al., 1989). This corresponds to hybridization conditions of Tm − 31 in 5× SSC, Tm − 34 in 2× SSPE (first wash), and Tm − 8 in 0.2× SSPE (final wash). Assuming a decrease in Tm of 1°C for 1% mismatch between Ch11 and Ch2, the Tm of the hybrid is estimated at 40°C, so the two clones would not be expected to cross-hybridize under these conditions. Experiments showed that there was less than 5% cross-hybridization between pCh2 and pCh11. The filters were exposed to x-ray film at −70°C.

For use in hybridizations, the cloned cDNA fragments were isolated from the plasmid by *Eco*RI digestion, electrophoresis in a 0.8% agarose gel, and use of the GeneClean II kit as recommended by the manufacturer (Bio 101, Inc., La Jolla, CA). The isolated fragment was labeled with [α-^{32}P]dATP by using a commercial kit (Stratagene) employing random primers and T7 DNA polymerase.

PCR Amplification and Cloning

Amplification of 0.5 μg of genomic DNA was carried out in a 50-μL reaction mixture that contained *Taq* DNA polymerase buffer supplemented with $MgCl_2$ to a final concentration of 1.5 mM, 100 μM of each dNTP, 0.2 μg of each degenerate primer, and 2.5 units of *Taq* DNA polymerase (BRL). The mixture was cycled 35 times in a Perkin-Elmer Cetus DNA Thermal Cycler as follows: 94°C for 1 min; 37°C for 1 min; and 72°C for 2.5 min; with a final 72°C extension of 7 min. The primers were designed according to the conserved chitinase nucleotide sequences from barley (Leah et al., 1991) and tobacco (Shinshi et al., 1988). The upstream degenerate primer was AAA/GGGNTTT/CTAT/CACNTA, of which the 5′ nucleotide corresponds to nucleotide 207 in the sequence of barley chitinase; the downstream degenerate primer was TGGTTT/CTGGATGACN, of which the 3′ nucleotide corresponds to nucleotide 612 in the sequence of barley chitinase (Leah et al., 1991). Following amplification, PCR products were analyzed by gel electrophoresis.

Screening of a Maize Leaf cDNA Library

A λ ZAP II cDNA library was made from poly(A)$^+$ RNA isolated from maize seedlings 48 h after spraying with 0.2% $HgCl_2$. The procedure used was previously described by Belanger and Kriz (1989). Screening of the library with the cloned, PCR-amplified maize chitinase (TA98) fragment as radiolabeled probe was performed under the conditions used in the Southern blots described above. Fifteen plaques continued to yield positive hybridization signals after the third round of screening, and four cDNA inserts of more than 1 kb were recovered from these clones as the recombinant pBluescript plasmid according to the manufacturer's protocols.

DNA Sequencing

Both strands of the chitinase cDNA fragment in the Bluescript vector were sequenced using a combination of commercial vector-specific primers and custom-designed oligonucleotides. Dideoxynucleotide sequence analysis (Sanger et al., 1977) of denatured double-stranded DNA templates or single-stranded DNA prepared from M13 templates (Yanisch-Perron et al., 1985) was performed with a modified T7 DNA polymerase (Sequenase; United States Biochemical Corp.). The DNA and deduced amino acid sequences were analyzed and assembled with the aid of MacVector computer software (IBI, New Haven, CT).

RESULTS

Amplification and Isolation of a Chitinase Genomic Sequence Fragment

A strategy utilizing the PCR was employed to amplify and isolate maize chitinase cDNA clones. The amino acid sequence of the barley protein (Leah et al., 1991) was compared with that of rice (Huang et al., 1991) to identify consensus sequences from which a pair of degenerate oligonucleotide primers were designed. Several products were detected in the PCR, which ranged from about 300 to 700 bp on ethidium bromide-stained agarose gels (data not shown). The DNA bands in the agarose gel corresponding to about 400 bp were purified and ligated to the dT-tailed *Eco*RV site of pBluescript SK. The resultant clones were sequenced and five were found to be homologous to chitinases from barley and rice. One of the PCR-derived clones (TA98) was then used to screen a maize cDNA library.

