Transgenic maize plants expressing the Totivirus antifungal protein, KP4, are highly resistant to corn smut

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Summary
The corn smut fungus, Ustilago maydis, is a global pathogen responsible for extensive agricultural losses. Control of corn smut using traditional breeding has met with limited success because natural resistance to U. maydis is organ specific and involves numerous maize genes. Here, we present a transgenic approach by constitutively expressing the Totivirus antifungal protein KP4, in maize. Transgenic maize plants expressed high levels of KP4 with no apparent negative impact on plant development and displayed robust resistance to U. maydis challenges to both the stem and ear tissues in the greenhouse. More broadly, these results demonstrate that a high level of organ independent fungal resistance can be afforded by transgenic expression of this family of antifungal proteins.

Introduction
The smut fungi are important agricultural pathogens responsible for significant crop yield losses. Corn smut, caused by a biotrophic fungus Ustilago maydis, is economically important in all countries where maize is grown. Yield loss because of corn smut is generally kept below 2% with available partially resistant field varieties of maize. Sweet maize in particular is more susceptible to this disease where yield losses can be as high as 20%. Hot and dry weather conditions are favourable for U. maydis that can attack maize during its early stage of development. However, corn smut occurs more frequently on maize ears, tassels and nodes than on leaves, internodes and aerial roots. If one considers that maize is the most economically important crop in the USA, generating $48.7 billion in 2009 with approximately 35 million hectares planted (2010 World of Corn, National Corn Growers Association, http://www.ncga.com) even a 2% loss is nearly $1.0 billion annually. In addition to domestic consumption, maize is a major profitable US export propelling USA as the largest maize exporter in the world for 2009. With the current increasing emphasis on biofuels, the role of maize in the USA agriculture is likely to increase to even greater importance.

To control corn smut disease, several methods have been recommended including crop rotation, sanitation, seed treatments, application of foliar fungicides, modification of fertility and biological controls (Pataky and Snetselaar, 2006). In spite of these frequently mentioned control tactics, host resistance is the only practical method of managing common smut in areas where U. maydis is prevalent. Currently, there is no maize line available that is immune to infection by U. maydis. A recent study suggested that U. maydis resistance in maize is a polygenic trait, where multiple quantitative trait loci contribute to plant resistance to corn smut (Baumgarten et al., 2007). We explored an alternative approach by introducing a component of a naturally occurring antifungal system into transgenic maize.

Our study focused on an ‘interstrain inhibition’ system found in U. maydis (Hankin and Puhalla, 1971; Day and Anagnostakis, 1973; Koltin and Day, 1975; Koltin, 1988). Interstrain inhibition in U. maydis is caused by antifungal proteins (killer toxins) secreted by double-stranded RNA Totiviruses (Hankin and Puhalla, 1971). It is estimated that ~1% of U. maydis found in nature secretes these killer toxins (Day, 1981). None of the three known killer strains of U. maydis are resistant to any toxin other than their own, and the three corresponding resistance genes are recessive and independent. Therefore, it has been suggested that transgenic crops expressing two or more different U. maydis killer toxins would be protected against all by a fraction of a percent of U. maydis strains (Kinal et al., 1995). Similar killer toxins have been identified in eight genera of yeast (Young, 1987). Specifically, we focused on the secreted KP4 protein of the interstrain inhibition system (Gu et al., 1994, 1995).

