Allozyme-Specific Modification of a Maize Seed Chitinase by a Protein Secreted by the Fungal Pathogen *Stenocarpella maydis*

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ABSTRACT

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Stenocarpella maydis causes both dry-ear rot and stalk rot of maize. Maize inbred lines have varying levels of resistance to ear rot caused by *S. maydis*. The genetic basis of resistance appears to rely on multiple genetic factors, none of which are known. The commonly used stiff-stalk inbred line B73 has been shown to be strongly susceptible to ear rot caused by *S. maydis*. Here, we report that the ChitA protein alloform from B73, ChitA-F, encoded by a known allele of the *chiA* gene, is susceptible to modification by a protein (Stm-cmp) secreted by *S. maydis*. We also identify a new allele of *chiA* (from inbred line LH82) which encodes ChitA-S, an alloform of ChitA that is resistant to Stm-cmp modification. Chitinase zymogram analysis of seed from a commercial field showed the

presence of both ChitA alloforms in healthy ears, and showed that ChitA-F but not ChitA-S was modified in ears rotted by *S. maydis*. The ChitA-F protein was purified from inbred line B73 and ChitA-S from LH82. ChitA-F was modified more efficiently than ChitA-S by *S. maydis* protein extracts in vitro. The *chiA* gene from LH82 was cloned and sequenced. It is a novel allele that encodes six polymorphisms relative to the known allele from B73. This is the first demonstration that the susceptibility to modification of a fungal targeted plant chitinase differs among inbred lines. These findings suggest that the LH82 *chiA* allele may be a specific genetic determinant that contributes to resistance to ear rot caused by *S. maydis* whereas the B73 allele may contribute to susceptibility.

Additional keywords: breeding, chitinase-modifying protein, class IV, *Diplodia*, disease resistance, zeamatin.

The genus *Stenocarpella* Syd. & P. Syd. (teleomorph unknown; *Diaporthe*) (9) contains two species that cause a dry rot of maize ears: *Stenocarpella macrospora* (Earle) B. Sutton (syn. *Diplodia macrospora* Earle) and *S. maydis* (Berk.) B. Sutton (syn. *Diplodia maydis* (Berk.) Sacc.). Maize diseases caused by *Stenocarpella* spp. are worldwide in their distribution. Although *S. maydis* is reported from humid zones wherever corn is grown, *S. macrospora* is most prevalent in humid subtropical and tropical zones where plants exhibiting dry-ear rot and stalk rot may also display symptoms of leaf striping (18,35).

In order to colonize maize ears and produce dry-ear rot, *Stenocarpella* spp. must overcome defense mechanisms present in the caryopsis. (From this point, we use the term seed, although we are referring to not only the embryo and endosperm but also the entire structure that would more accurately be called the caryopsis). One facet of seed defenses is a group of proteins termed chitinases (13). Chitinases are enzymes (EC 3.2.1.14) that degrade chitin, a polymer of the monosaccharide N-acetyl-glucosamine (GlcNAc). Chitin is a component of fungal cell walls. Plant chitinases are thought to defend against fungal invasion either directly, by inhibiting fungal growth (31), or indirectly, by cleaving fungal cell walls to produce small signaling molecules that bind plant receptors, resulting in a defense response (33,40).

Plant chitinases are divided into classes based on amino acid sequence and predicted structure (22). Plant class IV chitinases are composed of four regions: an amino terminal signal sequence that targets the protein for secretion, a chitin-binding domain that allows the protein to bind insoluble chitin with high affinity, a flexible linker region that separates the chitin-binding domain from the catalytic domain, and the catalytic domain that functions to cleave chitin. The SignalP algorithm predicts that the amino terminal signal sequence is removed by the action of signal peptidase I (4). This results in a mature secreted protein with an amino terminal chitin-binding domain, a flexible linker, and a carboxyl terminal chitinase domain.

Maize seed contain abundant amounts of two class IV chitinases: ChitA and ChitB (16). These chitinases, in purified form, were shown to inhibit the growth of fungi growing on agar plates, but only when tested against certain species. Recently, we reported that ChitA is modified by a secreted protein from *Bipolaris zeicola* termed chitinase-modifying protein (Bz-cmp) (21). Modification of purified ChitA by purified Bz-cmp reduces its apparent molecular weight when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, Bz-cmp destroys ChitA's chitin-binding ability while the chitinase activity is intact, indicating that Bz-cmp modifies the chitin-binding domain of ChitA.