Isolation and Sequence Analysis of Two Class I Chitinase cDNA Clones

A λ ZAP II cDNA library was screened with the 400-bp fragment of the TA98 chitinase clone. Approximately 250,000 plaques were screened at high stringency and 15 clones were hybridized to the PCR TA98 insert after three rounds of screening. Following plaque purification and in vivo excision of the pBluescript recombinant plasmid from the λ ZAP II, the cloned inserts were confirmed by Southern blot analysis. The four clones larger than 1 kb were sequenced, and two of the chitinase cDNA clones representing two different types of chitinases are described here.

The complete sequences of the two cDNA clones were determined. A full-length clone, designated pCh2, consisted of 1128 nucleotides with a 957-nucleotide open reading frame, which would encode a polypeptide of about 33.5 kD. This clone has 27 nucleotides in the 5′ untranslated region and 144 nucleotides in the 3′ untranslated region. One putative polyadenylation (AATAAA) signal is located at nucleotides 1105 to 1110. The pCh11 clone, which is not full length, since part of the 5′ end is lacking, consists of 1000 nucleotides with 173 nucleotides in the 3′ untranslated region. Two putative polyadenylation (AATAAA) signals are located at nucleotides 944 to 949 and 965 to 970, respectively. The cDNAs have different stop codons, i.e. TAG for pCh2 and TAA for pCh11. These cDNAs share about 70% identity at the nucleotide sequence level.

Primary Structures of Maize Chitinases and Homology to Other Chitinases

The deduced amino acid sequences of pCh2 and pCh11 are presented and compared to those of other chitinase genes in Figure 1. Both chitinase clone open reading frames exhibit strong codon bias (89 and 87%, respectively) for G or C in their third codon position, as is common for other nuclear-encoded maize genes (Campbell and Gowri, 1990). The pCh2 polypeptide contains a hydrophobic putative signal peptide of 21 amino acids at the N terminus, as well as hevein and catalytic domains. The preprotein consists of 319 amino acids. The primary structure of the pCh11 gene product is not complete, since the clone is not a full-length cDNA, so the clone lacks a signal peptide and the Cys-rich domain, as expected for a class I chitinase, but it does have a hinge region that is Pro-rich. The polypeptide encoded by this clone resembles that from the seed chitinase gene CHITD reported by Huynh et al. (1992). The deduced amino acid sequences of pCh11 and CHITD exhibit more than 90% identity (Fig. 1). Since CHITD, a class I chitinase, and pCh11 are so homologous, pCh11 would also appear to encode a class I chitinase.

