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Cultivar-Dependent Expression of a Maize Lipoxygenase Responsive to Seed Infesting Fungi

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Maize kernels are highly susceptible to *Aspergillus* spp. infection and aflatoxin (AF) contamination. Fatty acid signaling molecules appear to mediate the plant–fungal interaction by affecting the growth, development, and AF production of the fungus. In particular, fatty acid derivatives of the plant lipoxygenase (LOX) pathway are implicated in the *Aspergillus* spp.–seed interaction. The 9(*S*)-hydroperoxide derivative of linoleic acid promotes transcription of AF genes, whereas the 13(*S*)-hydroperoxide derivative decreases AF gene expression and production; both are sporulation factors. Our goal was to identify *LOX* genes responsive to *Aspergillus* spp. colonization and determine their specificities, 9(*S*)- or 13(*S*)-. Screening maize *LOX* expressed sequence tags (ESTs) identified one clone, *cssap 92*, which is highly expressed in *Aspergillus* spp.-infected seed susceptible to AF contamination and repressed in lines with resistance to AF contamination. The accumulation of *cssap 92* transcript was similar during *Fusarium* spp. infection. The cDNA clone has 94% identity to the previously described *L2* LOX gene from maize. Product-specificity analysis of the CSSAP 92 protein shows that it preferentially adds oxygen to carbon 9 of linoleic acid. Because 9(*S*)-hydroperoxy linoleic acid has been implicated as an aflatoxin-signaling molecule, it is possible that *cssap 92* could be used as a biomarker that is indicative of AF resistance in maize lines.

Additional keywords: *Aspergillus flavus*, *Fusarium verticillioides*, plant defense gene, *Zea mays*.

An intractable problem facing maize growers worldwide is contamination of crops by mycotoxins, particularly aflatoxin (AF), a potent carcinogen. During years with high temperatures and drought stress, the invasion of kernels by opportunistic seed infesting fungi of the *Aspergillus* genus, namely *Aspergillus parasiticus* and *Aspergillus flavus*, is prevalent (Payne 1992). These fungi readily produce AFB1, leading to substantial crop loss in developed countries and significant AF-associated health problems in those countries unable to implement detection and decontamination strategies. implement detection and decontamination strategies. Because traditional plant protection and breeding methods are not sufficient to prevent this disease, research efforts have turned to deciphering the molecular events regulating the *Aspergillus* spp.–seed interaction as a means to develop effective control measures.

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One research approach is the elucidation of genes and plant host gene products that might stimulate or repress *Aspergillus* spp. infection and/or AF contamination of seed. An emerging concept of the seed–*Aspergillus* spp.–AF interaction is the mediation of molecular cross-talk between the two organisms via fatty acid signaling. Burow et al. (1997) demonstrated that fatty acid derivatives of seed lipoxygenase (LOX) directly affect the production of AF in vitro. Specifically, LOX products 13(*S*)-hydroperoxy-9(*Z*),11 (*E*)-octadecadienoic acid (13*S*-HPODE), and 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13*S*-HPOTE) reduced levels of AF gene transcripts and 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9S-HPODE) extended the time period in which AF gene transcripts were detected. Downstream derivatives of 13*S*-HPODE and 13*S*-HPOTE, including methyl jasmonate (Goodrich-Tanrikulu et al. 1995) and various C6–C12 alkanal and alkenal volatiles (Doehlert et al. 1993; Zeringue et al. 1996) can also inhibit AF production. The effects of these compounds on AF production are distinct from their effects on fungal growth and development. In fact 9*S*- and 13*S*-HPODE as well as linoleic and linolenic acid elicit asexual sporulation in *Aspergillus* spp., presumably by mimicking endogenous *Aspergillus* spp. sporogenic factors (Calvo et al. 1999). Furthermore, Burow et al. (2000) have shown that expression of a peanut *LOX* gene, *pnlox1*, is stimulated by *A. parasiticus* infection and that levels of 9*S*-HPODE increase in *Aspergillus* spp.-infected peanut seed. Therefore, the fungus appears to receive morphological and chemical developmental signals from the host plant that are a direct consequence of fungal invasion.

Several lines of evidence suggest a significant role for the LOX pathway in plant–microbe interactions (Gardner 1991; Kolomiets et al. 2000; Kuhn and Thiele 1999). Lipoxygenases catalyze the addition of a peroxy group to either carbon 13 or 9 of the polyunsaturated linoleic and linolenic fatty acids. From these products, many metabolites are formed, including hydroxy fatty acids, derivatives of which have regulatory roles in plant metabolism. During wounding, methyl jasmonate and

C6 volatiles derived from the LOX pathway (Bate and Rothstein 1998) act as diffusible signals that initiate the plant defense response (Bohlman et al. 1998). Other products of the LOX pathway are known to have, or be precursors of, molecules with antimicrobial–insecticidal properties. For example, in potatoes, the elimination of LOX activity by antisense technology was reflected in the weight gain of insect pests feeding on the antisense potatoes (Royo et al. 1999). Antisense suppression of a *LOX* gene in tobacco allowed *Phytophthora parasitica* to successfully infect a resistant tobacco cultivar (Rance et al. 1998). In addition, *LOX* gene expression in plant tissues is spatially and temporally regulated and is often important for normal plant development (Dubbs and Grimes 2000a; Dubbs and Grimes 2000b; Eiben and Slusarenko 1994; Rodriguez-Concepcion and Beltran 1995; Van Mechelen et al. 1999; Wisniewski et al 1999).