KP4 is a single polypeptide of 105 amino acids produced by the UMV4 virus that infects the P4 strain of U. maydis (Park et al., 1994). It is the only U. maydis toxin not processed by Kex2p, and there is no sequence similarity to other toxins (Ganesa et al., 1991; Park et al., 1994). Although most of the yeast toxins are acidic (Bussey, 1972) and the Ustilago antifungal proteins, KP6 and KP1, have neutral pl values (Levine et al., 1979), KP4 is extremely basic, with a pl ~9.0 (Ganesa et al., 1989). KP4 is an α/β sandwich protein with a relatively compact structure (Gu et al., 1995). From a tenuous structural similarity to the scorpion toxin AaHII from Androctonus australis, it was suggested (Gu et al., 1995) and then subsequently shown (Gage et al., 2001) that KP4 inhibits calcium uptake in fungal cells. Our previous studies have shown that the KP4 protein inhibits calcium channels (Gage et al., 2001, 2002; Allen et al., 2008). This is a reasonable mode of action as calcium and calcium-dependent signalling is essential for normal growth as well as pathogenicity of various fungal plant pathogens (Rispail et al., 2009). Transforming economically important crops with the native U. maydis KP4 gene has been successfully applied in
the wheat defence against stinking smut (Clausen et al., 2000). In this study, we show that transgenic maize lines expressing the monocot codon-optimized chimeric KP4 gene containing a plant secretory signal sequence are highly resistant to corn smut disease caused by U. maydis.

Results

Expression of extracellular KP4 in transgenic maize

To express KP4 in the extracellular space of transgenic maize, a monocot codon-optimized gene containing the secretory signal peptide sequence of a plant defensin MsDef1 was placed under the control of a constitutive maize Ubi1 promoter (Figure 1) and introduced into maize inbred H99 (Sidorov et al., 2005). Forty-five R0 transgenic lines were generated. Nineteen of these lines contained the chimeric KP4 gene and expressed high levels of protein as determined by sandwich ELISA. These lines were backcrossed to maize inbred B73 once and subsequently selfed twice to generate BC1F2 plants. Ten transgenic lines containing a single insert were selected for further analysis. Of the lines tested (Table 1), only line 826 did not express KP4 protein as determined by ELISA. KP4 expression in homozygous transgenic lines 1040, 851 and 746 was determined to be 5.6, 6.6 and 5.8 ppm (fresh weight), respectively. As KP4 contains five disulfide bonds, we next addressed the issue of whether the protein was properly folded during export from the plant cells and biologically active. To that end, total leaf protein extracts of four transgenic lines 851, 746, 759 and 947 were tested for antifungal activity against U. maydis in a plate diffusion assay. From the data presented in Figure 2, it was clear that these transgenic lines expressed high amounts of biologically active KP4.

As we used a plant defensin secretory signal peptide sequence, we determined whether KP4 was indeed secreted in the transgenic lines (Tenras et al., 1995). The imbibed germinating seeds from transgenic lines 851 and 1040 and the non-transgenic H99/B73 BC1F2 control line were tested for their ability to secrete biologically active KP4 in a plate diffusion assay. The intact germinating seeds were used to enhance the contact area between the seed and the agar. This may cause some tissue damage that could release some KP4 from within the cell. However, it was thought that the larger contact surface might enhance the observed killing zone and that there was a limited amount of KP4 released from the damage. As shown in Figure 2b, the intact germinating seed from the line 1040 yielded a marked zone of inhibition indicating that antifungal KP4 protein was efficiently secreted. As expected, clear zone of inhibition was also observed around the cut germinating seed of this line, but not around the wild-type BC1F2 seed. The germinating seed of line 851 produced less pronounced zone of inhibition than that of line 1040. As shown in Table 1, this correlated well with the fact that line 1040 was slightly more resistant to U. maydis challenge than line 851. Perhaps transgenic maize line 1040 secretes KP4 better than line 851 even though the total KP4 expression level is essentially the same for these two lines. This is rather speculative as the killing zone assay is only a qualitative measure of KP4’s antifungal activity.

Corn smut resistance analysis of transgenic maize lines

As transgenic maize lines secreted bioactive KP4 protein, the ability of this protein to protect transgenic lines from the corn smut pathogen was assessed. As expected, clear zone of inhibition was also observed around the cut germinating seed of this line, but not around the wild-type BC1F2 seed. The germinating seed of line 851 produced less pronounced zone of inhibition than that of line 1040. As shown in Table 1, this correlated well with the fact that line 1040 was slightly more resistant to U. maydis challenge than line 851. Perhaps transgenic maize line 1040 secretes KP4 better than line 851 even though the total KP4 expression level is essentially the same for these two lines. This is rather speculative as the killing zone assay is only a qualitative measure of KP4’s antifungal activity.