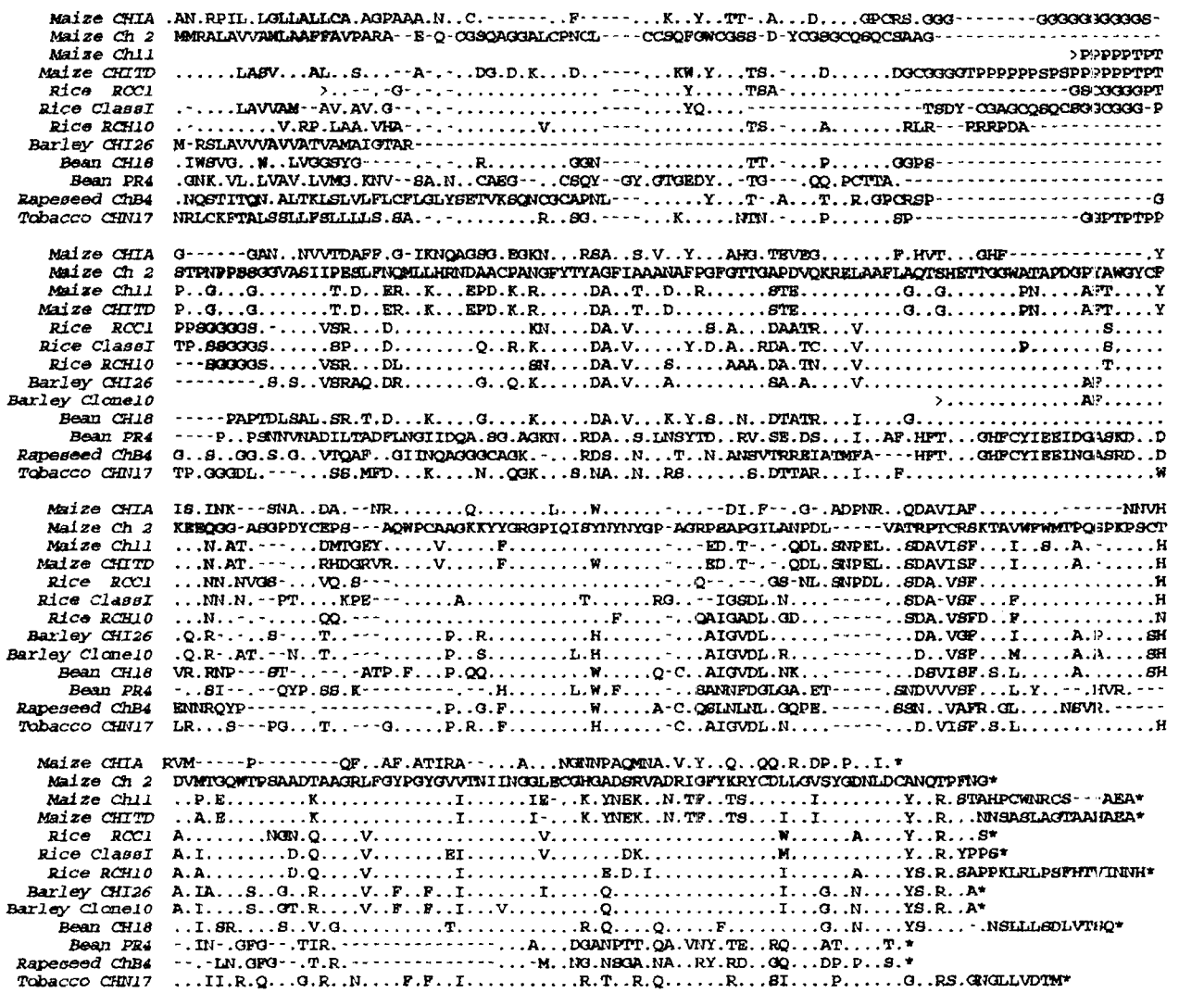

Figure 1. Comparison of the amino acid sequences of maize pCh2 and pCh11 and other monocot and dicot chitinase clones. The deduced amino acid sequence of pCh2 from maize is shown on the second line, which is used as the reference. The dots represent the identical amino acid residues. The dashes denote spaces required for optimal alignment. The asterisks indicate stop codons. Aligned amino acid sequences are presented for maize CHITA and CHITD (Huynh et al., 1992), rice RCC1 (Nishizawa and Hibi, 1991), rice class I (Huang et al., 1991), rice RCH10 (Zhu and Lamb, 1991), barley CHI26 (Leah et al., 1991), barley clone10 (Swegle et al., 1989), bean CH18 (Broglie et al., 1986), bean PR4 (Margis-Pinheiro et al., 1991), rapeseed CHB4 (Rasmussen et al., 1992), tobacco CHN17 (Shinshi et al., 1990), and tobacco Tach1 (Payne et al., 1990).

If one considers the presence of the Cys-rich domain and the hinge domain (Pro-/Gly-, and Pro-rich) as the definition of class I chitinases, the deduced peptides encoded by the pCh2 and pCh11 clones are very similar structurally to the basic chitinases of rice (Huang et al., 1991; Nishizawa and Hibi, 1991; Zhu and Lamb, 1991), bean (Broglie et al., 1986), and tobacco (Shinshi et al., 1990) (Fig. 2). However, the deduced amino acid sequences of the polypeptides encoded by pCh2, pCh11, and the CHITD clone of Huynh et al. (1992) show that they have acidic isoelectric points (Table I). Polypeptide analysis showed that the two rice chitinase clones also have acidic isoelectric points. In contrast, barley chitinase CHI26 is defined as a class II chitinase due to the lack of the Cys-rich domain, yet it has a basic isoelectric point. Although dicot plants have exceptions where the class I chitinases exhibit acidic isoelectric points (Table I), most of the data derived from monocot plants support the suggestion by Huynh et al. (1992) that maize chitinases, and probably those of rice, barley, and other monocot species in which chitinases have yet to be characterized, have greatly diverged from other dicotyledonous chitinases.

