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Is Catalase Activity One of the Factors Associated with Maize Resistance to *Aspergillus flavus*?

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Plant responses to biotic and abiotic stresses are usually accompanied by the release of reactive oxygen species including hydrogen peroxide. Hydrogen peroxide plays a direct role in defense and is involved in many signal transduction pathways that lead to the proliferation of other defenses. Because catalase helps to maintain reactive oxygen homeostasis during biotic and abiotic stress, its activity was measured in various cob tissues during maize ear development. Catalase activity was determined in immature and mature embryos, pericarp, and rachis tissues of maize lines that are resistant and susceptible to *Aspergillus flavus* infection. The effect of fungal inoculation on catalase activity was also measured. Over two years of field experimentation, a correlation was observed between resistance and the level of catalase-specific activity in immature embryos, which was significantly higher in resistant lines ($P < 0.0001$). Furthermore, catalase activity in the resistant lines was significantly higher in immature embryos from inoculated ears ($P = 0.0199$). No correlation was observed between resistance and catalase activity in other ear tissues. Levels of hydrogen peroxide, the catalase substrate, and salicylic acid in the embryo were also determined. The resistant lines showed lower levels of H_2O_2 ($P < 0.0001$) and higher levels of salicylic acid ($P < 0.0001$) as compared with the susceptible lines. Catalase 3 was sequenced from the aflatoxin-resistant (Mp313E) and -susceptible (SC212m) inbreds. The predicted amino acid sequence indicated that there was a 20-aa deletion in the resistant inbred that might affect enzymatic activity. Unlike many plant-pathogen interactions, it appears that lowering H_2O_2 levels helps to prevent *A. flavus* infection and subsequent aflatoxin accumulation.

Additional keywords: oxidative stress.

The production of reactive oxygen species (ROS) in response to pathogen attack is one of the hallmarks of plant defense. ROS are formed by the incomplete reduction of oxygen to water, resulting in the production of superoxide, hydroxyl radical and hydrogen peroxide. Generally, ROS accumulation leads to increased salicylic acid (SA) synthesis, the hypersensitive response (HR) and programmed cell death at the site of infection, and subsequent induction of pathogenesis-related genes (Dempsey et al. 1999; Lamb and Dixon 1997). In some cases, pathogen attack activates defenses distal to the infection site called systemic acquired resistance (Dempsey et al. 1999; Lamb and Dixon 1997). Hydrogen peroxide (H_2O_2), one of the ROS molecules formed, inhibits the growth and viability of diverse microbial pathogens (Kiraly et al. 1993; Peng and Kuc 1992; Wu et al. 1995) and, more importantly, is believed to act as a diffusible signal that induces downstream defense proteins. During the HR, H_2O_2 is the substrate for the oxidative cross-linking of proline-rich structural proteins and is a trigger for hypersensitive cell death (Bradley et al. 1992; Breusegem et al. 2001; Brisson et al. 1994; Lamb and Dixon 1997; Scandalios 1994; Xiong et al. 2002). The generation of H_2O_2 at the correct time and place hindered successful fungal penetration of plants in three different plant-fungal systems (Mellersh et al. 2002). It is assumed that, with H_2O_2 as well as with other ROS, signaling occurs when a target molecule perceives an increase in ROS concentration. The signal induces a change in gene expression, which can result in a change in oxidation state or transcription factor activation (Laloi et al. 2004) and ultimately lead to the activation of specific defenses against pathogens.

Although H_2O_2 is essential for signaling pathogen invasion and defense, the accumulation of excess H_2O_2 results in oxidative stress that can damage plant tissues. Two enzymes, ascorbate peroxidase and catalase (E.C. 1.11.1.6), remove H_2O_2 and modulate oxidative stress (Davletova et al. 2005); Mittler 2002; Mittler et al. 1999; Willekens et al. 1997). Catalase catalyzes the conversion of H_2O_2 to water and oxygen and regulates H_2O_2 concentration in tissues. This is essential because H_2O_2 is a relatively long-lived ROS that has the ability to diffuse widely from the site of its generation and penetrate certain biological membranes. If not eliminated, it can damage cells by reacting with other biomolecules, leading to oxidative stress (Dat et al. 2001). For example, H_2O_2 can react with unsaturated fatty acids and the bilayer lipid membranes converting the lipids into lipid peroxides that alter membrane quality, inactivate some membrane proteins, or spontaneously fragment into various toxic compounds, such as reactive electrophiles. Consequently, for a plant tissue to be healthy there must be the correct balance between H_2O_2 production and removal.

Catalases are important in plant development, defense, aging, and senescence (Yang and Poovaiah 2002); consequently, they are under strict temporal and spatial regulation. In addition, catalase expression is affected by environmental factors such as light (Boldt and Scandalios 1995), plant hormones (Guan and Scandalios 1998), ozone concentration (Ruzsa et al. 1999), temperature (Auh and Scandalios 1997), xenobiotics (Mylona

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et al. 1998), exogeneously applied H_2O_2 (Polidoros and Scandalios 1998), and wounding (Guan and Scandalios 2000). The role of catalase in pathogen defense has been investigated by either overexpressing or suppressing catalase in transgenic plants (Chamnongpol et al. 1998; Dat et al. 2001; Mittler et al. 1999; Vandenabeele, et al. 2004; Willekens et al. 1997). Generally, the suppression of catalase activity results in plants that have higher ROS levels and are primed to resist pathogen attack.

In maize, as in most plants, there are three catalases (Cat1, Cat2, and Cat3) encoded by genes on chromosomes 5, 1, and 4, respectively (Roupakias et al. 1979). The catalase isozymes share reasonably high sequence similarity at the nucleotide and amino acid levels (63 to 77%). The spatial and temporal expression of the isozymes has been characterized using the inbred line W64A (Acevedo and Scandalios 1992; Redinbaugh et al. 1988; Wadsworth and Scandalios 1989). Both Cat1 and Cat3 are expressed in whole kernels, while Cat2 was detected only in the late stages of kernel development (Acevedo and Scandalios 1992). It was demonstrated that Cat1 and Cat2 are located in the peroxisomes, but the subcellular location of Cat3 is unclear, and it might be localized in the mitochondria (Scandalios 1994). In *Arabidopsis*, Cat3 has a 16-aa calmodulin-binding domain in the carboxyl region of the protein (Yang and Pooviah 2002). When calmodulin binds this domain, catalase activity increases, thereby decreasing the H_2O_2 concentration. It is possible that calmodulin-binding could modulate the level of ROS in response to stress signals. Yang and Pooviah (2002) predicted that this sequence is present in Cat3 across a number of different species, including maize.