We also reported that commercial maize hybrids can produce two different forms of ChitA. These forms could result from either differences in the encoding genes—resulting in proteins with slightly different amino acid sequence—or differences in post-translational processing. Both forms of ChitA are equally susceptible to modification by Bz-cmp (21).

In the course of studying changes in seed chitinase content that result from inoculation with *S. maydis*, we discovered that this fungus also secretes a protein that modifies ChitA; however, it only efficiently modifies one form of ChitA. This finding

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prompted us to determine the difference between the two ChitAs. In this article, we show that they are allozymes—proteins encoded by different alleles of the same gene. In addition, we demonstrate that the previously reported ChitA allozyme is susceptible to modification by the protein secreted by *S. maydis* whereas the newly reported one is resistant. Our results imply that resistance to dry-ear rot caused by *S. maydis* might be genetically linked to the *chiA* gene.

MATERIALS AND METHODS

Fungal inoculum and culture conditions. *S. maydis* and *S. macrospora* cultures were obtained from the Agricultural Research Culture Collection, Peoria, IL. Five cultures, as indicated (Table 1), originated from the American Type Culture Collection (ATCC). Others were deposited by D. T. Wicklow after isolation and identification based on conidial micromorphology (34). They include: *S. maydis* NRRL 43670, NRRL 52415, NRRL 53563, NRRL 53565, NRRL 53566, and NRRL 53567 isolated from commercial maize seed produced in Illinois; NRRL 31249 and NRRL 53560 from Indiana; NRRL 13608 (=ATCC 10235) and NRRL 53564 from Kentucky; NRRL 53561 and NRRL 53562 from Nebraska; NRRL 13609 (=ATCC 16438) and NRRL 13615 from the United States; *S. macrospora* NRRL 13610 (=ATCC 26896) from Costa Rica; NRRL 13611 (=ATCC 42808) from Zambia; and NRRL 13612 (=ATCC 18606) from the United States (Georgia). Culture identities were confirmed by DNA sequencing of the internal transcribed spacer (ITS) region and domains D1 and D2 of the nuclear large subunit (28S) rDNA gene and comparison with sequences in the National Center for Biotechnology Information (NCBI) nonredundant database (1 June 2009) using the BLAST algorithm (23,41). DNA sequencing results were deposited in GenBank; sequence accession numbers are listed in Table 1.

Inoculation of maize ears. Maize ears were wounded by a single inoculation with *S. maydis* NRRL 43670 in the late milk to early dough stage of seed maturity (21 days after mid silk; 22 July 2006) for a commercial maize hybrid (FS 6873RR) grown in Kilbourne, IL. Fungal-penetrated toothpicks were prepared by inoculating potato dextrose agar with *S. maydis* and inserting sterile toothpicks. Following incubation to allow fungal colonization, toothpicks were removed from agar and used for plant inoculation. Twenty ears on plants within a single 7-m length of

row were inoculated in the mid-ear region by inserting a single wooden toothpick through the husks and developing seed, as far as the rachis (cob). Following natural dry down in the field (16% moisture content), ears were hand harvested (21 September 2006) and the visibly diseased seed from each ear were pooled and stored in a freezer at -7°C prior to preparation of protein extracts.

Fungal cultures and fermentation conditions. *Stenocarpella* isolates were grown as potato dextrose agar slant cultures (14 days, 25°C). A suspension of hyphal cells was prepared from these cultures giving a propagule density of $\approx 4 \times 10^4 \text{ ml}^{-1}$ served as inoculum. Fermentations were carried out in individual 500-ml Erlenmeyer flasks containing 50 g of whole maize seed. Distilled water (50 ml) was added to each flask, and the contents were soaked overnight before being autoclaved at 15 lb/in^2 for 30 min. Flasks were cooled and inoculated with 100 μl of hyphal-spore suspension. Cultures were fermented for 30 days at 25°C , harvested, and stored frozen at -80°C .