The hevein domain sequences shown in Figure 2 allows a comparison of the sequences of pCh2 and those of other gene products that contain this domain, including barley and rice lectin. The hevein domain of pCh2 shares 72% amino acid sequence identity with rubber hevein and CHITD, which

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Hevein          EQ-CGRQAGGKLCPNNL-CCSQYQYCGST-DEYC-SPDHNCQSN--CKD
Maize Ch 2      ..-..S....A....C.-....FGW...S-.-..G.G---...Q--.SA
Rice ClaseI     ..-..S....A....C.-......W..----------------------
Rice CH10       ..-..S....AV...C.-....FGW....S.-..GAG---...Q--.SR
Bean CH18       ..-.......A...GGN-.....G.....T.-..GPG---...Q--.GG
Tobacco Chi     ..-..S....AR..SG.-...KFGW..N.N.-..G-.G-....Q--.PG
Barley Lectin   ..R..E.GSNME.....-.....G...MGG.-..GKG---..NGA-.WT
Rice lectin     ..T..K.ND.MI..H..-.....G...MGR.-..GTG---...GAS.-T
Maize Seed CHITA  A.N..C.PNF-C.SKFGY.GTTDA...---.---G-----...GP-.RS
Maize Seed CHITB  A.N..C.PNV-C.SKFGY.GTTDE...---.---G-----...GP-.RS
Maize Seed CHITD  A.-..DG.D.....DC.-...KWG.....S.-..GDG---...Q--.DG
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Figure 2. Comparison of the amino acid sequences of the pCh2 hevein domain with hevein domains in other proteins. The amino acid sequence of rubber hevein (Broekaert et al., 1990) is used as the reference. Aligned amino acid sequences are rice CH10 (Zhu and Lamb, 1991), rice class I (Huang et al., 1991), bean CH18 (Broglie et al., 1986), tobacco chitinase (Shinshi et al., 1990), barley lectin (Lerner and Raikhel, 1989), rice lectin (Wilkins and Raikhel, 1989), and CHITA, CHITB, and CHITD from maize seed chitinases (Huynh et al., 1992). The dots represent the identical amino acid residues. The dashes denote spaces required for optimal alignment.

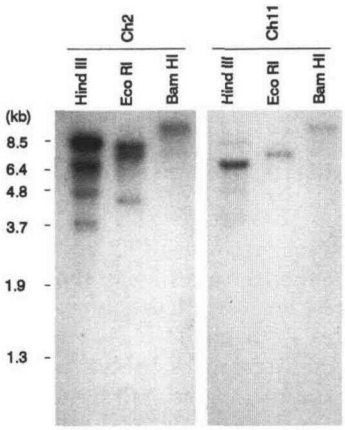

Figure 3. Southern blot analysis of maize genomic DNA hybridized with pCh2 and pCh11. Five micrograms of genomic DNA was digested with *Hin*dIII, *Eco*RI, or *Bam*HI and then fractionated on 0.8% agarose gels. Separated DNA was transferred onto nylon membranes and then hybridized with the radiolabeled pCh2 and pCh11 inserts. The mol wt standard was *Bst*E II-digested λ. The exposure time was 2 d, with an intensifying screen.

resembles pCh11. The other chitinase genes, CHITA and CHITB, obtained from maize seeds (Huynh et al., 1992) share only about 49% amino acid identity with the hevein domain. As shown in Figure 1, the hevein domain and catalytic domain of pCh2 are separated by a Gly- and Pro-rich hinge region. The amino acid sequence identities of the catalytic domains of pCh2 and other chitinases are 73% for rice (Huang et al., 1991; Nishizawa and Hibi, 1991; Zhu and Lamb, 1991), 76% for barley (Leah et al., 1991), 66% for bean basic chitinase (Broglie et al., 1986), 50% for bean acidic chitinase (Margis-Pinheiro et al., 1991), 51% for rapeseed acidic chitinase (Rasmussen et al., 1992), and 67% for tobacco basic chitinase (Shinshi et al., 1990).

Genomic Organization of the Chitinase Genes

To determine the complexity of the gene families encoding these two dissimilar class I chitinases, the pCh2 and pCh11 chitinase clones were used as probes to hybridize with genomic DNA that had been digested to completion with the restriction enzyme *Hin*dIII, *Eco*RI, or *Bam*HI. There are no *Hin*dIII, *Eco*RI, or *Bam*HI restriction sites in these two clones. Four or five hybridizing bands were found in pCh2-probed blots, suggesting that this gene is a member of a small gene family (Fig. 3). In contrast, the Southern blot probed with pCh11 showed only one distinct band in each digestion, indicating that pCh11 is probably encoded by a smaller gene