This present study was initiated to investigate the possible role of Cat3 in maize resistance to alfatoxin, which is a potent mycotoxin produced by the fungus *Aspergillus flavus* (Chen et al. 2004). Long-term research efforts are aimed at reducing aflatoxin accumulation due to fungal infection in the field during ear development (Anderson et al. 1975). Marker-assisted selection is one of the genetic strategies being used to develop maize inbreds that are resistant to *A. flavus* infection, aflatoxin accumulation, or both (Brooks et al. 2005). Several resistant inbred lines have been developed (Brooks et al. 2005; Windham and Williams 2002). This resistance is a complex, multigenic, quantitatively inherited trait (Brooks et al. 2005; Naidoo et al. 2002). For example, resistance in Mp313E is influenced by quantitative trait loci (QTL) on chromosomes 1, 2, 3, 4, and 5 (Brooks et al. 2005). Therefore, resistance to aflatoxin accumulation is believed to be a combination of two factors. The first is the plant's ability to resist fungal infection and colonization in the developing ear. The second is the plant's ability to provide an environment in the ear that is unfavorable for aflatoxin synthesis. It has been shown that toxigenic strains of *A. paraciticus* produce aflatoxin in response to oxidative stress (Jayashree and Subramanyam 2000) and aflatoxin accumulation in *A. flavus* can be reduced by adding phenol antioxidants to the culture medium (Kim et al. 2005, 2006). Walnuts, which naturally produce hydrolyzable tannins, are resistant to aflatoxin accumulation, suggesting that antioxidants in the seed play a protective role (Mahoney and Molyneux 2004). In maize, it has been shown that heat and drought stress, which also produce ROS, trigger aflatoxin production in *A. flavus*-infected ears (Chen et al. 2004; Payne 1998). Consequently, preventing oxidative stress in the ear might prevent alflatoxin accumulation even though it has been infected with the fungus. One major QTL that accounts for approximately 20% of the inbred Mp313E's resistance to aflatoxin accumulation was found on chromosome 4 (Brooks et al. 2005). One of the many genes that map in this region is *cat3*. Because of its role in regulating H_2O_2 homeostasis in cells and its map location, we postulated that Cat3 might contribute to maize resistance to *A. flavus* infection or aflatoxin accumulation. To test this, catalase activity, H_2O_2, and SA levels in developing maize ear tissues were measured. We focused on the embryo because of its importance in maize reproduction and reports suggested it is the primary location of cat3 in the developing kernel (Scandalios 1994). In addition to determining catalase activity, *cat3* from resistant and susceptible inbred lines was sequenced and the predicted amino acid sequences were compared.

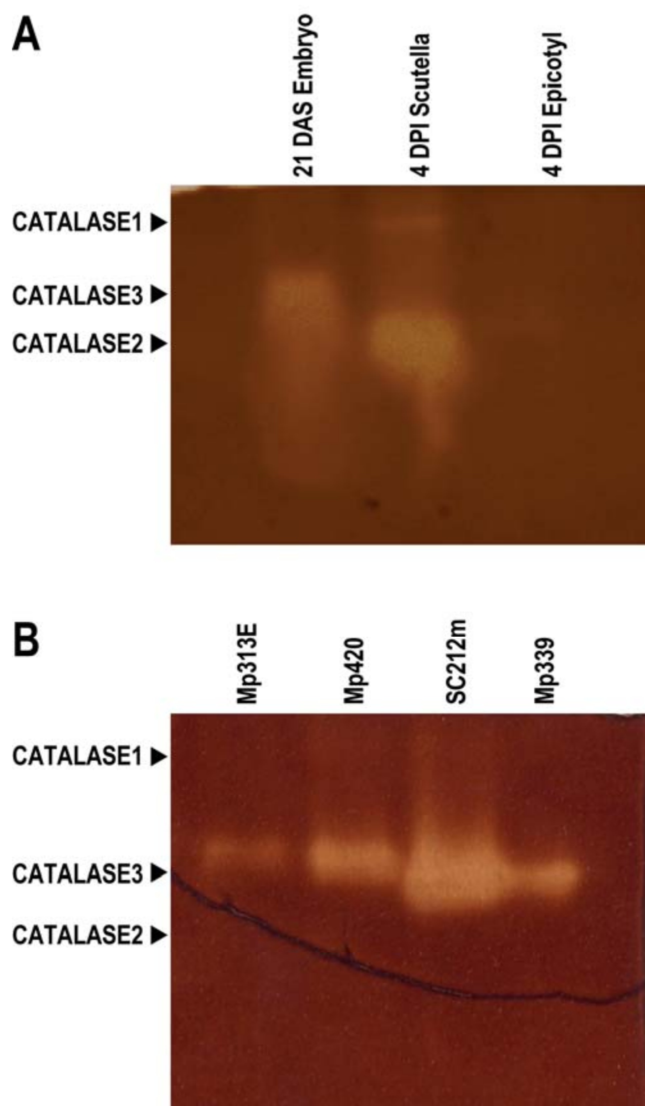

Fig. 1. A, Activity gel showing migration of catalase isozymes in protein extracts from embryos 21 days after silk emergence (DAS), scutella 4 days after imbibition (DPI), and 4-DPI epicotyls from Mp313E. The amount of protein loaded in each lane was 75, 60, and 20 ng for embryos, scutella, and epitcotyls, respectively. **B,** Catalase activity gel of protein extracts from 25-DAS embryos. The amount of protein loaded for Mp313E, Mp420, SC212m, and Mp339 was 75, 150, 750, and 375 ng, respectively.

RESULTS

Catalase activity in ear tissues from resistant and susceptible inbreds.

Initial experiments were conducted to determine which catalase isozyme was expressed in the embryo. Seeds from Mp313E were imbibed for 4 days, and extracts of the scutella and epicotyl were prepared. At this stage of development, the scutellum expresses Cat1 and Cat2 and the etiolated epicotyl expresses

Cat3 (M. Redinbaugh, *personal communication*). These samples and an extract from embryos 21 days after silk emergence (DAS) were electrophoresed on a nondenaturing gel and were treated with diaminobenzidine to visualize catalase activity (Fig. 1A). Despite overloading samples, it was difficult to visualize the Cat3 band in the epicotyl extract. However, the isozyme expressed by the embryo migrated between Cat1 and Cat2 in the region that is characteristic of Cat3 (Fig. 1A). This suggests that the major isozyme expressed in the developing embryo is Cat3.

Subsequently, in-gel activity staining was used to visualize catalase activity in immature embryos (25 DAS) from two resistant (Mp313E and Mp420) and two susceptible (SC212m and Mp339) inbreds (Fig. 1). These results also indicated that only one major isozyme, Cat3, was expressed in these embryos. A difference in migration pattern was evident between the resistant and susceptible lines. The isozyme from the two susceptible lines (SC212m and Mp339) migrated faster than the isozyme from the resistant lines. However, to visualize activity on the gel, the amount of protein loaded for Mp420, SC212m, and Mp339 was two-, ten-, and fivefold greater, respectively, than the amount loaded for Mp313E. Consequently, catalase from the two susceptible lines appeared to have lower specific activity than the two resistant lines.