Chitinase analysis of maize seed. Protein extracts were prepared from diseased and healthy seed. Seed (1 g) were soaked for 24 h at 4°C , dried, frozen in liquid nitrogen, and ground into a powder. The frozen powder was added to buffer (80 mM sodium acetate [pH 5.2] + 1 mM EDTA + 1 mM phenylmethylsulfonyl fluoride) at 4°C and mixed. Insoluble material was removed by centrifugation (5 min; 14,000 rpm; 4°C). Protein concentrations were determined by the dye binding technique of Bradford (6).

The chitinase content of the extracts was determined by denaturing chitinase zymography (38). For each extract and a mixed sample (equal volumes of both extracts were mixed and incubated for 1 h), 2 μg of total protein was combined with loading dye (80 mM Tris-Cl [pH 6.8] + 2% SDS + 0.02% bromophenol blue) and heated for 1 min at 60°C . Samples were loaded in a 12% SDS-PAGE gel (85 by 80 by 1.5 mm [length by width by height]) containing glycol chitin. Proteins were separated by electrophoresis at 150 V for 2 h. After electrophoresis, the gel was incubated for 16 h at 25°C in refolding buffer (100 mM sodium acetate [pH 5.2] + 1% Triton X-100). Zymograms were stained with fluorescent brightener 28 and imaged with a Versadoc (Bio-Rad, Hercules, CA). Organic extraction and ultrafiltration treatment of diseased seed extracts were performed as described previously (21).

Purification of maize seed chitinases. ChitA proteins were purified from maize inbred lines B73 and LH82 according to a published method (16). The ChitA proteins were previously

TABLE 1. *Stenocarpella maydis* and *S. macrospora* strains used in this study

NRRL ^a	Location	Substrate	GenBank ^b	Sample no. ^c
<i>S. maydis</i>				
43670	Cerro Gordo, Illinois	Maize seed	GQ167213	1
52415	Cerro Gordo, Illinois	Maize seed	GQ167214	2
53563	Cerro Gordo, Illinois	Maize seed	GQ167215	3
53565	Kilbourne, Illinois	Maize seed	GQ259129	4
53566	Kilbourne, Illinois	Maize seed	GQ167216	5
53567	Kilbourne, Illinois	Maize seed	GQ167217	6
31249	Indianapolis, Indiana	Maize seed	GQ167218	7
53560	Rochester, Indiana	Maize seed	GQ167219	8
13608 (=ATCC 10235)	Unreported, Kentucky	Maize	GQ428199	9
53564	Hopkinsville, Kentucky	Maize seed	GQ167220	10
53561	Crete, Nebraska	Maize seed	GQ167221	11
53562	Crete, Nebraska	Maize seed	GQ259130	12
13609 (=ATCC 16438)	Unreported, United States	Maize	GQ167222	13
13615	Unreported, United States	Maize	GQ167223	14
<i>S. macrospora</i>				
13610 (=ATCC 36896)	Costa Rica	Maize	GQ167224	15
13611 (=ATCC 42808;MRC 143) ^d	South Africa	Maize	GQ167225	16
13612 (=ATCC 18606)	Georgia, United States	Maize leaf lesion	GQ259128	17

^a ATCC = American Type Culture Collection.

^b GenBank accession number (National Center for Biotechnology Information).

^c Sample number in Figure 4.

^d MRC number (South African Medical Research Council PROMEC Unit culture collection).

identified by peptide mass fingerprinting (PMF) (21). In this article, the identity of the other 28-kDa protein, ChitB, and the 25-kDa protein, zeamatin, was determined by PMF. Samples of each protein were taken from an SDS-PAGE gel and submitted for tryptic digest and liquid chromatography tandem mass spectrometry analysis (NextGen Sciences Inc., Ann Arbor, MI). Peptides were identified by Mascot (Matrix Science, London) using the NCBI nonredundant database (5 May 2008). Protein identifications were validated using Scaffold (Proteome Software Inc., Portland, OR).

Cmp activity assays. Secreted proteins were extracted from fermentation culture. For each, 5 g of culture was thawed and combined with 5 ml of extraction buffer (10 mM sodium acetate [pH 5.2] + 0.2 mM PMSF + 1 mM EDTA + 2 mM ascorbic acid) in a 50-ml conical tube. Samples were incubated for 30 min at 4°C; protein extracts were separated from insoluble material by centrifugation (5 min; 14,000 rpm; 4°C). Protein concentrations were determined and all extracts were adjusted to a concentration of protein at 100 µg/ml by addition of buffer.