Considering the importance of *LOX* genes to plant development and defense and the putative role of LOX products in directing *Aspergillus* spp. developmental processes, our lab focuses on the characterization of *LOX* genes involved in the seed–*Aspergillus* spp. interaction. Previously, we described a peanut seed *LOX* gene responsive to *Aspergillus* spp. colonization that may be involved in the peanut seed–*Aspergillus* spp. interaction. Here, we describe a *LOX* gene active in the maize–*Aspergillus* spp. interaction. As AF biosynthesis is confined primarily to colonized embryonic tissues of the maize kernel (Guo et al. 1996; Keller et al. 1994), the focus of this study was to identify *Aspergillus* spp. responsive LOX present in the maize embryo and to determine their product specificity (13*S*-HPODE or 9*S*-HPODE). An examination of nine putative expressed sequence tag (EST)–*LOX* clones resulted in the identification of two embryo-expressed *LOX* genes. One of these, *cssap 92*, encodes a full-length LOX protein with high sequence identity to the previously described maize LOX protein, L2 (Jensen et al. 1997). The CSSAP 92 protein is a 9*S*-HPODE LOX whose transcript is expressed differentially in maize lines with different degrees of susceptibility to *A. flavus* infections.

RESULTS

Sequencing the EST *LOX* clone *cssap 92*.

Sequence comparisons among 21 putative maize EST *LOX* clones showed that nine were nonhomologous. Homologous clones are defined as having > 97% identity over the regions of overlap (sequences not shown). The nine *LOX* clones were individually radiolabeled and used to probe total RNA that was extracted over a 48-h time course from Tx772–Fr2128 (resistant to AF) maize embryos that had been i) inoculated with *A. flavus*, ii) not inoculated but allowed to germinate, and iii) mock inoculated (control). Seven failed to hybridize to any transcripts, indicating these are not ESTs of embryo-expressed *LOX* in this cultivar. The pattern of expression in the maize embryo for the remaining two clones, *cssap 92* and *chssh 76*, are shown in Figure 1. Both putative *LOX* genes are expressed in germinating tissues and the control but are absent (in the case of *chssh 76*) or present at reduced levels in embryos infected with *A. flavus*. This is seen most clearly when the *cssap 92* cDNA clone is used as a probe. Identical results were achieved with the 3′ end of *cssap 92* as a gene-specific probe for this transcript.

To ensure that *cssap 92* and *chssh 76* were unique *A. flavus*-responsive *LOX* genes, the 3′ end of *cssap 92* was radiolabeled and hybridized to the *cssap 92* and *chssh 76* cDNA clones. Available EST sequence data for the *chssh* 76 clone showed that it possessed the 3′ end of this gene. The 3′ fragment of *cssap 92* does not cross-hybridize to *chssh 76*. Conversely, the radiolabeled 3′ end of *chssh 76* hybridized to the *chssh 76* cDNA clone but not to *cssap 92* (data not shown). The 3′ radiolabeled end of *cssap 92* also was used to probe digested genomic DNA. In Southern hybridization analyses, the *cssap 92* probe hybridized only to one band, suggesting that it was specific to *cssap 92* (data not shown).

Because *cssap* 92 gave the strongest signal (Fig. 1), we concentrated on characterizing this gene. Sequence analysis of the 5′ and 3′ ends of the *cssap 92* EST clone indicated that the EST clone probably contained the full-length transcript of *cssap 92*. Sequencing of the internal portion of *cssap 92* showed that it appeared to encode for an allele of the previously published partial cDNA clone, *L2*, of maize (Jensen et al. 1997). Over the overlap region, *cssap 92* shares 94% identity with *L2* at the nucleotide and amino acid level (data not shown). The *cssap 92* EST clone appears to be a full-length transcript and, relative to the partial *L2* clone, has an extra 179 codons, including a putative ATG start site determined by sequence homology with barley *LOX1* and confirmed by obtaining active protein translated from this codon (see below).

cssap 92 is expressed differentially in embryos of maize cultivars infected with *Aspergillus flavus*.

There is a delay in the induction of expression of *cssap 92* and *chssch76* in *A. flavus*-infected embryos of Tx772–Fr2128