Catalase-specific activity was then quantified in immature and mature embryos, pericarp, and rachis tissues collected from the inbred lines Mp313E, Mp420, SC212m, Mp339, SC229, and Tx601. Maize ears used for experimentation were grown in the field during summer; hence, each experiment consisted of samples from one year. In the first year, the catalase activity of embryos collected 18, 21, and 25 DAS (which corresponded to 3, 6, and 10 days after inoculation [DAI]) was compared, and embryos from inoculated and uninoculated ears were analyzed at each timepoint. At least three replicates were planned for each timepoint (three inoculated and three controls), but some ears were not successfully pollinated, so some timepoints had fewer or no replicates. A replicate consisted of the embryos from one ear. If an ear did not yield sufficient material, embryos were pooled from several ears. However, embryos from a single ear were never divided to provide more than one replicate. Results of the enzyme activity assay (Fig. 2A) indicated that embryos from resistant lines (Mp313E and Mp420) had significantly higher catalase activity than the susceptible (SC212m) and intermediate (Tx601) lines ($P < 0.0001$) for all sampling dates. In addition, a significant difference ($P < 0.01$) in catalase activity was observed between the two resistant lines, with activity being higher in Mp420 than Mp313E. In the resistant inbred lines, catalase activity tended to increase during embryo development. Differences between genotypes ($P < 0.0001$) and between ears sampled at different ages ($P = 0.04$) were likewise significant. Embryos (21 DAS) from resistant plants that were collected six days after inoculation had significantly higher catalase activity than the uninoculated

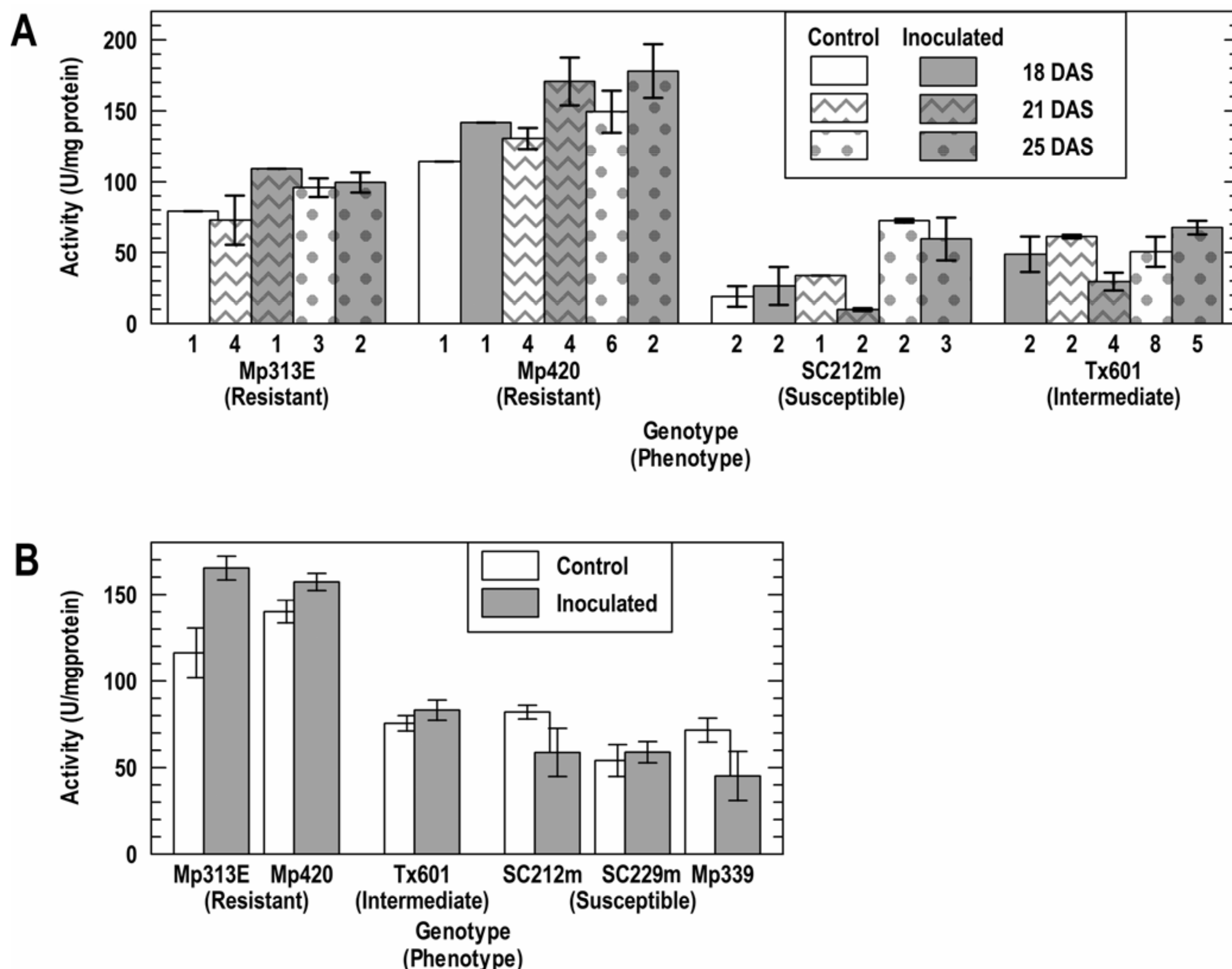

Fig. 2. Catalase activity in **A,** immature embryos 18, 21, and 25 days after silk emergence (DAS) in the first year and **B,** 25-DAS immature embryos in the second year of field experimentation. The number of replicates are indicated below the bars. Standard errors are shown.

control ($P = 0.05$). In the resistant line Mp420, catalase activity in the inoculated embryos tended to be higher at 3, 6, and 10 days after inoculation (18, 21, and 25 DAS). In the susceptible lines, a significant increase in activity was observed from 18 to 25 DAS ($P = 0.05$), but there were no significant differences between inoculated and uninoculated samples.