TABLE 2. Oligonucleotide sequences used in this study

Name	Sequence (5'–3')	Description ^a
<i>chiA</i> cloning		
Afwd1	CAGAAGCTTCACCGCAGCAACGAACACGAACCTTGG	<i>chiA</i> 5' UTR with <i>Hind</i> III
Arev1	GTCTCTAGATTTTCGCGAGGGTGCGACGCCGAGCAT	<i>chiA</i> 3' UTR with <i>Xba</i> I
<i>chiB</i> cloning		
Bfwd1	CAGAAGCTTCTGATCTCTATATAAGGGGCGGCAACC	<i>chiB</i> 5' UTR with <i>Hind</i> III
Brev1	GTCTCTAGACGAGGGTGCGATGCGACGCCGAGCACA	<i>chiB</i> 3' UTR with <i>Xba</i> I
Plasmid sequencing		
Afwd2	TTCGCCCATGGCGGGACGGAG	<i>chiA</i> exon1 forward
Arev2	GCTGATGTAGCAGAAATCTGA	<i>chiA</i> exon2 reverse
Bfwd2	CTTCTTCGCGCACGCCACGCA	<i>chiB</i> exon1 forward
Brev2	GATCTCGCTGATGTAGCAGAA	<i>chiB</i> exon2 reverse
T7	GTAATACGACTCACTATAGGGC	Vector specific
T3	AATTAACCCTCACTAAAGGG	Vector specific
<i>Stenocarpella</i> 28S sequencing		
28SFwd	GGAAGTAAAAGTCGTAACAAGG	28S sequencing forward
28SRev	GGTCCGTGTTTCAAGACGG	28S sequencing reverse

^a UTR = untranslated region.

Fig. 1. Ear rot of maize caused by *Stenocarpella maydis* and its effect on plant chitinase activity. **A**, Mature ears of maize that were toothpick inoculated with *S. maydis* NRRL 43670 at the milk stage of maturity (inner) compared with healthy ears (outer). **B**, Rotted ear caused by *S. maydis*. **C**, Denaturing chitinase zymogram of proteins from diseased and healthy ears. Healthy seed have two different ChitAs and a third unidentified chitinase (lane 2). Diseased seed do not have ChitA-F (lane 1). Adding extract from diseased seed before gel loading causes loss of ChitA-F from the healthy seed extract (lane 3). **D**, Activity is due to a protein. Diseased seed extracts were fractionated, mixed with healthy seed extracts, and analyzed by chitinase zymogram. Lane 1, healthy seed extract; lane 2, diseased seed extract mixed with healthy seed extract; lane 3, n-butanol extract mixed with healthy seed extract; lane 4, 10-kDa membrane flow-through mixed with healthy seed extract; lane 5, 10-kDa membrane retentate mixed with healthy seed extract. ChitA-S = slow migrating form of ChitA; ChitA-F = fast migrating form; ? = unidentified seed chitinase.

Each of the extracts was assayed for cmp activity by incubation with each of the three chitinase substrates. For each reaction, 1 µg of secreted proteins was combined with 10 µg of purified substrate in a volume of 20 µl. Reactions were incubated for 16 h at 25°C. Products were analyzed by Tricine-SDS-PAGE (12% T; 3% C) (29).

Cloning and sequencing of *chiA* and *chiB* genes. The *chiA* and *chiB* genes were cloned from genomic DNA of B73 and LH82. Genomic DNA was prepared from leaf tissue of 7-day-old seedlings using the cetyltrimethylammonium bromide procedure (11). Briefly, 1 g of seedling leaf tissue was combined with liquid nitrogen in a mortar and ground to a powder with a pestle. Genomic DNA was isolated from the powder and resuspended in a final volume of 100 µl of Tris-EDTA buffer. The two genes were amplified from each DNA prep by polymerase chain reaction (PCR) using the oligonucleotide primers listed in Table 2 and Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and the following conditions: an initial denaturing step (98°C, 15 s), 35 amplification cycles (96°C, 30 s; 55°C, 45 s; and 72°C, 60 s), and a final extension (72°C, 10 min). PCR products were incubated with restriction endonucleases (*EcoRI* and *XbaI*) to cleave the oligonucleotide encoded termini. Digested PCR products were then ligated into the pBluescript KS(+) plasmid (Stratagene, La Jolla, CA), and transformed into *Escherichia coli* DH5α. The cloned genes were sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA) and a

combination of two vector-specific and two internal gene primers. Both strands were sequenced.