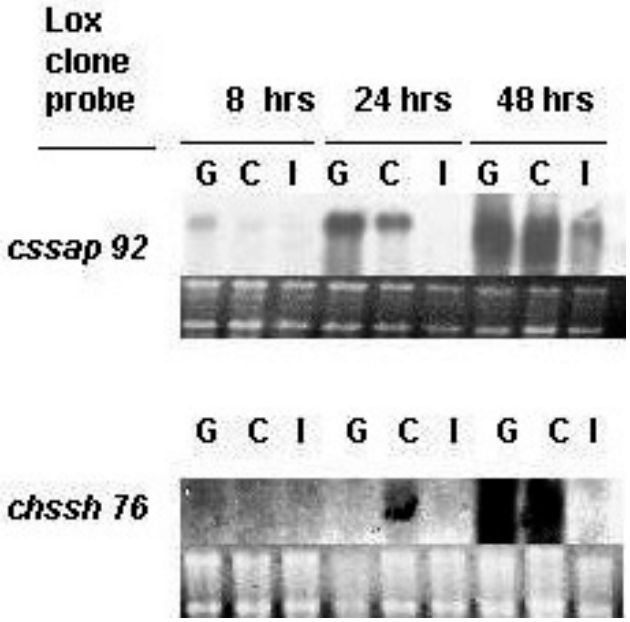

Fig. 1. Transcript analysis of two putative, embryo-expressed lipoxygenase (*LOX*) genes with radiolabeled *LOX* cDNA clones as probes. RNA was extracted from corn embryos of the backcross line Tx772–Fr2128 at the time points indicated. Total RNA (5 μg) was loaded in each lane. G = RNA from an uninfected, germinating embryo. C = Uninfected, nongerminating control. I = Embryonic RNA from corn infected with *Aspergillus flavus* 12-S. The experiment was repeated three times, and results shown are typical of three separate experiments. Lower panel shows ribosomal RNA stained with ethidium bromide to indicate loading of RNA samples.

compared to the control embryos (Fig. 1). To investigate whether this dampening of *LOX* expression (in comparison to control) was a conserved feature of the maize kernel response to *Aspergillus* spp. infections, we examined *cssap 92* expression in several lines of maize, varying in degrees of susceptibility to *Aspergillus* spp. colonization and AF contamination. Figure 2 shows the expression of *cssap 92* in the control and *A.* flavus-infected embryos of Asgrow 404 (Monsanto, St. Louis, MO, U.S.A.), a commercial seed line susceptible to AF contamination (J. Betran, *personal communication*). In this line, there are similar levels of *cssap 92* expression in the control and infected embryos, up to 48 h postinoculation. At 48 h and beyond, however, *cssap 92* expression increases dramatically in the infected embryos compared with the control. This is in contrast to the decreased *cssap 92* expression in the infected embryos of Tx772–Fr2128 at 48 h (Fig. 1).

Figure 3 shows the expression of the *cssap 92* gene at 48 h postinoculation with *A. flavus* in three maize lines that show some resistance to AF contamination (Campbell and White 1995; J. Betran, *personal communication*): Tex6, MASqK and Tx772, and the susceptible line Tx714. Once again, we see that the patterns of *cssap 92* expression differs between the various maize lines. Although expression of *cssap 92* in the control embryos does not show any consistent pattern between the resistant or susceptible lines, it is interesting to note that in infected embryos, a definite pattern can be seen. *cssap 92* mRNA accumulation is observed in infected embryos of susceptible lines but is not expressed or reduced in message accumulation during infection of resistant lines at the time points examined.

cssap 92 is expressed in root and stem tissue of maize and induced by wounding and methyl jasmonate.

To examine organ-specific expression in healthy plants, *cssap 92* mRNA accumulation was also examined in leaf, root, and stem tissues. Expression of *cssap 92* transcripts was not detected in young leaves but, similar to *L2* (Jensen et al. 1997), was detected in root tissue. In addition, *cssap 92* expression was observed in stem tissue (data not shown).

An antiserum capable of recognizing L2 and L1 LOX isoforms was used (Jensen et al. 1997) to detect putative L2 protein in maize tissues after treatment with methyl jasmonate. Because Jensen et al. (1997) used a different corn line in their studies (W64A), and we observed differential expression of *cssap 92* in different lines in response to *Aspergillus* spp. infection, we examined the expression of *cssap 92* after treatment of embryos with methyl jasmonate and wounding in Asgrow 404 and TX772–Fr2128. Our findings, similar to those of Jensen et al. (1997), showed that *cssap 92* was expressed under all of these conditions in both lines (data not shown).

cssap 92 is expressed differentially in maize lines in response to *Fusarium verticillioides.*

Another serious mycotoxigenic fungus that frequently infects maize kernels is *F. verticillioides* (formerly called *Fusarium moniliforme*). To address the question of whether the expression of *cssap 92* in response to *A. flavus* infection is a specific or general response to pathogen invasion, we studied the expression of this gene after inoculation with a strain of *F. verticillioides* isolated from maize. At 48 h postinoculation, the levels of expression of *cssap 92* in Asgrow 404 is higher for *A. flavus*-infected seed than in *F. verticillioides*-infected seed (Fig. 4). *cssap 92* is more highly expressed in *A. flavus*- and *F. verticillioides*-infected seed than in uninfected control seed.

In Tx772–Fr2128, at 24 h postinoculation, *cssap 92* expression is seen only in the uninfected control seed. At 48 h postinoculation, similar levels of *cssap 92* expression are seen in all three seed treatments.

In vitro expression of CSSAP 92 and analysis of its products.