Table I. *Isoelectric points of monocot and dicot plant chitinases based on the amino acid composition deduced from the cDNA or genomic sequences*

Clone Names	Class I		Class II		Reference
	Acidic	Basic	Acidic	Basic	
Monocots					
Maize CHITA		9.2			Huynh et al., 1992
Maize CHITB		9.8			Huynh et al., 1992
Maize CHITD	4.9				Huynh et al., 1992
Maize pCh2	5.9				*This article*
Maize pCh11	4.7				This article; based on partial sequence
Rice class I	5.0				Huang et al., 1991
Rice RCH10	6.3				Zhu and Lamb, 1991
Barley CH126				9.4	Leah et al., 1991
Dicots					
Bean CH18		9.2			Broglie et al., 1986
Bean PR4	4.5				Margis-Pinheiro et al., 1991
Rapeseed ChB4		9.1			Rasmussen et al., 1992
Tobacco DHN 17		9.5			Shinshi et al., 1990

family or by a single gene. Under the high-stringency hybridization conditions used here, cross-hybridization with related genes should be minimized but cannot be ruled out completely.

Induction of Chitinase mRNA in Seedlings by Mercuric Chloride

Northern blot analysis with pCh2 and pCh11 as probes was used to monitor the expression of the chitinase genes in seedlings that were treated with the putative induction chemicals ethylene from ethephon, salicylic acid, and mercuric chloride. The chitinase probes hybridized specifically to a single mRNA band that corresponded in size to the cloned chitinase sequence of 1.1 kb (Fig. 4). There was a dramatic increase in chitinase mRNA corresponding to pCh2 after treatment with mercuric chloride, as reported by Nasser et al. (1990). The northern blots in Figure 4 demonstrate the increases in transcripts corresponding to pCh2 chitinase, which were much more intense than those observed with the pCh11 probe. The exposure time of the pCh2-probed northern blot was 2 d, whereas it took about 3 weeks for the signals on the blots probed by pCh11 to become barely visible. The transcripts encoding these two chitinases were not induced by ethylene or salicylic acid. This indicates that the response to ethylene is different from that found in rice (Nishizawa and Hibi, 1991) and dicotyledonous plants such as bean (Broglie et al., 1986), where the induction was slight and strong, respectively.

Expression of Chitinase Genes in Embryos upon Germination and the Effects of ABA Treatment

Northern blot analysis of RNA extracted from maize seeds germinated in either water or 100 μM ABA showed that both pCh2 and pCh11 transcripts were present in embryos from d 1 to 3 (Fig. 5). Different regulation of these two chitinases is apparent because the levels of pCh2 mRNA were slightly higher than those of pCh11. The phytohormone ABA had little effect on the induction of either chitinase gene under these conditions. The results were consistent with those reported from barley seeds, where ABA, which is involved in seed dormancy and germination, did not induce chitinase mRNA in barley aleurone layers (Leah et al., 1991).

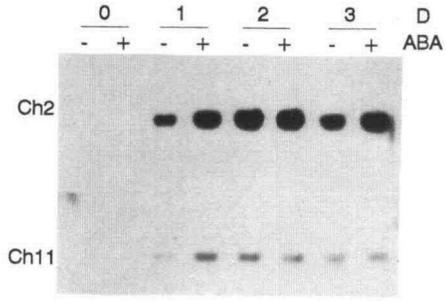

Figure 5. Effect of seed germination and the phytohormone ABA on the Ch2 and Ch11 transcripts in maize embryos. After seeds imbibed water and 100 μM ABA, they were transferred to Petri dishes containing layers of filter paper saturated with water or ABA, respectively. Embryos were harvested at the indicated days after imbibition (D), and northern blot analysis was performed using radiolabeled Ch2 and Ch11 as the probes. For d 0, embryos were removed 2 h after imbibition. The exposure time was 2 d for Ch2 and 3 d for Ch11, with intensifying screens.