The PCR amplification, cloning, and sequencing were repeated three times. In two instances, one of three sequences had a single nucleotide mismatch; it was assumed that this was due to a DNA polymerase error during amplification and the nucleotide in the other two clones was assumed to be correct. The *chiA* sequence from LH82 is available from GenBank (accession no. GQ856537).

Computational methods. The specificity of the four cloning primers (Afwd1, Arev1, Bfwd1, and Brev1) was confirmed by using the blastN algorithm (2) to search the maize genome for sequences with distant homology (<http://www.maizegenome.org/blast>). The novelty of the ChitA sequence from maize inbred line LH82 was confirmed by using the Tblastn (2) algorithm to search both the nonredundant and non-human non-mouse expressed sequence tag databases (5 August 2009) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequence alignment of ChitA allozymes and ChitB was generated with Clustal W (36). The alignment image was created with the EMBOSS programs emma and showalign (25).

RESULTS

Stenocarpella ear-rot of maize. To look for changes in maize chitinase activity in response to infection by *S. maydis*, we first

Fig. 2. Purification of ChitA and ChitB from maize inbred line B73. **A**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of chitin affinity separation. Most maize seed proteins did not bind chitin (lane 2). Chitin resin was washed with 20 volumes of binding buffer (not shown) followed by incubation with intermediate pH buffer (lane 3). Then, low-pH buffer was added and bound proteins were eluted (lane 4). **B**, Cation exchange chromatography. Proteins were bound to a cation exchange column and eluted with a linear, increasing-salt gradient (gray line). Eleven fractions—that span the two major peaks—were collected for analysis (dark boxes). **C**, SDS-PAGE analysis of fractions. Each of the major peaks contain a protein with an apparent molecular mass of 28 kDa. The mass of each marker protein, in kilodaltons, is indicated to the left of each gel.

generated diseased seed. Ears of maize growing in a commercial field near Kilbourn, IL were inoculated with *S. maydis* NRRL 43670 during early seed development and were harvested at maturity. The inoculated ears had disease symptoms consistent with ear rot caused by *S. maydis*: bleached husks (Fig. 1A); white mycelial growth between seed and cob, incomplete filling of the cob, and evidence of premature seed germination (vivipary) (Fig. 1B).

The chitinase content of the diseased and healthy seed was determined by denaturing chitinase zymography (Fig. 1C). Healthy seed contain three chitinases (lane 2). The top two bands are different forms of the ChitA protein and the bottom band is an unidentified seed chitinase (21). Diseased seed contain two chitinases (lane 1); ChitA-F is absent as a result of *S. maydis* infection. In order to demonstrate that the absence of ChitA-F in diseased seed was due to direct, post-translational modification, healthy and diseased seed extracts were mixed and incubated prior to gel loading. Incubation resulted in loss of ChitA-F from the healthy sample, demonstrating that a molecule produced by *S. maydis* which was still active in the diseased seed extracts was modifying ChitA-F (lane 3).

By fractionating the diseased seed extracts prior to mixing with healthy seed extracts, it was determined that loss of ChitA-F signal was due to a protein (Fig. 1D). When diseased seed extract was mixed with n-butanol, activity did not transfer into the organic phase, as would be expected if activity was due to a small molecule or peptide (lane 3). When passed through a membrane designed to retain proteins >10 kDa, activity was absent in the flow-through (lane 4) and present in the retentate (lane 5). These results indicated that activity was due to a protein >10 kDa, which we named Stm-cmp.

Purification of ChitA-S, ChitA-F, and ChitB. In order to study Stm-cmp activity in vitro, we first purified ChitA-S and ChitA-F. ChitA-S and ChitA-F can be individually purified from maize inbred lines that only contain one of the proteins (21). Seed from maize inbred B73, which is commonly used in producing commercial hybrids and is the genetic model used for sequencing the maize genome, contains ChitA-F but not ChitA-S. ChitA-F was purified from seed by a combination of chitin affinity separation and cation-exchange chromatography (Fig. 2). SDS-PAGE analysis of proteins that result from affinity separation showed two protein bands—one with a mass of 28 kDa and a second with a mass of 25 kDa (Fig. 2A, lane 4). Cation exchange chromatography revealed that the 28-kDa band contains two different proteins that elute at different salt concentrations. We previously identified the more weakly bound 28-kDa protein as ChitA (21). In order to identify the other two proteins, we performed PMF on both the 25-kDa protein and the more tightly bound 28-kDa protein (Table 3). The 28-kDa protein is ChitB, an isozyme of ChitA that is encoded by a different gene (16); the 25-kDa protein is zeamatin, an antifungal protein found in maize seed that is known to bind β -1,3-glucans (30).