In order to determine the oxidation products of CSSAP 92, a full-length cDNA was expressed in a bacterial expression system. Protein extracts from bacteria containing pRAW2, the pET-30a expression vector containing the entire *cssap 92* coding region, had measurable hydroperoxidizing activities at pH 6.5 to 7.5 (data not shown), and maximum activity was observed at pH 7.0. No activity was seen from extracts of bacteria containing only the pET-30a cloning vector or pRAW1 (encoding CSSAP 92 truncated at the N terminus) at this pH level. Activity at neutral pH suggests that the major product of

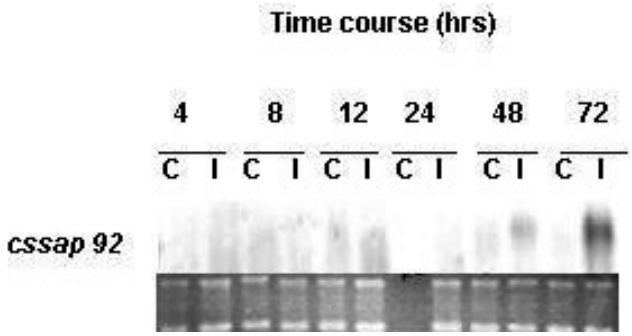

Fig. 2. RNA from control (C) and *Aspergillus flavus*-infected (I) embryos of Asgrow 404 seeds (Monsanto, St. Louis, MO, U.S.A.) was extracted at 4, 8, 12, 24, 48, and 72 h time points. Total RNA (5 µg) was loaded in each lane. RNA was probed with *cssap 92*. The experiment was repeated three times, and results shown are typical of three separate experiments. Lower panel shows ribosomal RNA stained with ethidium bromide to indicate loading of RNA samples.

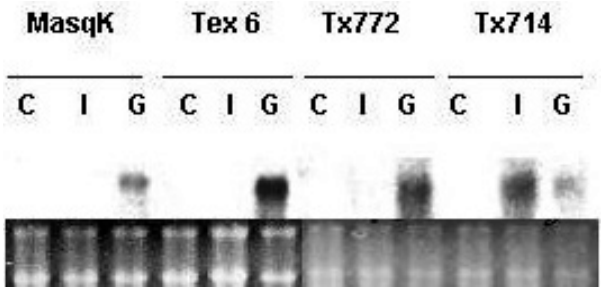

Fig. 3. The 0.26-kb 3′ end of the *cssap 92* gene was used to probe total RNA of embryos from corn lines MASqK, Tex 6, Tx772, and Tx714. Total RNA (5 µg) was loaded in each lane. C = Uninfected, nongerminating control. I = *Aspergillus flavus*-infected. G = Germinating. RNA was extracted from corn embryos at a single time point, 48 h postinoculation. The experiment was repeated three times, and results shown are typical of three separate experiments. Lower panel shows ribosomal RNA stained with ethidium bromide to indicate loading of RNA samples.

CSSAP 92 would be 9*S*-HPODE. In addition, the protein contains the bipartite Thr-Val (TV) motif, which was shown by Hornung et al. (1999) to specify 9*S*-HPODE production. Examination of the hydroperoxy linoleic acid products confirms that this is, indeed, the case because full-length CSSAP 92 protein produced 96% 9*S*-HPODE (Table 1). The small amount of 13*S*-HPODE that was present was largely racemic, with some tendency toward (R), indicating that some of the isomer arose from autoxidation or LOX-catalyzed autoxidation (Table 1). The (*E,E*)-diene isomers arise from rearrangements of peroxyl radicals, which are generally racemic and were not examined for (*R,S*)-stereo configuration. Interestingly, the CSSAP 92 protein lacking the N terminus was weakly active at alkali pH 12.5, suggesting that it would mainly produce 13*S*-HPODE. Indeed, 13*S*-HPODE is the major reaction product of the truncated protein at this pH level (Table 1), whereas the full-length clone was not active.

Analysis of HPODE in vivo and AF production in two maize lines inoculated with *A. flavus*.

To couple expression data of *cssap 92* (and other *LOX* genes) with activity and product specificity of the protein in situ, a series of control and infected seeds (Asgrow 404 and Tx772–Fr2128) were examined for LOX activity at 0, 12, 24, 48, and 72 h postinoculation. The 9,10-ketol (analyzed as methyl 9,10-diOTMS derivative) was a major product in all cases (data not shown). The 9,10-ketol arises from allene oxide synthase (AOS) action on 9*S*-HPODE (Gardner et al. 1970). Trace amounts of 12,13-ketols derived from AOS action on 13*S*-HPODE were detected in some samples. Therefore, the LOX proteins present in the mature seed are mainly 9*S*-HPODE producers.

Although 9*S*-HPODE apparently is present in seed under all conditions, the *cssap 92* transcript encoding a 9*S*-HPODE producer nonetheless increases during infection in AF-susceptible seed and is reduced in AF-resistant seed. We wanted to see whether this difference in *cssap 92* expression correlated with measurable differences in the concentration of AF produced by *A. flavus* growing on AF-resistant or AF-susceptible lines. The amount of AF present in *A. flavus*-infected Asgrow 404 and Tx772–Fr2128 seed at 0, 24, 48, 72, and 120 h postinoculation is shown in Table 2. AF was detected earlier (72 h) in the Asgrow 404 seed, but by 120 h, there was no significant difference in AF accumulation in the two lines.