Chitinase Induction in Maize Kernels after Infection with *A. flavus*

A modified pinboard device was used both to damage and to infect kernels with *A. flavus* spores to mimic field infection. Northern blot analysis of total RNA extracted from aleurone layers and embryos harvested 9, 16, and 23 d after inoculation showed that control (untreated) kernels did not express

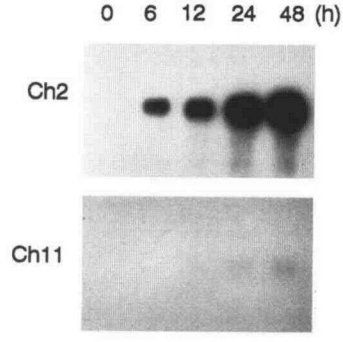

Figure 4. Northern blot analysis of the pCh2 and pCh11 transcripts in response to mercuric chloride. Plants were grown in pots in a growth chamber. At the age of about 7 d, the plants were sprayed with solution containing 0.2% mercuric chloride and then covered with plastic bags. Control plants were untreated and also covered with plastic bags. Leaves were harvested at the times indicated and total RNA was isolated. Ten micrograms of total RNA was subjected to northern blot analysis and the pCh2 and pCh11 fragments were used as radiolabeled probes. The exposure time was 2 d for pCh2 and 21 d for pCh11, with intensifying screens.

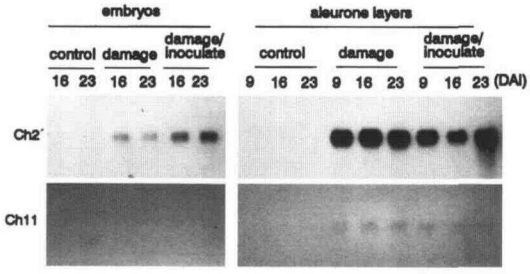

Figure 6. The effects of development and *A. flavus* infection on pCh2 and pCh11 mRNA accumulation in B73 × Mo17 aleurone layers and embryos. Kernels were either damaged with a pinboard or damaged/inoculated with inoculum of mixed isolates prepared from lyophilized cultures of *A. flavus*. In treated materials, aleurone layers and endosperm tissues were separated from only-damaged or damaged-plus-infected kernels after harvesting. Aleurone layers were the pericarp tissue enriched with aleurone layers. Ten micrograms of total RNA was subjected to northern blot analysis with the radiolabeled pCh2 and pCh11 as probes. The exposure time was 2 d for pCH2 and 7 d for pCh11, with intensifying screens. DAI, Days after inoculation.

either of the chitinase genes corresponding to pCh2 and pCh11 (Fig. 6). The damaged and infected kernels did contain chitinase mRNA in the aleurone layers and embryos, with the majority being present in the aleurone layers. Levels of pCh2 mRNA were higher than those of pCh11 mRNA in both aleurone layers and embryos. Only low levels of transcripts of both chitinases were detected in embryos. Furthermore, neither chitinase mRNA was found to be induced in endosperm tissues by damage or infection by the fungus or in damaged kernels within 24 h (data not shown).

Chitinase Induction in Maize Callus after Challenge with *A. flavus*

Regenerable maize calli derived from the hybrids Mo17 × LB31 and LB31 × Mo17 were challenged with *A. flavus* in agar plates containing growth medium. After 1 week of co-incubation without actual contact between the fungus and callus, RNA was isolated from the callus and subjected to northern blot analysis. There was a dramatic induction of pCh2 mRNA by fungal co-incubation in both genotypes tested (Fig. 7). Induction of pCh11 mRNA was not detected in these experiments. Untreated control callus did not have measurable levels of the transcripts corresponding to either Ch2 or Ch11. These results further indicate the possible different roles of the different chitinases in the plant-defense reactions, as suggested by Mauch and Staehelin (1989) for acidic and basic chitinases.

DISCUSSION

The present study describes the isolation and primary characterization of two cDNA clones from maize leaves, pCh2 and pCh11, with sequences similar to those of other chitinases. Based on the nucleotide and deduced amino acid sequences of the full-length pCh2 clone and the partial pCh11 clone, they both apparently encode class I chitinases with the following features: (a) a highly hydrophobic signal peptide of 21 amino acids; (b) a Cys-rich domain (also called the hevein domain) of 40 amino acids (missing from the partial pCh11 clone); (c) a hinge domain of 24 amino acids with a Pro- and Gly-rich region; and (d) a catalytic domain. The pCh11 clone is similar, but not identical, to one of three chitinase genes (CHITD) described in maize seeds (Huynh et al., 1992), which has a Pro-rich hinge domain (Fig. 1). Based on the available deduced amino acid sequences, pCh11 and CHITD share 90% identity (Fig. 1), indicating that they are members of a gene family but are not different alleles of the same gene. It has been shown that the maize seed chitinases, CHITA and CHITB, are significantly different in their biochemical and in vitro antifungal activity properties, even though they show 87% amino acid sequence homology (Huynh et al., 1992). Huynh et al. (1992) concluded that maize chitinases are more divergent than other plant chitinases. The sequences of the chitinase genes we obtained from maize leaves also support a similar observation, because only 61% identity was found in the amino acid sequences of the pCh2 and pCh11 catalytic domains (Fig. 1). The homology between pCh2 and rice chitinases is much higher than among maize chitinases, i.e. between 71 and 74% (Fig. 1). The comparison of the hevein domain within the maize chitinase gene family in Figure 2 also shows greater divergence. Although the chitinase genes pCh2 and CHITD demonstrate significant homology to rubber hevein, the other maize chitinase genes, such as CHITA and CHITB, share less identity in the hevein domains (Fig. 2).