![Figure 1](https://example.com/figure1.png)

**Figure 1** (a) For expression in transgenic maize, a chimeric KP4 gene was designed where the nucleotide sequence encoding the 105-amino acid KP4 was fused to the carboxyl terminus of the 27-amino acid signal peptide sequence of a plant defensin, MsDef1. (b) The monocot codon-optimized chimeric KP4 gene was synthesized (GenScript Corporation, Piscataway, NJ), placed under the control of the maize Ubi1 promoter/intron/tobacco etch virus mRNA leader sequence, and cloned into the plant expression vector pZP212 (Hajdukiewicz et al., 1994). The resulting construct was introduced into Agrobacterium tumefaciens strain EHA101 for maize transformation.
Smut disease was then determined (Figure 3). Ten transgenic maize lines were tested against *U. maydis* infection in the greenhouse. In general, all transgenic maize lines expressing KP4 developed normally when compared to the nontransgenic control line BC1F4 (Figure 3e,f). Seven-day-old seedlings were inoculated with a mixture of the wild-type *U. maydis* KP4-sensitive strains 1/2 and 2/9 (Gold et al., 1997). These strains are commonly used wild-type laboratory strains of *U. maydis* (Brefort et al., 2009). Both strains, 1/2 (same as strain 521) (Kronstad and Leong, 1989) and the near-isogenic strain 2/9 (Gold et al., 1997) generated galls in nontransgenic maize plants.

Disease symptoms were observed and scored at 10 days post-inoculation (dpi). To determine whether plants resisted or simply delayed *U. maydis* infection, transgenic plants were scored for disease symptoms at 21 dpi. Disease symptoms were absent at 21 dpi in several transgenic maize lines, while the wild-type line BC1F4 exhibited plant death (Figure 3d). Five KP4 transgenic lines, 851, 947, 1040, 885 and 972, demonstrated strong resistance to this fungus. Four KP4 transgenic lines, 746, 759, 810, and 923, showed moderate resistance to this fungus. One KP4 transgenic line, 826, did not confer resistance to *U. maydis* infection.

### Table 1: Disease symptoms caused by *Ustilago maydis* infection on KP4 transgenic maize

<table>
<thead>
<tr>
<th>Events (maize lines)</th>
<th>Disease score</th>
<th>Disease index</th>
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</thead>
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<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>947*</td>
<td>49</td>
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</tr>
<tr>
<td>1040*</td>
<td>59</td>
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<td>40</td>
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<td>60</td>
<td>12</td>
</tr>
<tr>
<td>810†</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td>923†</td>
<td>40</td>
<td>12</td>
</tr>
<tr>
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<td>759†</td>
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<tr>
<td>826</td>
<td>40</td>
<td>12</td>
</tr>
<tr>
<td>BC1F4 (wt)</td>
<td>60</td>
<td>11</td>
</tr>
<tr>
<td>Non-infected 947</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Non-infected wt</td>
<td>20</td>
<td>2</td>
</tr>
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</table>

Results from three pathogenicity experiments using stem inoculations. The disease scores are as follows: 0 = no symptoms; 1 = anthocyanin and/or chlorosis; 2 = small leaf galls; 3 = small stem galls; 4 = basal stem galls; 5 = plant death. 851, 746, 759, 947, 1040, 810, 826, 885, 923, and 972 are 10 independently generated KP4 transgenic maize lines. BC1F4 (wt) is the wild-type (wt) maize line from which KP4 transgenic plants were generated. Symptoms were scored 10 dpi. Values with different superscripts in the disease index column are significantly different (*P* ≤ 0.05) in Duncan’s multiple range test. The number of plants used differed because not all seeds germinated or (in the case of uninfected plants) so many replicates were not needed.