DISCUSSION

AF contamination of oil seed crops by *Aspergillus* spp. is a grave concern for farmers in Africa, the Asian subcontinent, and the southern United States where conditions are often optimal for AF production (Payne 1992). One way to address this problem is to develop a better understanding of the molecular processes underlying the *Aspergillus* spp.–seed interaction. Studies in recent years have implicated a lipid-mediated signaling process important in the *Aspergillus* spp.–aflatoxin–seed interaction. Products of lipoxygenases, identified as having a role in plant defense against aggressive pathogens such as *P. parasitica* (Rance et al. 1998) and *Pythium irregulare* (Staswick et al. 1998), also might have a role in the *Aspergillus* spp.–seed–AF interaction problem. Studies by Burow et al. (1997) have shown that 13*S*-HPODE reduces the expression of AF gene transcripts, whereas 9*S*-HPODE increases the time during which these biosynthetic genes are expressed. Furthermore, derivatives of the 13*S*-LOX pathway have been shown to inhibit AF production in vitro (Goodrich-Tanrikulu et al. 1995; Zeringue et al. 1996). Recently, Burow et al. (2000) have shown that a *LOX* gene in the mature peanut seed is expressed during infection by *A. parasiticus*, thus establishing the first case of an *Aspergillus* spp.-responsive LOX. This study aimed to characterize *LOX* gene response and product specificity in *Zea mays*, another important oil seed crop highly susceptible to *Aspergillus* spp.-infection and AF contamination.

Transcript analysis indicated that at least two distinct *LOX* genes respond to *A. flavus* colonization. We made the novel observation that one of these genes, *cssap 92*, is expressed differentially in maize lines showing different degrees of susceptibility to AF contamination (Figs. 1, 2, and 3). The corn lines Tx772–Fr2128, Tx722, Tex6, and MASqK showed low levels of expression of the *cssap 92* transcript in infected embryos. These four lines show some resistance to AF in the field (Campbell and White 1995; J. Betran, *personal communication*). Asgrow404 and Tx714 are susceptible to AF contamination in the field and have increased expression of the *cssap 92* transcript in infected embryos.

To determine whether *cssap 92* expression was affected by different fungal pathogens, we infected Asgrow 404 (AF susceptible) and Tx772–Fr2128 (AF resistant) with the fumonisin-producing fungus *F. verticillioides* (Fig. 4). In both lines, *cssap 92* had the same pattern of expression in response to both fungi. This suggests that the differential expression of *cssap 92* that was seen in the different maize lines is a general mode of response to seed pathogens. In Asgrow 404, however, the level of gene expression was less during *F. verticillioides* infection than during *A. flavus* infection at the same time point. A future goal for our laboratory is to examine whether there is an interaction between *cssap 92* expression, genetic background, and different fungal pathogens.

Sequencing the *cssap 92* EST clone showed that the full-length transcript is approximately 94% identical to *L2* at the

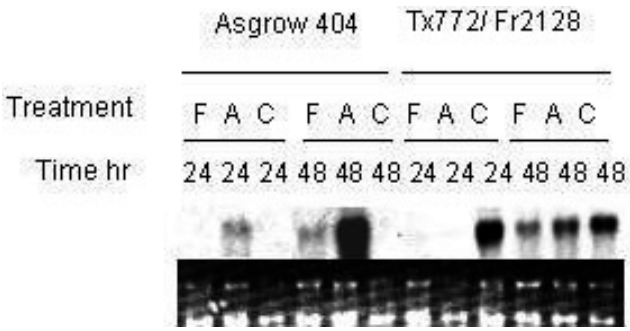

Fig. 4. Asgrow 404 (Monsanto, St. Louis, MO, U.S.A.) and Tx772–Fr2128 seeds were challenged with *Fusarium verticillioides* (F), *Aspergillus flavus* (A), and a water control (C). RNA was extracted from the embryos of these seeds at 24 and 48 h postinoculation. Total RNA (5 μg) was loaded in each lane. RNA was probed with the 0.26-kb 3′ end of the *cssap 92* gene. The experiment was repeated three times, and results shown are typical of three separate experiments. Lower panel shows ribosomal RNA stained with ethidium bromide to indicate loading of RNA samples.

nucleotide level (results not shown). This suggests that *cssap 92* is a possible allelic variant of *L2*, which is thought to be a 9*S*-HPODE producing LOX, based on the crude extract examination of corn seed (Gardner 1970) and amino acid sequence. Both proteins have the TV motif (Hornung et al 1999) to determine 9*S*-HPODE specificity. To accurately determine the nature of the product specificity of CSSAP 92, the protein was purified and reacted with linoleic acid. The purified CSSAP 92 protein has a pH optimum of 7.0, indicative of type 2 LOX (Shibata 1996). Product analysis confirmed that *cssap 92* produced mainly 9*S*-HPODE (96%). Intriguingly, an N-terminal truncated version of this protein, PRAW1, has very low but measurable activity at alkaline pH levels and produces mainly 13*S*-HPODE. From the structure of soybean LOX-1, it has been determined that the N-terminal region of the protein forms a beta-barrel structure (Boyington et al. 1997; Prigge et al. 1997) shown to target lipid body LOX to liposomes and lipid bodies in cucumber seedlings (May et al 2000). Without structural studies, one can only speculate as to why deleting this region would affect the specificity of CSSAP 92 in vitro. With 13*S*-specific LOXs, H-removal and O_2 insertion occurs on opposite sides of the substrate to 9*S*-specific LOXs (Funk et al. 1987; Lehman 1994). At high pH levels, the substrate molecule enters the active site in an orientation that favors 13*S*-HPODE production, presumably because the carboxylate anion prevents its insertion into the hydrophobic pocket of LOX in the inverse orientation. As the pH is lowered, the molecule reverses its orientation, leading to more 9*S*-HPODE production (Gardner 1989). Deleting the N-terminal residues of CSSAP 92 could lead to a change in protein conformation and activity such that PRAW1 is active only at high pH levels. At elevated pH levels, the substrate molecule might enter the active site only in an orientation that leads to 13*S*-HPODE production.