The purification was repeated with seed from inbred line LH82, which contains ChitA-S but not ChitA-F. Both ChitAs and ChitB appear the same when denatured by addition of SDS, dithiothreitol, and heating at 100°C followed by SDS-PAGE separation and protein staining (Fig. 3A). They behave differently when partially denatured by addition of SDS, heated at 60°C, and assayed for activity in chitinase zymograms (Fig. 3B). ChitA from LH82 produces ChitA-S signal and ChitA from B73 produces

ChitA-F; ChitB does not efficiently degrade the substrate. This inability of ChitB to degrade substrate in zymogram gels indicates that it was not detected in zymogram analysis of maize seed. In addition, because its sequence is closely related to that of ChitA, it might also be a substrate of Stm-cmp; therefore, we included it as a third substrate in subsequent cmp reactions.

In vitro cmp activity of proteins secreted by *Stenocarpella* fermentation cultures. In order to confirm that Stm-cmp activity is commonly present in maize isolates of *S. maydis*, determine the substrate specificity of Stm-cmp in vitro, and determine whether the related maize pathogen *S. macrospora* has cmp activity, we grew fermentation cultures on autoclaved corn and tested them for cmp activity. For each of 17 cultures, an extract of secreted proteins was made and incubated with each of the three maize chitinase substrates. Each reaction was analyzed by SDS-PAGE to determine the amount of cmp activity.

All isolates of *S. maydis* secreted Stm-cmp (Fig. 4). The amount of activity varied and was very low in protein extracts from NRRL 53563 (sample 3) and NRRL 53564 (sample 10). We repeated the experiment, starting with a second set of cultures, and obtained a different set of activity levels, including high amounts of activity in both NRRL 53563 and NRRL 53564 (not shown); therefore, the difference in activity was due to experimental variation rather than isolate-specific differences. Unlike the level of Stm-cmp activity, the preferential modification of ChitA-F relative to ChitA-S did not vary. All of the *S. maydis* protein extracts modified ChitA-F more efficiently than ChitA-S in both experiments. Additionally, ChitB was modified by Stm-cmp. Modification of ChitB by Stm-cmp was also more efficient than that of ChitA-S.

The isolates of *S. macrospora* also modified the three proteins (Fig. 4, bottom gel). When compared with the *S. maydis* samples, the level of cmp activity was lower. Also, the substrate preference was different. NRRL 13610 (sample 15) preferentially modified ChitB while both ChitAs were less

Fig. 3. Polyacrylamide gel analysis of the two purified ChitAs and ChitB. **A**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis. ChitAs from inbred lines LH82 and B73 and ChitB have equivalent electrophoretic mobility. **B**, Chitinase zymogram. ChitA from LH82 produces the ChitA-S form while ChitA from B73 produces ChitA-F. ChitB degrades the glycol chitin substrate poorly. Lane 1 = ChitA purified from inbred line LH82; lane 2 = ChitA from B73; lane 3 = ChitB from B73.

TABLE 3. Peptide mass fingerprinting to identify seed proteins that bind colloidal chitin

Protein	Unique peptides	Unique spectra	Residues identified	Coverage (%)
ChitB	12	18	159/280	57
Zeamatin	8	10	107/206	52

The cDNA sequences for *chiA* and *chiB* were initially reported by Huynh et al. (16) (GenBank accession nos. M84164 and M84165). Both sequences match genome sequence data reported

Amino acid sequence analysis of LH82 ChitA. To identify the amino acids that differentiate LH82 and B73 ChitA—and to compare both ChitAs to the ChitB isozyme—we aligned the protein sequences and created an alignment image (Fig. 6). The two ChitAs have six polymorphisms: the secretion signal of LH82 ChitA is one amino acid longer (P1), the chitin-binding domains differ at one amino acid (P2), the linker of LH82 ChitA is three glycine residues shorter (P3), and the chitinase domains have three amino acid differences (P4, P5, and P6). The sequence of ChitB, which is modified by Stm-cmp more efficiently than LH82 ChitA but less efficiently than B73 ChitA, does not match that of either ChitA at P1, P2, or P3; in the chitinase domain, the amino

acid sequence of ChitB matches B73 ChitA at all three polymorphic sites.