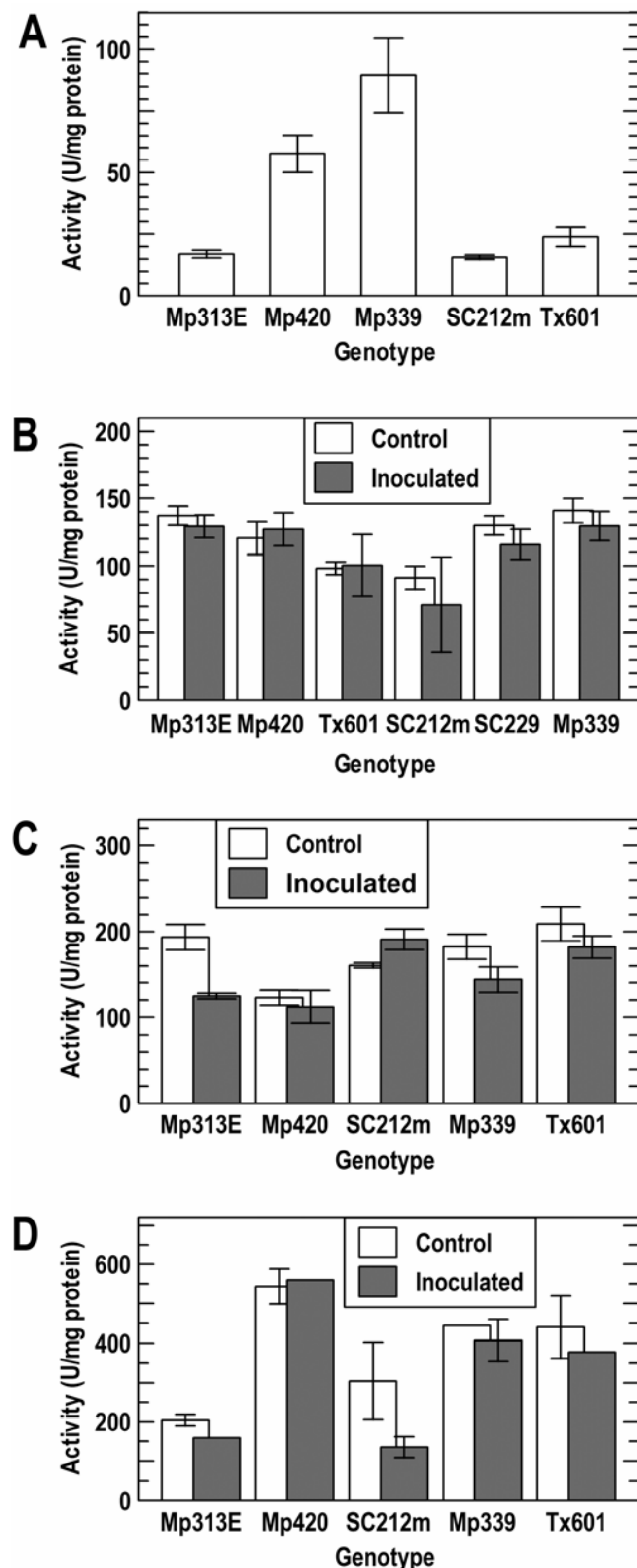

Fig. 3. Catalase activity in mature embryos measured for two years. **A,** Year 1 and **B,** year 2. **C,** In pericarp 25 days after silk emergence (DAS) and **D,** in 25-DAS rachis tissues. Each datapoint represents three replicates. Standard errors are shown.

To determine if differences in catalase-specific activity between resistant and susceptible lines were consistent between years, we selected the timepoint that showed the highest activity (25 DAS) in the first year and collected embryos at that stage in the second year. Also, during the second year of field experimentation, embryos from two additional susceptible lines, SC229m and Mp339, were included in the study. As in the first year, embryos from resistant lines exhibited significantly higher catalase-specific activity than those from susceptible lines ($P < 0.0001$) (Fig. 2B). Inoculation resulted in higher catalase activity in the two resistant inbred lines ($P = 0.002$) but had no effect on the susceptible lines. When results from both years of experimentation were pooled, catalase activity differed significantly by year ($P = 0.0099$), phenotype ($P < 0.0001$), and genotype ($P = 0.01$). However, catalase activity in the resistant lines did not change significantly from the first year to the second year in 25-DAS embryos, suggesting that the environment had little effect on catalase activity in these lines. The resistant lines also showed a significant increase in catalase activity upon inoculation ($P = 0.0199$). The effect of inoculation on catalase activity in susceptible lines, on the other hand, varied from year to year ($P = 0.0188$), suggesting either the absence or inconsistency of such a response in these lines.

We also determined the amount of catalase activity in mature embryos (45 DAS) collected from resistant and susceptible inbreds. In this case, there was no correlation between resistance and catalase activity in either year (Fig. 3A and B). This suggests that catalase functions in resistant embryos in a development stage during which they are most likely to be infected by *A. flavus* in the field. Catalase activity in pericarp and rachis tissue collected 25 DAS was also determined. Despite the generally high levels of activity found in rachis tissues, no correlation was observed between resistance and catalase activity in pericarp (Fig. 3C) or rachis tissues (Fig. 3D).

H_2O_2 levels in 25-DAS embryos.

Since H_2O_2 is the substrate for catalase, its level in 25-DAS embryos from inoculated and uninoculated ears of the inbred lines Mp313E, Mp420, SC212m, Mp339, and Tx601 was determined (Fig. 4). The steady-state H_2O_2 levels in resistant lines were lower than those in susceptible lines ($P < 0.0001$). This suggests that higher catalase activity results in lower H_2O_2. The inoculation state, on the other hand, had no effect on the steady-state level of H_2O_2 in either the resistant or susceptible lines.

SA levels in 25-DAS embryos.

Because SA has been implicated in the regulation of catalase activity in vivo, the levels of SA in inoculated samples and uninoculated controls of inbred lines Mp313E, Mp420, SC212m, Mp339, SC229, and Tx601 were quantified to determine if there was a relationship between resistance and SA levels (Fig. 5). Significantly higher levels of SA (approximately 100 ng per gram of fresh weight) were found in the two resistant lines than in susceptible lines ($P < 0.0001$). In fact, SA was only detected in one of the four susceptible lines tested (Mp339). The concentration in Mp339 was approximately threefold less than in the resistant inbreds. Jasmonic acid (JA) was also found in the embryos (data not shown), but there were no significant differences among the lines.

Sequence comparison of Cat3 from resistant and susceptible inbreds.

Because it has been postulated that there is a calmodulin-binding domain in Cat3 and that calmodulin binding increases its activity (Yang and Pooviah 2002), we wanted to determine

if there was an alteration in this region of Cat3 between resistant and susceptible inbreds that might account for the difference in specific activity. The complete *cat*3 cDNA sequences including 3′ and 5′ untranslated regions were obtained from Mp313E and SC212m. The predicted amino acid sequences from these two lines were compared with that of Cat3 from the maize inbred W64A (Fig. 6). Although we have not evaluated the resistance of W64A to *A. flavus*, the Maize Genetics and Genomics Database does not include it as a resistant phenotype. The predicted amino acids sequences indicated that Cat3 from Mp313E had 475 aa, whereas Cat3 from SC212m and W64A had 496. A comparison of the derived amino acid sequences of Cat3 from SC212m and W64A reveals a 99.6% similarity, with only two amino acid differences between the two lines. A comparison of Cat3 from Mp313E with that of the two susceptible inbreds indicated approximately 94% similarity. There were five amino acid substitutions between Mp313E, W64A, and SC212m (Fig. 5). The primary difference between the amino acid sequence of the resistant and susceptible lines was a 20-aa deletion in the carboxy-terminal region of Mp313E. This deletion spans amino acids 401 to 421 and is approximately 20 aa upstream from the putative calmodulin-binding site located at amino acids 442 to 455 (Yang and Poovaiah 2002). There are seven proline residues in this region, and four of these are grouped as doublets. The presence of the prolines might increase the rigidity of protein in this region. Although the putative calmodulin-binding site is intact in all three inbreds, the upstream deletion in Mp313E may change the enzyme's conformation, alter the ability of the site to bind calmodulin, and increase its specific activity.