* Lines that were homozygous at the time of testing.
† Lines that were hemizygous at the time of testing.
strains directly into maize ears was done upon the appearance of silks (Figure 3g,h). Two weeks later, plant tumours or galls were observed in the ears of the wild-type line (BC1F4) and transgenic line 826 (Figure 3i), while the ears of the KP4 transgenic lines 851, 746, 759, 947, 1040, 810, 885, 923 and 972 remained healthy and disease free (Figure 3j).

Correlation between expression levels and resistance

Corn smut resistance observed in various transgenic lines correlated well with the expression of KP4. As shown in Table 1, lines 759, 810 and 923 that showed partial resistance to U. maydis were hemizygous. In contrast, line 826 was completely susceptible to infection. To ascertain whether this is because of differences in the mRNA levels, quantitative RT-PCR was performed on line 826 and homozygous lines 746, 1040, 851, 885, 923 and 972 remained healthy and disease free (Figure 3i).

The protein expression levels of a number of individual transgenic maize plants was determined using ELISA assays and compared with their respective disease scores. As shown in Figure 5, there is excellent correlation between KP4 expression and resistance to U. maydis infection. Importantly, these results demonstrated that constitutively expressed KP4, throughout the maize plant, was capable of preventing infection in all organs while recent results suggest that naturally occurring immunity to this disease is encoded by a number of genes in an organ-dependent manner (Baumgarten et al., 2007; Skibbe et al., 2010).

Figure 3 KP4 transgenic plants resist Ustilago maydis infection. Wild-type maize plants (BC1F4) at 10 dpi show disease symptoms of (a) anthocyanin and chlorosis, (b) leaf galls and (c) basal galls. (d) Wild-type maize seedlings 21 days poststem inoculation results in death (white arrow 1), while the KP4 transgenic plants remain symptomless (line 947; white arrow 2). (e, f) Three-month-old transgenic maize plants (line 947) expressing KP4 protein have no observable developmental differences in comparison with the wild-type (BC1F4). (g, h) Maize ears prior to Ustilago maydis inoculation. (i) Galls appear 14 days postinoculation in the wild type (arrow 3), while the KP4 transgenic maize remain free of disease for the duration of the experiment (j).
In this work, we generated several transgenic maize lines expressing extracellular KP4. High level expression of KP4 was obtained using the monocot codon-optimized mature KP4 protein coding sequence. The chimeric KP4 gene also utilized a secretory signal peptide sequence of a plant defensin gene for efficient secretion of the protein to the extracellular space. The resistance of 10 independent maize lines expressing KP4 was compared with that of the parental maize inbred line H99/B73 (BC1F2). Pathogenicity assays showed strong resistance to *U. maydis* infection in nine KP4 transgenic lines, but not in line 826. Transgenic lines 851, 947, 1040, 885 and 972 showed robust resistance to this fungus (Table 1) with no observed galls and little chlorosis and/or anthocyanin on the leaves. Lines 746, 759, 810 and 923 showed partial resistance to *U. maydis* infection with observed leaf, stem and basal galls. At 21 dpi, chlorosis and/or anthocyanin symptoms were reduced or completely absent in KP4 transgenic maize plants, while the wild-type maize line (BC1F2) exhibited plant death. This suggests that plants resisted *U. maydis* infection and not simply delayed infection. ELISA and *in vitro* antifungal assays of the disease-susceptible line 826 showed absence of the KP4 protein. Surviving KP4 transgenic maize plants were transplanted into large pots, and in the next 3 months, they developed normal ears and tassels similar in appearance to the wild-type plants. This suggests that initial inoculation of transgenic KP4 lines at the early life stage (7-day-old plantlets) did not affect development into mature plants. Upon the appearance of silks, mature KP4 transgenic plants were inoculated with the fungus directly into maize ears. Two weeks later, plant tumours or galls were observed in the ears of the wild-type maize line BC1F2 and in 826, while the ears of the remaining KP4 transgenic maize lines (851, 746, 759, 947, 1040, 810, 885, 923, 972) were healthy and largely disease free. This suggests that transgenic mature plants continued KP4 production, which enabled them to resist *U. maydis* infection. Interestingly, partially resistant KP4 lines did not develop ear galls, suggesting that KP4 production in this tissue was sufficient to protect transgenic plants from *U. maydis* infection. However, a few ears of the KP4 transgenic maize lines did show ear rot (data not shown), which may be caused by other invading pathogens.