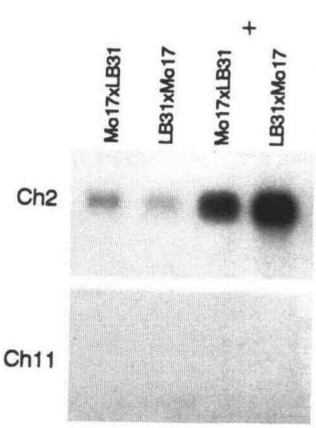

Figure 7. Induction of pCh2 and pCh11 mRNA in maize callus co-cultured with *A. flavus*. Control callus did not receive any treatment (−). The treated callus tissue (+) was co-cultured with fungal hyphae produced from spores placed in the center of the plates of callus growth medium and was removed after 7 d, before the hyphae contacted the callus. Each lane contains 10 μg of RNA, which was transferred to a nylon membrane and was probed with the pCh2 and pCh11 inserts. The exposure time was 2 d for pCh2 and 7 d for pCh11, with intensifying screens.

Huynh et al. (1992) showed that lack of chitin-binding domains, i.e. the hevein domain, did not influence the antifungal activities of either CHITA or CHITB. However, the antifungal activities of the proteins encoded by pCh2 and CHITD remain to be determined, even though they both have high homology in the chitin-binding sequence (Fig. 2). It is also true that class II chitinase, which lacks both the hevein and hinge domains, can effectively digest chitin (for instance, barley chitinase pCHI26, as shown by Leah et al., 1991).

Although almost all dicot class I chitinases have basic isoelectric points, there are two reported exceptions where acidic chitinases have Cys-rich domains in their N-terminal regions: in bean (Margis-Pinheiro et al., 1991) and in garlic (van Damme et al., 1993). Likewise, some monocot class I chitinases do not always have basic isoelectric points, as in the case of dicot chitinases. Therefore, we can conclude that monocot class I chitinase does not necessarily have a basic isoelectric point, as suggested for dicot chitinases from sequence information (Shinshi et al., 1990). However, it is not known if some monocot chitinases are different from their dicot counterparts or if our examples are exceptions. As chitinase nucleotide and protein sequences from monocot plants accumulate, this question should be answered. On the other hand, in studies of a tobacco β-1,3-glucanase gene (Shinshi et al., 1988), it has been found that some sequences necessary for targeting the proteins to the vacuole are located at the C terminus. Recent studies of tobacco pathogen-related proteins in transgenic plants confirmed that the vacuolar

pathogenesis-related proteins might have the targeting information in a short C-terminal propeptide (Melchers et al., 1993). This peptide is then removed during or after transport into the plant vacuole, indicating its importance in this process. Although there is no homology between the C termini of the pCh2 and pCh11 chitinase genes and the C terminus of the tobacco β-1,3-glucanase gene, there is a 14-amino acid C-terminal extension in Ch11 (Fig. 1), which could be important for this process (Neuhaus et al., 1991). Since these two types of maize chitinases show different expression patterns, as demonstrated by their responses to chemical treatments and expression in germinating seeds, there may be differences in the mechanisms controlling the regulation of gene expression.

