Engineering Fungal Resistance in Crop Plants using Antifungal Proteins from Viruses

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Impact of corn smut on crop yields

Smut fungi are important agricultural pathogens that are responsible for significant losses in crop yield. Corn smut, caused by a biotrophic fungus *Ustilago maydis*, is economically important in all countries where maize is grown. Sweet maize in particular is more susceptible to this disease, where losses can be as high as 20%. Yield loss due to corn smut is generally kept below 2% in current partially-resistant field varieties of maize. However, since maize is the most economically important crop in the USA—generating $48.7 billion in 2009, with approximately 35 million hectares planted—even a 2% loss is nearly $1.0 billion annually. In addition to domestic consumption, the US was the largest maize exporter in the world in 2009. Since maize plays a major role in current biofuel production, the importance of maize in US agriculture is only expected to increase.

Hot and dry weather conditions favor *U. maydis* attack on 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. 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. 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* and no single gene that confers resistance. We explored an alternative approach by introducing a component of a naturally occurring antifungal system into transgenic maize.

Antifungal proteins made by the Totiviruses

Our recent study*1* focused on an "interstrain inhibition" system found in *U. maydis*[2]. Interstrain inhibition in *U. maydis* is due to antifungal proteins (killer toxins) produced by double-stranded RNA Totiviruses that persistently infect particular strains of *U. maydis*. These proteins are secreted by the fungal host and kill other competing strains of corn smut that are not infected by that particular virus. Specifically, we focused on the secreted KP4 protein. KP4 is a single polypeptide of 105 amino acids produced by the UMV4 virus that infects the P4 strain of *U. maydis*. KP4 is the only *U. maydis* toxin not processed by Kex2p, and it has no sequence similarity to other toxins[3]. Although most yeast toxins are acidic, and the *Ustilago* antifungal proteins KP6 and KP1 have neutral pI values, KP4 is extremely basic, with a pI ~9.0. KP4 is an α/β sandwich protein with a relatively compact structure[4]. Due to a tenuous structural similarity to the scorpion toxin AaHII from *Androctonus australis*, it was suggested[4] and then subsequently shown[5] that KP4 blocks calcium channels in fungal cells[4-6]. This is a reasonable mode of action since calcium and calcium-dependent signaling is essential for normal growth as well as pathogenicity of various fungal plant pathogens. We have now demonstrated 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*[1].

We generated several genetically engineered lines of maize that produce extracellular KP4. KP4 was expressed to high levels using the monocot codon-optimized mature KP4 protein coding sequence and a signal peptide sequence of a plant defensin gene to secrete the protein to the extracellular space. The resistance of ten independent maize lines expressing KP4 was compared to the parental maize inbred line H99/B73 (BC1F4). Pathogenicity assays showed strong resistance to *U. maydis* infection that was directly correlated with expression levels of the protein. The more highly expressing lines showed robust resistance to this fungus, with no observed galls and little chlorosis and/or anthocyanin on the leaves. At 21 dpi of *U. maydis* challenge, chlorosis and/or anthocyanin symptoms were reduced or completely absent in KP4 genetically engineered maize plants, while the wild-type maize line (BC1F4) exhibited plant death. Surviving KP4 transgenic maize plants were transplanted into large pots and in the next three months developed normal ears and tassels similar in appearance to wild-type plants. This suggests that transgenic plants resisted *U. maydis* infection, not simply delayed infection, and 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 again inoculated with the fungus directly into maize ears. Two weeks later, plant tumors or galls were observed in the ears to levels inversely proportional to KP4 expression. This suggests that transgenic mature plants continued KP4 production throughout their life cycle, affording resistance to *U. maydis* infection. Interestingly, partially resistant KP4 lines that had intermediate expression of KP4 did not develop ear galls, suggesting that KP4 production in this tissue was sufficient to protect transgenic plants from *U. maydis* infection.

With regard to commercial application of this technology, there is growing evidence that these proteins are safe for human and animal consumption. Since the KP4 protein blocks calcium channels, 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 less than 60s in artificial stomach fluid, and its amino acid sequence is not similar to any known allergens[7]. 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. KP4 does not affect fungal soil communities, wheat-infesting insects such as aphids, and "standard" soil arthropod *Folsomia candida*[8]. It can therefore be assumed that KP4 transgenic maize will not have any deleterious effects on humans, plants, insects, and bacterial and fungal soil communities. However, as additional safety measures, we are considering several gene containment strategies. For example, since maize silk is the major route of *U. maydis* infection, a silk-specific promoter such as SLG may be useful to drive KP4 expression and limit expression to the most sensitive tissue.

In summary, our study shows that transformation of maize with KP4 can generate constitutive antifungal activity against corn smut in the whole plant. It is estimated that ~1% of *U. maydis* found in nature secrete these killer toxin. None of the three known killer strains of *U. maydis* (P1, P4, and P6) 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 but a fraction of a percent of corn smut strains. In addition, KP4 also exhibits some antifungal activity against other maize fungi, *Fusarium graminearum*[9], *F. verticillioides*, and *F. 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[5]. 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 due to demand for more food, feed, and biofuels. Applying our novel control method could significantly reduce annual farm yield losses due to corn smut and potentially other fungi.

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