In order to confirm the novelty of the LH82 ChitA sequence, we searched the NCBI nonredundant database (5 August 2009). Although there weren't any exact matches, a number of ChitA sequences from *Zea mays* subsp. *parviglumis* were close. The sequence diversity of ChitA proteins in this annual teosinte, which is the ancestor of modern maize (19), has been studied, and 94 sequences are available from GenBank (20,37). A summary of the *Z. mays* subsp. *parviglumis* sequences at each of the maize polymorphic sites is presented in Table 4.

We also searched for matches of LH82 ChitA to expressed sequence tags. We found one sequence that spans the amino terminal 60% of the protein sequence and is an exact match: GenBank accession no. FL407643. This sequence is from an expressed sequence tag project that used several hybrids from Pioneer Hi-Bred International, Inc. (Johnston, IA) (1).

DISCUSSION

Previously, we reported that *B. zeicola*, a fungal pathogen of maize, secretes a protein called Bz-cmp that modifies two forms

Fig. 5. Cloning and sequencing of *chiA* and *chiB* genes. **A**, Two genes were polymerase chain reaction amplified from genomic DNA of LH82 and B73. Oligonucleotide primers were designed to bind the untranslated region of each gene, with restriction sites added for plasmid cloning. **B**, *chiA* gene from LH82, which encodes ChitA-S, is a novel allele. Boxes indicate the start and stop codons, respectively. The intron sequence is underlined.

of ChitA produced by a commercial maize hybrid (21). In diseased seed, both ChitA proteins were modified. In vitro, purified Bz-cmp modified both ChitA proteins. In this study, we have shown that *S. maydis* secretes a protein, Stm-cmp, that efficiently modifies one of these two forms of ChitA. Zymogram analysis of chitinases from diseased and healthy seed showed that diseased seed lack ChitA-F while ChitA-S is still present. We purified ChitA-F from B73 and ChitA-S from LH82 and tested secreted proteins from *S. maydis* cultures for Stm-cmp activity in vitro and observed that modification of ChitA-F was more efficient than that of ChitA-S. In vitro analysis also showed that the isozyme ChitB is susceptible to Stm-cmp modification. We cloned and sequenced the ChitA-encoding *chiA* gene from LH82 and found that it is a novel allele; therefore, ChitA-S and ChitA-F are allozymes. Six polymorphisms differentiate the proteins. This is the first demonstration that the susceptibility to modification of a fungal-targeted plant chitinase can vary and is determined by genetic diversity within alleles of a gene.

What is the function of ChitA and ChitB? Maize seed contain large amounts of these class IV chitinases, suggesting that they play a significant role in protecting seed against attack from pathogens during germination (16). The accumulation of ChitA and ChitB during seed maturation may contribute to the increase in resistance to ear rot caused by *S. maydis* that was reported to occur when ears were inoculated 28 days after mid silk (8). *S. maydis* infection of stalk, leaf, and shank tissues is initiated following conidial germination and penetration of epidermal cell walls and host cytoplasm through the formation of an appressorium and enzymatic degradation (5). ChitA might function to inhibit the spread of fungal hyphae, either by degrading fungal cell wall chitin or by releasing chitin elicitors from fungal cell walls to trigger plant defense responses, including production of pathogen response proteins and triggering of the hypersensitive response, a form of programmed cell death that is a major component of plant immunity (14,17). Modification of ChitA to either suppress direct growth inhibition or suppress induction of

Fig. 6. Sequence alignment of ChitAs and ChitB. The ChitA allozymes have six polymorphisms, labeled P1 through P6. The predicted site at which the secretion signal sequence is removed, as determined by the SignalP algorithm, is indicated. The B73 ChitA sequence is shown in the top row and was used as the reference sequence; the LH82 ChitA sequence and ChitB sequence are shown in the second and third rows. Dots indicate homology to the reference sequence. Dashes indicate gaps. Uppercase letters indicate nonconservative amino acid changes. Lowercase letters indicate conservative amino acid changes.