Analysis of the predicted amino acid sequence indicated that Cat3 from Mp313E had a molecular mass of 54.7 kDa, an isoelectric point of 6.06, and a charge of –8.49 at pH 7, while Cat3 from both SC212m and W64A had a predicted molecular mass of 56.8 kDa, an isolectric point of 6.49, and a charge of –3.49 at pH 7. The charge differences between Mp313E and SC212m may account for the mobility differences in the activity gel (Fig. 1). If the 20-aa sequence is deleted from SC212m or W64E Cat3 in silico, the predicted isoelectric point and the charge at pH 7.0 changes to 6.14 and –7.49, respectively. This suggests that the 20-aa deletion alters the electrophoretic mobility of Cat3 from Mp313E. P-Sort bioinformatic analysis (Nakai and Horton 1999) was used to predict subcellular location of Cat3. The analysis predicted with greater than 70% certainty that Cat3 was localized to the peroxisome for all three inbreds.

DISCUSSION

Catalase plays a key role in maintaining H_2O_2 homeostasis in cells and has been implicated in ROS signaling in response to pathogen attack. Thus, following identification of a QTL on chromosome 4 near the cat3 map location (Brooks et al. 2005), we hypothesized that Cat3 was involved in maize resistance to either *A. flavus*, aflatoxin accumulation, or both. Our first assumption was that catalase activity would be lower in embryos from resistant plants, resulting in the increased accumulation of H_2O_2 and triggering defense against *A. flavus* infection. This was not the case; catalase activity was significantly higher in resistant lines, and H_2O_2 levels were lower.

Generally, the oxidative burst is an early response to pathogen attack. But *A. flavus* is a weak pathogen of maize and might not trigger high levels of ROS production. *A. flavus* produces aflatoxin in response to oxidative stress (Kim et al. 2005), and the inclusion of phenolic antioxidants in media prevents its accumulation (Chipley and Uraih 1980; Kim et al. 2006). Recent reports show that application of abscissic acid, a mediator of plant abiotic stress, or H_2O_2 to maize leaves activates a mitogen-activated protein kinase, which enhanced the expression of antioxidant genes, including Cat1 (Zhang et al. 2006). In addition, catalase activity was increased. Since aflatoxin contamination of maize typically occurs in climates in which plants undergo heat and drought stress during grain filling and maturation (Chen et al. 2004), our hypothesis is that oxidative stress in the ear tissues resulting from these environmental stresses triggers aflatoxin accumulation when the fungus is present. Consequently, to reduce aflatoxin accumulation, the kernel must strike a balance in the oxidative state that is sufficient to inhibit fungal growth but low enough to prevent oxidative stress that triggers aflatoxin synthesis. High catalase activity in the embryo may help maintain this balance by preventing the production of excess H_2O_2 and thereby reducing aflatoxin accumulation. It may also prevent oxidative damage to the embryo that would make it more susceptible to fungal colonization.

Comparison of the catalase-specific activities in different ear tissues revealed a correlation between resistance and activity only in immature embryos. Since the fungus typically infects

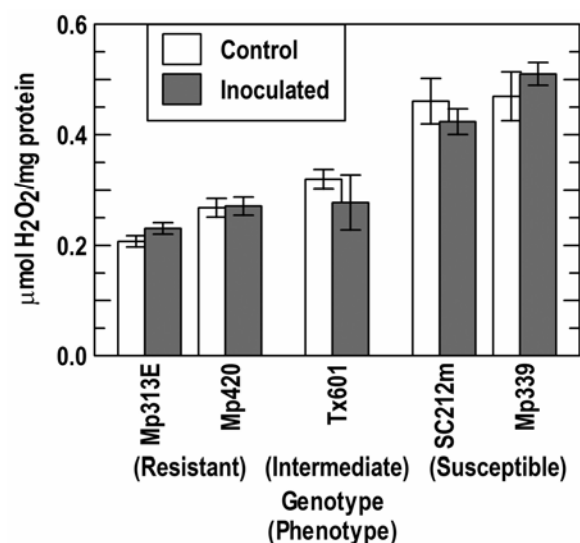

Fig. 4. Steady-state H_2O_2 concentration in embryos 25 days after silk emergence. Each datapoint represents five replicates. Standard errors are shown.

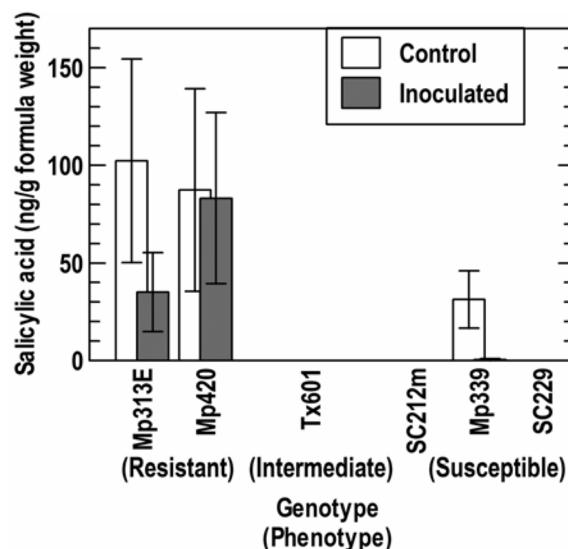

Fig. 5. Salicylic acid levels in embryos 25 days after silk emergence. Each datapoint represents ten replicates. Standard errors are shown.

the ear approximately 15 DAS and proliferates during ear development and maturation, it seems likely that catalase plays a role in regulating H_2O_2 levels during embryo development that may not be necessary in the mature embryo. In the resistant lines, there was little variability in catalase activity at 25 DAS in two years of field experimentation, suggesting that this mechanism is consistent from year to year. Similarly, the resistant lines reacted to fungal inoculation with a 23% increase in catalase activity ($P = 0.02$), which did not occur in the susceptible lines.

When visualized on a gel, it appeared that only one major isozyme of catalase appeared to be expressed in embryos at 25

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W64A        1 MTMDPTKFRPSSSHDTTVTTTNAGAPVWNDNEALTVGPPRGPILEDYHLIEKVAHFARER
SC212m      1 MTMDPTKFRPSSSHDTTVTTTNAGAPVWNDNEALTVGPPRGPILEDYHLIEKVAHFARER
Mp313E      1 MTMDPTQFRPSSSHDTTVTTTNADAPVWNDNEALTVGPPRGPILEDYHLIEKVAHFARER
Consensus   1 MTMDPTkFRPSSSHDTTVTTTNAgAPVWNDNEALTVGPPRGPILEDYHLIEKVAHFARER

W64A       61 IPERVVHARGASAKGFFECTHDVTSLTCADFLRAPGVRTPVIVRFSTVIHERGSPETIRD
SC212m     61 IPERVVHARGASAKGFFECTHDVTSLTCADFLRAPGVRTPVIVRFSTVIHERGSPETIRD
Mp313E     61 IPERVVHARGASAKGFFECTHDVTSLTCADFLRAPGVRTPVIVRFSTVIHERGPPETIRD
Consensus  61 IPERVVHARGASAKGFFECTHDVTSLTCADFLRAPGVRTPVIVRFSTVIHERGsPETRID