With regard to commercial application of this technology, there is growing evidence that these proteins are safe for human and animal consumption. As the KP4 protein inhibits *U. maydis* growth and also blocks L-type voltage-gated calcium channels (Gu et al., 1995), a major concern has been whether KP4 transgenic maize is safe for use by humans and animals, and whether it is safe for the environment. It has been shown that KP4 protein degrades in <60 s in artificial stomach fluid, and its amino acid sequence is not similar to any known allergens (Schlaich et al., 2006). In the same study, it was also shown that KP4 does not affect viability or subcellular structures of human, plant, insect and hamster cell lines. Various bacterial and fungal strains have been tested *in vitro* and none appeared to be susceptible to KP4 except for the specific genera of the order Ustilaginales causing smut and bunt (Schlaich et al., 2006, 2007). Furthermore, KP4 transgenic wheat was shown to not affect fungal soil communities, wheat-infecting insects, such as aphids, and ‘standard’ soil arthropod *Folsomia candida* (Widmer, 2007). It can therefore be assumed that KP4 transgenic maize will not have any deleterious effects on humans, plants, insects, bacterial and fungal soil communities. Currently, we are considering several gene containment strategies. For example, as maize silk is the major route of *U. maydis* infection (Christensen, 1963; Snetselaar et al., 2001), we propose to use a silk-specific promoter, such as SLG promoter (Liu et al., 2008), to drive KP4 expression, thus addressing the food safety issue and gene flow of transgenic plants. Another alternative would be to generate maize plants expressing KP4 in the chloroplasts of transgenic plants, which is a promising approach for transgene containment.

In summary, our study shows that transformation of maize with KP4 can generate constitutive antifungal activity against corn smut in the whole plant. In theory, the eventual combination of KP4 with the other Totivirus-encoded antifungal proteins, KP1 and KP6, should inhibit all strains of *Ustilago*.
In addition, KP4 also exhibits some antifungal activity against other maize fungi, Fusarium graminearum (Spelbrink et al., 2004), Fusarium verticilloides and Fusarium proliferatum (unpublished data). To this end, greenhouse and field trials are underway to determine the level of protection against these pathogenic fungi in transgenic maize expressing KP4. This work is aided by the fact that the atomic structure of KP4 is known, and the active site has been partially defined via mutagenesis (Gage et al., 2001). Therefore, this transgenic approach has great potential to improve maize resistance to a broad spectrum of fungal pathogens. As American farmers intend to plant 88.8 million acres of maize in 2010 [Prospective Plantings, Released March 31, 2010, by the National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA)], the need for maximizing maize production increases owing to demand for more food, feed and biofuels. Applying our novel control method could significantly reduce annual farm yield losses caused by corn smut and potentially other fungi.

### Experimental procedures

#### Construction of KP4 expression vector

As _U. maydis_ is a biotrophic pathogen, it was thought necessary to target the expression of KP4 to the apoplast in transgenic maize. To this end, the KP4 signal peptide sequence was replaced with the 27-amino acid secretory signal peptide sequence of a plant defensin, MsDef1 (Figure 1a). This signal peptide was previously shown to facilitate transport of proteins to the apoplast in transgenic plants (Gao et al., 2000). To obtain a high level of expression of KP4 in all organs of transgenic maize, the 407-bp full-length chimeric KP4 gene was chemically synthesized using the monocot-preferred codons and cloned as a _Nco I-Xba I_ fragment between the tobacco etch virus (TEV) mRNA leader sequence and CaMV 35S polyadenylation signal in pRTL2 (a gift from Dr Tom Clemente, University of Nebraska). The 5′TEV mRNA leader/KP4/cauliflower mosaic virus 35S polyA3′ cassette was removed from pRTL2 on a 667-bp _Xho I-Pst I_ fragment. This restriction fragment and 1.9-kb _Hind III-Sal I_ fragment containing the maize _Ubi1_ promoter were cloned into the unique _Hind III_ and _Pst I_ sites of the vector p2P212 (Ha- jdukieiwicz et al., 1994). Thus, the full-length chimeric KP4 coding sequence was placed under the control of the maize _Ubi1_ promoter/Intron/TEV mRNA leader signal sequence and terminated by the cauliflower mosaic virus 35S 3′ UTR (Figure 1b). The p2P212 vector containing this chimeric gene was transferred to _Agrobacterium tumefaciens_ strain EHA101 for transformation of maize.