We predicted that the differences in *cssap 92* transcript levels (and possibly the amount of 9*S*-HPODE) in Asgrow 404 and Tx772–Fr2128 kernels could be reflected by differences in mycotoxin production. Table 2 shows that Asgrow 404 seeds have measurable quantities of AF at earlier time points (72 h) than seeds of Tx772–Fr2128. Because these lines are not isogenic, this difference could be the result of differences in how *A. flavus* grows and develops on different maize seeds. Whatever the reason, it is clear that Asgrow 404 is contaminated with more AF per gram of embryo at earlier time points than Tx772–Fr2128. This is consistent with the earlier appearance of the *cssap 92* transcript in Asgrow 404. At 120 h postinoculation, the AF level in both lines is very similar, suggesting that *cssap 92* expression in Tx772–Fr2128 is no longer delayed and contributes to AF production in the seed. To definitively make this conclusion, we need to compare AF production in corn lines that are isogenic to Asgrow 404 but deleted in *cssap 92*. The fact that Asgrow 404 expresses *cssap 92* under infection conditions affords the potential for increased formation of 9*S*-HPODE and its major metabolite, 9,10-ketol. Although 9*S*-HPODE increases AF gene expression, the effects of 9,10-ketol on AF production are not known. Nevertheless, these metabolites may explain increased AF gene expression in vitro. The differential expression of *cssap 92* could explain why in laboratory and field situations, Asgrow 404 exhibits more susceptibility to AF contamination than does Tx772–Fr2128.

In summary, we believe to have uncovered a potential role for the *cssap 92* gene encoding a 9*S*-HPODE producing LOX in its contribution toward AF contamination. Expression of *cssap 92* clearly responds to *A. flavus* infestation. Moreover, its expression is also different in resistant and susceptible maize lines, with expression strongest during infection of susceptible lines. Because this work was performed under laboratory conditions, it most closely resembles the reaction of postharvest seeds to colonization during storage conditions. In future studies, we would like to examine the expression of *cssap 92* as a biomarker denoting resistance or susceptibility to AF in the field. Because *cssap 92* also responds to infection by *F. verticillioides*, it would be interesting to determine whether *LOX* expression plays a role in the *Fusarium* spp.–maize interaction.

MATERIALS AND METHODS

EST-*LOX* clones.

Twenty-one EST cDNA pSPORT (Promega, Madison, WI, U.S.A.) clones encoding putative *LOX* genes from a number of different maize libraries were received from J. Duvick of Pioneer Hi-Bred International (Des Moines, IA, U.S.A.). Sequence data of the 5′ and 3′ ends facilitated comparisons of all clones. The Sequencher software program (Gene Codes, Ann Arbor, MI, U.S.A.) allowed the determination of homologous and nonhomologous clones on the basis of the available sequence, with homologous clones determined as having > 97% identity over the overlap region.

Sequencing and sequence analysis.

Sequencing the full-length *LOX* cDNA clone *cssap 92* was performed with the automated sequencing service provided by the Gene Technologies Department at Texas A & M University. The nucleotide sequence of both strands was determined. Sequences were aligned with Sequencher software. The full-length *cssap 92* sequence was compared with known *LOX* genes in the GenBank database with the BLAST program.

Table 1. Analysis of regio and stereo configurations of hydroperoxy-9(*Z*), 11(*E*)-octadecadienoic acid (HPODEs) produced by a full-length CSSAP 92 (pRAW2 construct) as their methyl HODEs at pH 7.0 and by a truncated CSSAP 92 (pRAW1 construct) at pH 12.5[a]

	13-HODEs (% of total)			9-HODEs (% of total)		
	9(*Z*),11(*E*)-diene	9(*E*),11(*E*)-diene		10(*E*),12(*Z*)-diene		10(*E*),12(*E*)-diene
Extract	13(*S*)	13(*R*)	13(*R,S*)	9(*S*)	9(*R*)	9(*R,S*)
pRAW2 (pH 7.0)	0.27	0.65	0.63	95.7	0.4	2.4
pRAW1 (pH 12.5)	50.7	12.1	8.7	11.6	10.3	6.6

[a] Incubations of linoleic acid with pRAW1 and pET30a extracts gave only trace amounts of HPODEs at pH 7.0. pRAW2 extracts produced only trace amounts of HPODEs at pH 12.5.