W64A      121 PRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAFKPNPRSHVQEYWRVFDFLSH
SC212m    121 PRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAFKPNPRSHVQEYWRVFDFLSH
Mp313E    121 PRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAFKPNPRSHVQEYWRVFDFLSH
Consensus 121 PRGFAVKFYTREGNWDLLGNNFPVFFIRDGIKFPDVIHAFKPNPRSHVQEYWRVFDFLSH

W64A      181 LPESLHTFFFLFDDVGVPSDYRHMEGFGVNTYTFVSAAGKAQYVKFHWKPTCGVRCILTD
SC212m    181 LPESLHTFFFLFDDVGVPSDYRHMEGFGVNTYTFVSAAGKAQYVKFHWKPTCGVRCILTD
Mp313E    181 LPESLHTFFFLFDDVGVPSDYRHMEGFGVNTYTFVSAAGKAQYVKFHWKPTCGVRCILTD
Consensus 181 LPESLHTFFFLFDDVGVPSDYRHMEGFGVNTYTFVSAAGKAQYVKFHWKPTCGVRCILTD

W64A      241 EEAALVGGRNHSHATQDLYDSIAAGSFPEWTLYVQVMDPDTEEQYDFDPLDDTKTWPEDL
SC212m    241 EEAALVGGRNHSHATQDLYDSIAAGSFPEWTLYAQVMDPDTEEQYDFDPLDDTKTWPEDL
Mp313E    241 EEAALVGGRNHSHATQDLYDSIAAGSFPEWTLYVQVMDPDTEEQYDFDPLDDTKTWPEDL
Consensus 241 EEAALVGGRNHSHATQDLYDSIAAGSFPEWTLYvQVMDPDTEEQYDFDPLDDTKTWPEDL

W64A      301 LPLRPVGRLVLDRNVDNFFNENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLG
SC212m    301 LPLRPVGRLVLDRNVDNFFNENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLG
Mp313E    301 LPLRPVGRLVLDRNVDNFFNENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLG
Consensus 301 LPLRPVGRLVLDRNVDNFFNENEQLAFGPGLVVPGIYYSDDKMLQCRVFAYADTQRYRLG

W64A      361 PNYLMLPVNAPRCAHHNNHYDGAMNFMHRDEEVRDYYPSRHAPLQAAPPTPLPPRPVAGR
SC212m    361 PNYLMLPVNAPRCAHHNNHYDGAMNFMHRDEEVRDYYPSRHAPLQAAPPTPLPPRPVAGR
Mp313E    361 PNYLMLPVNVPRCAHHNNHYDGAMNFMHRDEEVRDYYPSRH...................
Consensus 361 PNYLMLPVNaPRCAHHNNHYDGAMNFMHRDEEVRDYYPSRHaplqaapptplpprpvagr

W64A      421 REKATIRKPNDFKQPGERYRSWDADRQDRFVRRFADSLGHPKVSQELRSIWIDLLAKCDA
SC212m    421 REKATIRKPNDFKQPGERYRSWDADRQDRFVRRFADSLGHPKVSQELRSIWIDLLAKCDA
Mp313E    398 .EKATIRKPNDFKQPGERYRSWDADRQDRFVRRFADSLGHPKVSQELRSIWIDLLAKCDA
Consensus 421 rEKATIRKPNDFKQPGERYRSWDADRQDRFVRRFADSLGHPKVSQELRSIWIDLLAKCDA

W64A      481 SLGMKIATRLNMKANM
SC212M    481 SLGMKIATRLNMKPNM
Mp313E    457 SLGMKIATRLNMKPYM
Consensus 481 SLGMKIATRLNMKpnM
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Fig. 6. The derived amino acid sequence of *cat3* from W64A (susceptible), SC212m (susceptible), and Mp313E (resistant). Identical amino acids are in black and those that differ are in white. The putative calmodulin-binding regions is enclosed in a black box. The 20 amino acids that are absent in Mp313E are represented by dots. The accession number for *cat3* from W64A is X12539.

DAS in the inbred lines. Given that Cat3 is observed at the onset of kernel development (Acevedo and Scandalios 1992) and in coleoptiles, we infer that the isozyme that was expressed was Cat3. The isozymes Cat1 and Cat2 were not detected on the activity-stained gel, and it is unlikely that they contributed significantly to the observed catalase activity. Likewise, it was observed that Cat3 from the two susceptible lines migrated faster than Cat3 from the resistant lines. In the case of Mp313E, this was probably to due the 20-aa deletion, which altered its isoelectric point and charge at pH 7. We do not have sequence data for Cat3 from Mp420, and its altered mobility might be the result of a change in molecular weight or charge, or both, or expression of a different allele. Both the activity gel and catalase activity measurement indicated that the two resistant inbreds had higher specific activities than the two susceptible inbreds. In the case of Mp313E, this might have been caused by the 20-aa deletion, which could alter the enzyme's conformation and, hence, its activity.

Steady-state levels of H_2O_2 in immature embryos from resistant inbreds were lower than those in susceptible inbreds, which supports the catalase activity data. Since only steady-state H_2O_2 levels were measured, we do not know if there was an initial oxidative burst in the embryo in response to *A. flavus*. If there was a burst, it may have been attenuated by the higher catalase activity in the resistant lines. Transgenic tobacco lines with reduced catalase activity were found to be more responsive to pathogen attack (Chamnongpol et al. 1998; Mittler et al. 1999). Although higher H_2O_2 levels might enhance disease resistance, they also make the plant more susceptible to abiotic stresses (Willekens et al. 1997). Tobacco plants deficient in Cat1 activity had more light-induced leaf necrosis, a compromised ascorbate-glutathione cycle, and increased peroxidase activity (Willekens et al. 1997). These plants were more sensitive to high light and salt levels, ozone, and paraquat-induced oxidative stress (Vandenabeele et al. 2004; Willekens et al. 1997), and it was suggested that catalase serves as a H_2O_2 sink that protects the leaves from oxidative damage. A catalase-deficient mutant of barley was unable to overcome stress conditions in the field and had reduced survival and yield (Acevedo et al. 2001).

In addition to having less H_2O_2, the resistant embryos had three- to fourfold more SA than the susceptible inbred Mp339. The remaining inbreds had no detectable SA. In tobacco, SA binds to catalase and inhibits its activity (Conrath et al. 1995). This allows H_2O_2 and ROS to accumulate and signals the production of various defense-related proteins. In monocots, the response of catalase to SA appears to be different. Rice catalase A, which is expressed in leaves, was insensitive to SA inhibition (Chen et al. 1997). In contrast, SA in roots inhibited catalase B, which shared more sequence similarity with the SA-binding tobacco catalase. Guan and Scandalios (1995) also investigated the effect of SA on the three maize catalases. When CAT2-null maize embryos were cultured on medium containing SA, total catalase activity in the scutella increased in response to low concentrations of SA. This appeared to be due to an increase in Cat1 activity, as there was little change in Cat3 activity in response to SA. This suggests that maize catalases respond differently to SA than those of tobacco. Of the three maize catalases, Cat3 has the least similarity to the SA-binding tobacco catalase (Guan and Scandalios 1995), and it is unlikely that SA affects its activity.