The differences of the maize chitinase genes are also reflected in the genomic organization of these genes. Southern blots probed with pCh2 and pCh11 showed that more bands are present with pCh2 than with pCh11 (Fig. 3), indicating a larger gene family for pCh2 than for pCh11. This result indicates that the differences in gene numbers for pCh2 and pCh11 may possibly explain why the expression level of pCh2 was much higher than that of pCh11 in response to different stresses. Restriction maps show that at least three other cDNA clones isolated in the course of this study belong to the pCh2 family (data not shown). Rescreening the cDNA library made from mercuric chloride-treated leaf mRNA to identify the full-length clone with homology to pCh11 was unsuccessful even under low-stringency screening conditions, indicating that the expression level of this clone is very low under those conditions. However, *A. flavus*-infected aleurone layers and germinating embryos showed a greater abundance of pCh11 mRNA than did leaves (Figs. 5 and 6).

The possibility of different roles for pCh2 and pCh11 chitinases in vivo might be indicated by the different expression levels observed when plants were treated with mercuric chloride (Fig. 4). Mercuric chloride has been shown to induce a set of pathogenesis-related proteins in maize, including chitinase and β-1,3-glucanase (Nasser et al., 1990). Northern blot analysis of RNA isolated from maize seedlings treated with mercuric chloride showed a strong and linear induction of pCh2 mRNA, but only a slight induction of pCh11 mRNA. The transcripts encoding these two chitinase clones were not induced by the other chemical treatments, such as ethylene and salicylic acid (data not shown), showing that the responses are different when compared to rice (Nishizawa and Hibi, 1991) and dicotyledonous plant chitinases, such as that from bean (Broglie et al., 1986). Ethylene was shown to be a weak and a potent inducer of rice and bean chitinase expression, respectively. The differences observed here and those from rice and bean may indicate that different sets of genes are involved in plant defense-related reactions. Thus, the induction of maize chitinases is different from that of dicot plants, and this also contrasts with the results where ethephon and salicylic acid were able to induce rice chitinase mRNA (Nishizawa and Hibi, 1991).

In the experiments conducted by Huynh et al. (1992), where up to 5 μg of poly(A)$^+$ RNA was used in northern blots, the accumulation of mRNA for one of the chitinase clones (CHITA) was demonstrated in maize seeds, roots, and shoots. However, when only 1 μg of poly(A)$^+$ RNA was used, only seeds that had imbibed showed visible hybridization, which is consistent with our data obtained from germinating embryos (Fig. 5). This indicates that maize embryos constitutively express both pCh2 and pCh11 chitinase genes without environmental stimuli. The differential regulation and expression of pCh2 and pCh11 suggest that each might have some specific role under each condition.

Inoculation of developing kernels with *A. flavus* resulted in a large induction of chitinases in both aleurone layers and embryos (Fig. 6), with the pCh2 mRNA levels being much higher than those of pCh11. These genes were not induced in endosperm tissues (data not shown). Infection of kernels with fungi utilized physical damage of tissue prior to inoculation, but northern blot analysis of RNA from kernels that were only damaged revealed no chitinase transcripts after 2, 4, 6, and 24 h of treatment (data not shown). This might indicate that wounding alone did not induce the chitinase genes, or that 24 h of wounding was not long enough to induce the defense reaction. Since these experiments were conducted in the field, it is possible that natural infection caused the induction of chitinase mRNAs in the damaged-only kernels after 9 d. Embryos were found to have far less of both chitinase mRNAs than aleurone layers (Fig. 6). The lower level of chitinase induction in embryos might indicate that fungal infection of kernels could occur through the silks, thus bypassing the aleurone layer.

The evidence indicates that maize and probably other cereal chitinase genes are more divergent than those from other plants that have been described, although the gene products need to be tested in vitro for their antifungal activity. Further study of this divergence and its importance are especially needed because information concerning cereal chitinases is very limited. Individual chitinases may each play a particular role in vivo under a given condition, as indicated by the observations: (a) the pCh2 multiple-gene family and the pCh11 single-gene family suggest different roles in the defense-related process; and (b) these two genes are both induced in the event of fungal infection but not when challenged by mercuric chloride, nor are they induced in callus exposed to the fungus, and the genes are expressed in a tissue-specific manner. More study is needed to determine the exact role of the different maize chitinases.

ACKNOWLEDGMENTS

We wish to thank Dr. D. White of the Department of Plant Pathology at the University of Illinois for providing control and treated field-grown ears and Dr. Aree Waranyuwat for providing fungi-treated callus for our study.

Received November 22, 1993; accepted March 13, 1994.
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The GenBank accession numbers for nucleotide sequences of pCh2 and pCh11 are L00973 and L16798, respectively.

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