TABLE 4. Number of *Zea mays* subsp. *parviglumis* ChitA sequences that match maize inbred line B73 or LH82 at the six polymorphic sites

ChitA polymorphism ^a	ChitA region	Peptide sequence ^b		Sequences matching ^c		
		LH82	B73	LH82	B73	Neither
P1	Secretion signal	G(L) ₃ A	G(L) ₂ A	5	70	19
P2	Chitin-binding domain	TDDYC	TDAYC	43	51	0
P3	Linker region	S(G) ₁₁ S	S(G) ₁₄ S	6	4	84
P4	Chitinase domain	VVSDA	VVTD A	41	52	1
P5	Chitinase domain	VNKYP	VNAYP	16	78	0
P6	Chitinase domain	HR LMP	HRVMP	13	79	2

^a Six differences in ChitA sequence from maize inbred lines B73 and LH82 as shown in Figure 6.
^b Amino acid sequence at polymorphic sites. Differences are underlined.
^c Determined by visual inspection of GenBank sequences in nonredundant database (5 August 2009).

maize defense responses would promote *S. maydis* endophytic-biotrophic infection.

Stenocarpella spp. are the most important ear rot pathogens in nearly all countries where maize is produced (26). Over several decades, plant pathologists and breeders have offered evidence that maize resistance or susceptibility to *S. maydis* is associated with both qualitatively and quantitatively inherited traits, the former presumably based on single dominant or recessive genes and the latter providing intermediate levels of resistance based on multigenic inheritance. Quantitative resistance to *S. maydis* in which resistance is additively inherited remains the main focus in breeding programs (10,27,28,39). At the same time, dominance gene effects have also contributed to variation in *Stenocarpella* ear rots of maize (10,24).

Although we lack a full understanding of ChitA's biological function, if we assume that *S. maydis* modifies ChitA to facilitate invasion of maize ears, then the *chiA* allele found in LH82 should contribute to resistance against *S. maydis* (ear rot) and the B73 allele should contribute to susceptibility. Although resistance to ear rot caused by *Stenocarpella* spp. is reported to be inherited independently from resistance to other ear rot pathogens (15), it can be noted that the proprietary inbred line LH82 was associated with intermediate resistance to both *Fusarium graminearum* (seedling blight) (12) and *Phaeosphaeria maydis* (Henn.) Rane, Payak & Renfro (leaf spot) while inbred lines related to B73 were particularly susceptible (7). Might inbred lines reported to be resistant to *Stenocarpella* ear rot also possess resistant alleles of *chiA*? Dorrance and co-workers (10) reported that the public inbred line B73 and its direct descendants are highly susceptible to *Stenocarpella* ear rot in maize whereas four inbred lines are good sources of resistance. Furthermore, these results were indicative of dominant gene action or epistasis, and agreed with Wiser et al. (42), who had earlier suggested that susceptibility may be dominant over resistance, reporting that crosses between two susceptible parents yielded progeny that were more susceptible than either parent. A high level of susceptibility for inbred line B73 was confirmed by Rossouw et al. (28), who reported that B73 showed pronounced negative specific combining ability in combination with Mo17 and other inbred lines selected as providing resistance in previous resistance evaluations. Dominance gene effects made the major contribution to variation in ear rot of maize caused by *S. macrospora* in the crosses studied using selected maize populations for breeding purposes in the mid-altitude (1,280 m) agroecological zone in Nigeria (24). The evaluation of disease resistance or susceptibility was based on visual assessment of disease severity for individual ears at harvest. The authors concluded that, given the minor contribution of additive effects in resistance to *S. macrospora*, more rapid advances can be made in a breeding program using a method that emphasizes the dominance and epistatic gene effects. Reports identifying maize inbred lines as new sources of resistance to ear rots caused by *S. maydis* (28,39) or *S. macrospora* (24) are based on traditional breeding programs, and no mapping studies of loci conferring resistance to this disease have been carried out (3). Furthermore, no genes have been shown to convey resistance to *Stenocarpella* ear rot in maize. In the present study, we have identified different alleles of *chiA* as potential sources of resistance to susceptibility to ear rot caused by *S. maydis* and *S. macrospora*.

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