If SA does not alter the activity of Cat3 in the maize embryo, it could protect the embryo from oxidative stress. Endogenous levels of SA in rice leaves are much higher than those in tobacco or *Arabidopsis*, and consequently, rice is insensitive to exogenous SA treatment (Yang et al. 2004). Transgenic rice plants expressing *nahG,* the gene that encodes salicylate hydroxylase, were SA-deficient and contained elevated ROS levels. They were also were hypersensitive to oxidative stress. In these plants, SA probably does not act as a signal for defense-gene expression, but it may protect the plant from oxidative stress caused by various abiotic and biotic factors (Yang et al. 2004). The SA concentration in the resistant maize embryos was not as high as that in rice leaves (Yang et al. 2004), but ROS levels are likely to be lower in the developing embryo than in the photosynthetic tissues. The combination of higher catalase activity and SA concentration and lower H_2O_2 levels in resistant maize embryos could be detrimental to fungal colonization and aflatoxin accumulation in response to oxidative stress. Lines of Mp313E that are deficient in Cat3 are being generated by back-crossing into Cat3 null lines, and in the future, we will be able to genetically test the role of Cat3 in this complex resistance mechanism.

MATERIALS AND METHODS

Plant materials.

Aflatoxin-resistant maize inbred lines Mp313E and Mp420, susceptible inbred lines SC212m, Mp339, and SC229, and the intermediately resistant inbred Tx601 were used for this study (Windham and Williams 2002). Both Mp313E and Mp420 were selected for reduced *A. flavus* infection (Scott and Zummo 1990, 1992), and they usually have significantly lower aflatoxin levels than the susceptible lines (Windham and Williams 2002). Seeds were planted in the field either on 23 April 2001, 17 April 2003, or 20 April 2004, and the emergence of silk was tagged. The plants were self-pollinated. At 15 DAS, half of the tagged ears were spray-inoculated through the silk channel with a suspension of *A. flavus* (strain GAP 2-8) spores in water (9×10^7 conidia/ml). Ears were collected at 18, 21, and 25 DAS, corresponding to 3, 6, and 10 DAI for the inoculated ears. Embryos were excised from the kernels intact, to avoid elevation of catalase levels in response to wounding, and were placed on wet paper towels to prevent dehydration until all the embryos in an ear were collected. Mature (at least 45 DAS), quiescent embryos from inoculated and uninoculated ears were likewise collected by excision with a scalpel. Pericarp was peeled from each kernel individually. The rachis was cleaned extensively of kernel tissues and was sliced in approximately 3-mm sections. The tissues were frozen in liquid nitrogen immediately after collection and were stored at –80°C for protein and nucleic acid extractions. To visualize the different catalase isozymes on a gel, scutella (Cat1 and Cat2) and epicotyl (Cat3) were collected from mature Mp313E seeds 4 days after imbibition (DPI). They were surface sterilized with 10% Chlorox and were rinsed three times with autoclaved water. The seeds were soaked in autoclaved water for 18 h and were placed on top of germination paper inside sterile Magenta boxes in the dark for 4 days. At the end of the fourth day, epicotyls were trimmed from the top of the seedlings and the scutella were excised. Tissues were processed immediately and the remaining plants were stored at –80°C.

Protein extraction and quantification.

Samples (200 mg) of tissues were ground either in liquid nitrogen (pericarp and rachis) or in 200 µl (embryos and scutella) or 20 µl (epicotyl) of 50 mM potassium phosphate buffer, pH 7.0. Each 200-mg embryo sample contained 5 to 20 embryos, depending on their age. For the pericarp and rachis tissues, 200 µl of the phosphate buffer was added after grinding. The extract was vortexed for 10 s and was centrifuged for 15 min twice; the pellet was discarded and the supernatant was retained each time. The resulting extract was kept on ice for protein quantification and catalase activity assays. Protein was

quantified using the Micro protein determination kit (Sigma-Aldrich, St. Louis) according to the manufacturer's directions. Absorbance at 750 nm was measured using a Hitachi 100-80A spectrophotometer.

Catalase activity assay.

For catalase assays, embryos, pericarp, and rachis from year 2001 and embryos from year 2003 were used. Activity was quantified based on the rate of disappearance of the substrate H_2O_2 from a reaction medium containing the protein extract (Beers and Sizer 1952). The H_2O_2 concentration in the reaction medium was quantified by the change in absorbance at 240 nm, which was measured using a Cary 3C UV-visible spectrophotometer and the Cary WinUV kinetics program (Varian Inc., Palo Alto, CA, U.S.A.). A cuvette containing 25 µl of crude extract (never frozen) and 875 µl of 50 mM potassium phosphate buffer, pH 7.0, was placed in the spectrophotometer and the reading was set to zero. A total of 100 µl of 0.2 M H_2O_2 was then added to the extract solution and the absorbance was monitored for 2 min. The activity was calculated based on the initial reaction rate during the first 0.25 min after H_2O_2 injection, when substrate concentration was not limiting and the reaction velocity was maximal. Catalase activity was visualized on gels following procedure modified from Clare and associates (1984). Total extract (2, 5, or 25 µl) was loaded on a standard nondenaturing polyacrylamide gel (10% separating and 3.5% stacking), and the samples were electrophoresed for 18 h. The upper buffer consisted of 50 mM Tris and 50 mM glycine at pH 8.9, while the lower buffer was 100 mM Tris at pH 7.9. The gel was soaked in 50 mg of horseradish peroxidase per milliliter (Sigma-Aldrich) in 50 mM potassium phosphate, pH 7.0, at room temperature for 45 min and was rinsed with the phosphate buffer twice. The gel was then treated with 5.0 mM H_2O_2 for 10 min. The gel was rinsed twice with water, after which staining with 0.5 mg of diaminobenzidine (Sigma) per milliliter in phosphate buffer was performed. The staining step took a few hours to overnight. The gels were replicated eight times and one was chosen as a representative gel.