#### Maize transformation

Maize inbred line H99 was transformed using a previously described protocol (Sidorov et al., 2005) except immature embryos were used as starting material in this case instead of mature seeds. The primary transgenic maize plants containing the KP4 gene (R0) were identified by PCR and out-crossed to non-transgenic public inbred B73 to generate BC0F1 seeds. Based on the Mendelian segregation of 1 : 1 indicative of a single insert, the BC0F1 plants were self-pollinated twice to generate BC2F2 lines. Homozygous lines were identified using the Invader Reagent kit (Third Wave Technologies, Madison, WI) and used subsequently for corn smut resistance evaluations. All plants were grown under the standard greenhouse growth conditions used for maize.

#### ELISA assays

KP4 expression levels were determined using the sandwich ELISA method. Antibodies were raised against purified KP4 using the services at Sigma Genosys (The Woodlands, TX). The antibodies were purified from antiserum using Protein G affinity chromatography, and this purified material was used for both primary and secondary antibodies. The enzyme-linked secondary antibodies were prepared by conjugating horseradish peroxidase (HRP) using the SureLINK HRP Conjugation kit (KPL Corporation, Gaithersburg, MD, catalogue #84-00-01). For the antigen capture, 100 µL of a 10 µg/mL solution of primary polyclonal antibodies purified from KP4 rabbit antiserum was added to each well and incubated for 2 h at room temperature or overnight at 4°C. The plate was washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and blocked with 5% milk prepared in 15 mM Na2CO3, NaHCO3, pH 9.6 for >2 h. Leaf extracts were made by homogenizing 50 mg of frozen tissue in 1 mL 50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% Tween-20, and 0.1% BSA, pH 7.4. Extracts were diluted 1 : 50 in the same buffer and loaded on the assay plate. A standard curve using purified KP4 was added in triplicate. A 1 : 1000 dilution of secondary antibody of KP4 polyclonal antibodies conjugated to HRP was used (SureLINK HRP Conjugation kit 84-00-01). The HRP substrate, 3,3′,5,5′-tetramethylbenzidine, was added, 1 N HCl used to stop the reaction and the plate was read on a spectrophotometer (SpectraMax M2; Molecular Devices Inc., Sunnyvale, CA) at OD450. The ELISA was validated to ensure accuracy of expression level quantitation. Extraction was optimized by homogenizing leaf tissue by varying the tissue : buffer ratios and by testing different buffers and additives. Matrix effects were accounted for by adding purified KP4 to control tissue extract (non-transformed) and using these samples for the standard curve. A spike and recovery experiment was performed where unhomogenized control tissue and buffer were spiked with purified protein, subjected to normal extraction rigours and analysed for protein recovery.

### KP4 antifungal activity assay

Plant material was prepared for antifungal activity by grinding 50 mg of fresh leaf material in 1 mL of PBS. As a positive control, a 20 µg/mL solution of purified KP4 was used. For the agar-based killing zone assay, KP4-sensitive P2 cells were grown overnight in complete _U. maydis_ media. P2 cells (~1 mL culture per 100 mL agar) were added to warm complete _U. maydis_ media containing 2% bacto agar and poured into 100 × 20 mm culture dishes. Once the agar solidified, wells were cut into the agar, and 20 µL of the test solutions was added to each well. The plates were then incubated at 30 °C for <36 h. KP4 activity presents a clear zone of growth inhibition around the point of application.