Plant materials and treatments.

The maize cultivars used in this study were Asgrow 404 and Tx714, lines that are highly susceptible to AF in the field, and Tx772–Fr2128, Tex6, MASqK, and Tx772, lines that have shown some resistance to AF contamination in the field (J. Betran, *personal communication*). Asgrow 404 is a commercial hybrid. Tx772, Tx714, and Tex6 are inbred lines. Tx772–Fr2128 is a hybrid of the two inbred lines Tx 772 and Fr2128. MASqK is a derived line from the MASqK population, developed in Georgia. Prior to treatment, kernels were sterilized by immersion in 10% Clorox (Oakland, CA, U.S.A.) for 3 min, followed by immersion in sterile water for 3 min. Seeds were inoculated with either *A. flavus* strain 12-S (provided by P. Cotty, USDA–ARS, New Orleans, LA, U.S.A.) or a *F. verticillioides* isolate (provided by T. Isakeit, Department of Plant Pathology and Microbiology, Texas A & M). To sterilize, kernels were immersed for 30 min in 0.1% Tween 80 containing 10^6 spores per ml. Control kernels were immersed in Tween water for 30 min. Neither Tween 80 or 20 affected *LOX* gene expression (data not shown). Corn kernels were wounded with a sterile needle, piercing near the center of the embryo to a depth of approximately 1 mm. To determine the effects of methyl jasmonate on *LOX* gene expression, corn kernels were treated with a racemic mixture (Bedoukin Research, Danbury, CT, U.S.A.), as previously described for peanut seed (Burow et al. 2000). All treated and control seed were incubated in the dark at 30ºC in petri dishes containing moist filter paper and a water reservoir. Samples were collected at the indicated time points.

For all RNA studies, only the maize embryo was used. The embryo was removed by wrapping the seed in the folds of a paper towel, striking carefully with a hammer to crack the kernel. The endosperm, aleurone layer, and scutellum were discarded, and the embryo was frozen in liquid nitrogen. Embryos were stored at –80ºC.

RNA and DNA extraction.

RNA was isolated from kernel embryos, leaf, root, and stem with Trizol reagent (GIBCO-BRL, Rockville, MD, U.S.A.), following the manufacturer's protocol with modifications. Two embryos (approximately 150 mg) were used per extraction or 150 mg of leaf, root, and stem, which were lyophilized overnight and ground in liquid nitrogen. Trizol (1 ml) was added, the resulting aqueous phase was extracted with phenol–chloroform and chloroform–isoamyl alcohol, and the RNA was precipitated with isopropyl alcohol. The RNA was resuspended in water and reprecipitated in 8 M LiCl, followed by a second reprecipitation in 3 M NaOAc and resuspension in 22 µl of water. All solutions were prepared with diethyl pyrocarbonate-treated water.

Genomic DNA was isolated from young leaf tissue following the urea extraction procedure of Chen and Dellaporta (1993). Two grams of leaf was used per 6 ml of lysis buffer.

Northern blot analysis.

RNA (5 µg) was separated on a 1.2% agarose–1.5% formaldehyde gel and transferred to Hybond membrane (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Following prehybridization and addition of the probe, the membranes were hybridized overnight at 60ºC and washed with increasing stringency, up to 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate, at 60ºC.

Probes were obtained from the EST clones by releasing the *LOX* inserts from the pSPORT1 cDNA cloning vector with the directional cloning restriction sites *Sal*I (at the 5′ end) and *Not*I (at the 3′ end). The inserts were labeled with ^{32}P by the random primer method (Sambrook et al. 1989). To analyze *cssap 92* expression, digestion of the *cssap 92* cDNA clone with *Sal*I and *Not*I yielded two fragments: a 234-bp *Sal*I fragment from the 5′ end of the gene and an approximately 2.6-kb *Sal*I–*Not*I fragment. Both fragments were purified, radiolabeled, and used as probes in the RNA analysis. To confirm that the 2.6-kb *Sal*I–*Not*I fragment was hybridizing only to the *cssap 92* transcript, RNA blots were stripped and probed with an approximately 0.2-kb *Kpn*I–*Not*I fragment containing the more variable 3′ untranslated regions (UTR) end of the *cssap 92* gene.

Southern blot analysis.

Maize genomic DNA (2 µg) were digested with *Eco*RI, *Hin*dIII, or *Xba*I and separated on an 0.8% agarose gel by electrophoresis. The DNA was transferred to Hybond membranes after denaturation in 0.4 M NaOH solution. Membranes were probed with the 0.2-kb *Kpn*I–*Not*I fragment containing the 3′ UTR of the *cssap 92* gene in conditions described above for RNA blots.