Table 1. Primers used in sequencing and polymerase chain reaction

Primer name	Primer sequence (5' – 3')	Primer location
Ucat3m	GGCCTCGCGGTCCCATC CT	131 to 149
Lcat3m	CGCCTTCACGAATCGGT CCTGT	1356 to 1377
Cat3L-U	TGATGGACCCGGCACA GGAGGAGCAGTA	845 to 872
Cat3L-L	TTCATGGCGCCGTCGTA GTGGTTGTTGT	1,145 to 1,172
Mp313E-5_3Sp6F.1	CTGCGTCGCGTGGCTGT GGTTC	765 to 786
Mp313EcDNAF2	GTGAGCGCGGCGGGGA AGG	664 to 682
SC212mcDNASp6R.1	TGTACGCGCAGGTGAT GGA	833 to 851
SC212mcDNAT7F.1	AGGAGTACTGGCGGGT GTTC	526 to 546
cat3-3'ERI	CGGAATTCCACCACAA CAACCACTACGAC	1,143 to 1,161
cat3-3'XhoI	CCGCTCGAGCGGCTATT CAGCAGAAGCATCAC	1,506 to 1,525
cat3-3	TTTTTTGCTGCAGGTGT CTT	1,753 to 1,771
cat3int1462f[a]	TGAGGGGGATTGAGGG GGTTTT	2,329 to 2,350
Cat3int1462r[a]	AGTCAGCGCCTCGTTGT CGTTC	2,790 to 2,769

[a] These primers were designed from the catalase 3 gene sequence (L05934).

H_2O_2 quantification.

The 25-DAS embryos from year 2004 were used for this analysis. The Correlate-assay colorimetric H_2O_2 kit (Assay Designs, Inc., Ann Arbor, MI, U.S.A.) was used to measure H_2O_2 concentration. Approximately 500 mg of fresh 25-DAS embryos were ground in 500 µl of 50 mM phosphate buffer, pH 6.0. The mixture was vortexed for 10 s and was centrifuged for 30 min twice, each time discarding the pellet and recovering the supernatant. Half of the supernatant was reserved for protein quantification, while the other half was passed through a 5,000 molecular weight cut-off column filter (Millipore Corp., Billerica, MA, U.S.A.), to eliminate potential interference from particulates; 50 µl of the filtered supernatant was used directly in the assay. A set of standards was run for each assay, and the standard curve was fit to a four-parameter logistic equation as recommended by the manufacturer. A Softmax Pro plate reader (Molecular Devices Corp., Sunnyvale, CA, U.S.A.) was used to measure the optical density at 550 nm, and the corresponding software was employed to perform all the calculations.

SA and JA quantification.

Embryos collected in 2003 were used for this assay. To extract and detect JA and SA, a previously described method (Tooker and De Moraes 2005), which was modified slightly from Schmelz and associates (2003, 2004) was used. Briefly, we derivatized carboxylic acids to methyl esters, which were isolated using vapor phase extraction and were analyzed by gas chromatographic-mass spectrometry (GC-MS), with isobutane chemical ionization using selected-ion monitoring. We quantified amounts of methyl jasmonate (meJA) and methyl salicylate (meSA) using standard curves made with the pure compounds (Sigma-Aldrich), relying on internal standards to confirm derivatization and recovery. We also processed samples without the derivatization agent, to verify that the recovered meJA and meSA were not originally present in analyzed material but were derived from the carboxylic acids. To confirm the identity of meJA and meSA in the samples, extracts were analyzed by GC-MS with electron ionization, comparing retention times and spectra with that of the pure compound.

Nucleic acid extraction.

Total RNA was extracted from 25-DAS embryos using the RNEasy plant kit (Qiagen, Inc., Valencia, CA, U.S.A.) in accordance with the manufacturer's recommendations, including the optional DNase treatment. Genomic DNA was extracted from 5 g of young leaves using 10 ml of a CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris pH 8.0, 20 mM EDTA). The extract was purified with chloroform and was precipitated, using an equal volume of 2-propanol. The precipitated DNA was washed with 70% EtOH and was air-dried. Plasmid DNA from clones was isolated using the Qiaprep spin miniprep kit (Qiagen, Inc.).

cDNA, 5′ and 3′ ends amplification, and cloning.

cDNA and 5′-end DNA were amplified from total RNA using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, U.S.A.), and 3′-end DNA was amplified from genomic DNA. The resulting polymerase chain reaction (PCR) reaction mixtures were electrophoresed on agarose gel, and the desired DNA fragments were recovered by excision from the gel, were purified using Qiaquick gel extraction kit (Qiagen Inc.), and were cloned, using either the pGEM-T easy vector system (Promega, Madison, WI, U.S.A.) or the TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA, U.S.A.).

PCR.

All PCR assays were performed in an Eppendorf PCR machine. For reactions that did not involve cDNA amplification, either Platinum *Taq* DNA polymerase (Invitrogen) or Ex *Taq* polymerase (Takara, Panvera, CA, U.S.A.) was used. The catalase 3-specific primers used are shown in Table 1.

Sequencing and sequence alignment.

The Prism 310 genetic analyzer was used for all sequencing with the Prism BigDye terminator cycle sequencing kit (ABI, Foster City, CA, U.S.A.). The sequences obtained were verified against the existing National Center for Biotechnology Information database using the Basic Local Alignment Tool (Altschul et al. 1990). The Lasergene (DNAStar, Madison, WI, U.S.A.) suite of analytical tools was employed for all nucleic and amino acid sequence analyses and comparisons. In all cases, alignment was performed using the ClustalW algorithm.

Cloning of cat3 from *Zea mays* cDNA and genomic DNA.

Cloning of catalase 3 was performed using i) cDNA amplified from total RNA from 25-DAS immature embryos, and ii) PCR of genomic DNA extracted from young leaves. The lines Mp313E (resistant) and SC212m (susceptible) were used to clone the coding and untranslated regions. The first group of primers used was designed from the Cat3 mRNA sequence from line W64A from GenBank (L05934.1); additional primers were designed as sequence information from actual cloning became available. A list of the primers used is shown in Table I. Primer pair Ucat3m and Lcat3m was utilized to clone 1,246 bp of the coding region of Mp313E. The same primer set was unsuccessful in amplifying a similar region in SC212m; hence, the pair Ucat3m and Cat3L-L was used to amplify a 1,041-bp fragment of the coding region. To clone the cDNA ends, primers Cat3L-U and Cat3L-L were initially used to amplify the 3' and 5' ends, respectively, from the two lines, together with their corresponding primers from the kit. Only the 5′ ends of Mp313E and SC212m were successfully cloned from Cat3L-L. Initial sequencing of the clones was performed using Sp6, T7, M13F, M13R, and T3 primers. The partial sequences obtained from the ends of the cloned cDNAs were used to design primers more specific to each line, and primer walking was performed to sequence through the cDNA. After several unsuccessful attempts to clone the 3′ ends from the cDNA of both lines, we designed another set of primers with attached restriction enzymes at their 5′ end (cat3-3′ERI and cat3-3′XhoI). This primer pair spans the second intron of catalase 3 and should produce a 385-bp fragment from cDNA and a 478-bp band from genomic DNA. This primer pair was used to amplify the 3′ end in the genomic DNA. In addition, another reverse primer was designed at the most 3′ end of catalase 3 (cat3-3′) to again pair with cat3-3′ERI to complete the 3′ untranslated region of the gene.

Data analysis.

All data analyses were performed using Excel (Microsoft, Redmond, WA, U.S.A.) and SAS (SAS Institute Inc., Cary, NC, U.S.A.). The procedure glm (general linear model) was utilized for all analyses of variance and the Tukey method for separation of means.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Maize genetics and genomics database: www.maizegdb.org