As the well-documented defensin export signal (Terras et al., 1995) was placed at the N-terminus of KP4, it was assumed that this antifungal protein was targeted to the apoplast. To test for this, germinating seeds were placed onto the P2 containing agar plates as described earlier to detect secretion of active KP4. For these experiments, maize kernels from...
representative transgenic lines 851, 1040 and wild-type (BC,F4) were surface sterilized with 70% (v/v) ethanol for 1 min, followed by washing in a sixfold diluted commercial hypochlorite solution containing 0.1% (w/v) sodium dodecyl sulfate (SDS) for 10 min, and washed with autoclaved distilled water five times for 5 min each. These kernels were dried before starting the experiment. maize kernels were soaked in autoclaved distilled water overnight at RT. The next day, some of the kernels from each genotype were incised at the top using a sterile blade (keeping the embryo intact) and other set was plated without incision. While the incision likely damaged some of the tissue, it was hoped that it might improve the contact between the seeds and the agar. The plates were incubated in the dark at RT for 2 days before photographing.

Plant infections and pathogenicity assays
Ustilago maydis KP4 sensitive wild-type strains 1/2 (mating type a1/b1) and 2/9 (mating type a2/b2, near isogenic to 1/2) were grown in potato dextrose broth to an OD600 of 1.0 (2 days before photographing. and the agar. The plates were incubated in the dark at RT for 10 min, and washed with autoclaved distilled water five times with 1 x 10^6 cells/mL. These strains were mixed in equal amounts immediately prior to inoculation. Maize plants were established in a fine grade composted pine bark mixed with vermiculite in a 3:1 ratio in 9-cm plastic pots under 16 h light at 28°C and 8 h dark at 20°C in a Conviron growth chamber. Using a hypodermic needle and syringe, 3.0 mL of the cell suspension was injected into the stems of 7-day-old maize plants. Initial disease symptoms were observed 7–10 days postinoculation (dpi). Disease symptoms were recorded at 7, 10, 14 and 21 dpi using the method of Gold et al., (Gold et al., 1997). maize plants were grown in 24-cm plastic pots and grown under the same conditions as mentioned earlier for 80–90 days. Upon the appearance of silks, maize ears were inoculated with 3.0 mL of the fungal cell suspension. Ear symptoms were observed 14 dpi. All data were submitted to analysis of variance (ANOVA) and Duncan’s multiple range test via Dsaastat statistical software, version 1.0192 (Onofri, 2006).

RNA isolation and quantitative RT-PCR analysis
Leaf tissue of 3-week-old homozygous transgenic maize lines (746, 1040, 851 and 947) along with wild-type control BC,F4 was used for isolation of total RNA using Trizol reagent (Invitrogen, Carlsbad, CA). RNA quantification was done using NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA), and one microgram equivalent of total RNA was then reverse transcribed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen) following manufacturer’s instructions. The resulting cDNA was diluted five times and amplified using Promega GoTaq® qPCR master mix (Promega Corporation, Madison, WI) on the AB StepOne Plus Real time PCR system (Applied Biosystems, Carlsbad, CA). Expression of maize cytosolic glyceraldehyde-3-phosphate dehydrogenase 2 (GAPC2) gene was used as an endogenous control for normalization of the mRNA target. Primer pair sequences used for amplifying GAPC2 were forward, TGGAGATCTCTCAGAAGG; reverse, AGTGTGTGCTCAGCATTGG and for KP4 forward, TGCTCTGCTTCTCCTGTCTGT; reverse, TACGGACGTGTCCATGTCA. Real-time run protocol composed of initial denaturation and polymerase activation (95°C for 5 min), 40 cycles of amplification and quantification (95°C for 15 s, 60°C for 10 s, 72°C for 25 s with single fluorescence measurement), and melting curve programme (60–95°C with fluorescence read every 0.3°C). The threshold cycle (CT) was calculated by the StepOne Plus software to indicate significant fluorescence signals rising above background during the early cycle of the exponential amplification phase of the PCR amplification process. The resulting data were analysed using relative quantitation based on the ΔΔCt (delta-delta-CT) method.

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