Expression of the *cssap 92 LOX* gene in *Escherichia coli.*

The full-length *cssap 92* cDNA clone was ligated into the expression vector pET-30a (Novagen, Madison, WI, U.S.A.). This was achieved by isolating a 2.6-kb *Sal*I–*Not*I fragment of *cssap 92*, removing the *Sal*I–*Not*I fragment from the pET-30a vector, and subcloning the *cssap 92* fragment directly into pET-30a to give pRAW1. pRAW1 encodes for 2.6 kb of the *cssap 92* and lacks approximately 0.24 kb of the 5′ coding region, including the putative start site. Next, approximately 0.3 kb of the 5′ end of *cssap 92* was amplified from the pSPORT1 vector with *pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) to introduce a unique *Nco*I site at the ATG codon. The polymerase chain reaction (PCR) product and pRAW1 were digested with *Nco*I and *Sal*I, and the PCR product was ligated into pRAW1 to give pRAW2. Plasmid pRAW2 encodes the full-length *cssap 92* coding region, in-frame with the pET-30a start site, preceded by the oligomeric histidine

Table 2. Aflatoxin concentration was measured with the indirect competitive enzyme-linked immunosorbent assay method for embryos extracted from Asgrow 404 (Monsanto, St. Louis, MO, U.S.A.) and Tx772–Fr2128 seeds contaminated with *Aspergillus flavus*[a]

Sample	Hours postinoculation	AF concentration
Tx772–Fr2128	12	0
	24	0
	48	0
	72	0
	120	32.8 ± 8.8
Asgrow 404	12	0
	24	0
	48	0
	72	10.4 ± 3.1
	120	30.3 ± 3.4

[a] Amounts are given in ppm.

purification tag. pRAW1 and pRAW2 were maintained in DH5α *E. coli* cells and transformed into the BL21 (DE3) strain of *E. coli* for induction of the CSSAP 92 protein. Induction was carried out following previously described protocols (Steczko et al. 1992). The best results were obtained when cells were incubated at 15ºC for 20 h, with 300 rpm shaking and 1 mM isopropyl-β-D-thiogalactopyranoside.

Analysis of the oxidation products of pRAW2 and pRAW1.

Total protein was extracted from bacteria containing pRAW1, pRAW2, and the pET-30a vector as described previously (Steczko et al. 1992). LOX activity of the extracts was assayed spectrophotometrically. Extracts were stored in dry ice prior to incubation with linoleic acid. Protein extracts were thawed and an aliquot diluted in 24 ml of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0 and 50 µl of 48 mM methanolic linoleic acid at 25ºC (final pH of 6.9). The solution was stirred magnetically in a 125-ml Erlenmeyer flask for 15 min, after which time the pH level of the reaction was adjusted to 4.0 with 1 M oxalic acid. $CHCl_3$–methanol (75 ml, 2:1, vol/vol) was added immediately to extract the products. The hydroperoxide derivatives contained in the bottom $CHCl_3$ layer were analyzed for positional and stereochemical properties, as described previously (Gardner et al. 1998).

Analysis of the oxidation products of endogenous maize LOXs.

Maize seeds from Asgrow 404 and Tx772–Fr2128 lines were inoculated with *A. flavus* or mock inoculated and ground in a coffee grinder. Four maize seeds per time point were combined in each case. HEPES (50 mM, pH 7.0) was added at a rate of 10 ml of maize meal per gram and the slurry homogenized for 1 min at full speed. After centrifugation at 10,000 *g* for 20 min, the fat layer was aspirated and a 0.25 ml aliquot of supernatant was stored at −20ºC for protein concentration determination. The remainder of the supernatant was added to a 125-ml Erlenmeyer flask, followed by the addition of HEPES buffer (pH 7.0) to give a maize extract–HEPES buffer ratio of 1:1.5 (vol/vol). The solution was stirred magnetically at a constant temperature of 25ºC. The substrate solution contained 140 mg of linoleic acid in 5 ml of methanol and was added by injection to the maize–HEPES buffer mix at a rate of 5 µl of methanolic linoleic acid solution per 5 ml of maize–HEPES mix to give a final concentration of 0.1 mM linoleic acid. After 15 min of incubation at 25ºC with stirring, the reaction was stopped by adjusting the pH to 4.0 with oxalic acid and extracting the products with a volume of $CHCl_3$–methanol (2:1 vol/vol), which is equivalent to three times the volume of the reaction mix. The $CHCl_3$ extract could be saved at −20ºC until product analysis. Products were analyzed by gas chromatography mass spectrometry, as described in Burow et al. (2000), and the α-ketol products were examined as methyl ester–trimethylsilyloxy derivatives after $NaBH_4$ reduction, giving two separable diastereoisomers for each α-ketol, e.g., two separable methyl di-9,10-trimethylsilyloxy-12(*Z*)-octa-decenoates.

AFB1 quantitation.

Maize seeds from Asgrow 404 and Tx772–Fr2128 lines inoculated with *A. flavus* were ground under liquid nitrogen with a mortar and pestle and transferred to a 1.5-ml Eppendorf tube. The sample was weighed and 500 µl of 70% MeOH was added. The sample was vortexed vigorously, spun briefly in a centrifuge, and left to sit overnight at room temperature in the dark. Following a brief vortex and centrifugation, an aliquot of the MeOH layer was diluted 1:5 with 0.01 M phosphate-buffered saline and 50 µl of the diluted samples were used for enzyme-linked immunosorbent assay (ELISA) measurements. AFB1 concentration was determined by indirect competitive ELISA with standards of known AFB1 concentrations. Treatments were performed in triplicate and analyzed with the SAS statistical software package (SAS Institute, Cary, NC, U.S.A.).

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