A Potential New Method to Control Aflatoxin and Other Mycotoxins in Corn

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Transgenic maize development. The main objective of this study, which began in mid-May 2009, is to express an α-amylase inhibitor from hyacinth bean in corn to reduce both infection and aflatoxin accumulation by *Aspergillus flavus*. The α-amylase enzyme of *A. flavus* is necessary for the fungus to digest kernel starch and produce aflatoxins. Hyacinth bean contains α-amylase inhibitors that block the α-amylase activity of *A. flavus*, and inhibit spore germination and fungal growth as well. A secondary objective of this study was to confirm the presence of an additional amylase inhibitor in Western Soapberry and begin identification of this protein.

The development of transgenic lines. The hyacinth bean inhibitor gene B01 was inserted into the pCAMBIA vector 1300S between the CaMV 35S promoter and NOS terminator and its integrity confirmed by DNA sequence analysis. This construct was inserted into corn line HiII using biolistic transformation along with plasmid pTF101.1 containing the bar gene for bialaphos resistance. The callus events (43) were regenerated into plantlets under bialaphos selection and thirty of these were regenerated to plants. Of these regenerated lines, twenty lines were confirmed to have the B01 transgene (Table 1). These lines were crossed with the standard pollen donor line B73. These transgenic lines have been tested for transgene expression levels using real-time RT-PCR. Over 20 T0 lines of one AILP family member were regenerated and transferred to soil and grown in the greenhouse to maturity so as to produce T1 seed.

Table 1. Actions in the development of transgenic maize lines expressing hyacinth bean α-amylase inhibitor.

Action	Number
Bialaphos-resistant callus lines	**43**
Callus lines regenerated to T0 plants	**30**
Callus lines with transgene confirmed by PCR	**20**
Total Transformed T0 plants	**74**
Seeds of transgenic T0 maternal parent x B73 pollen	**1101**
Seeds of B73 maternal parent x pollen from transgenic line	**512**
Transgenic lines screened by Q-RT-PCR	**20**
Transgenic lines screened by Western Blot analysis	**11**

Antibody production and western blot analysis. In order to measure the levels of hyacinth bean protein produced in these transgenic corn lines, an antibody was developed. First the inhibitor was produced in bacteria, purified, and then sent to Cocalico Biologics Inc. for the immunization of a rabbit. This antibody, specific for the hyacinth bean inhibitor, was used to analyze the expression levels of the inhibitor in the second generation transgenic lines. A representative western blot displaying varying expression levels of the hyacinth bean inhibitor in T1 lines is shown in **Figure 1**. The darker the band, the greater the level of protein in the transgenic line, while B73, representing the untransformed control line, has no band. The same antibody has been used in numerous western blots (data not shown) to monitor the expression level of the transgene in all subsequent generations that have been carried forward.

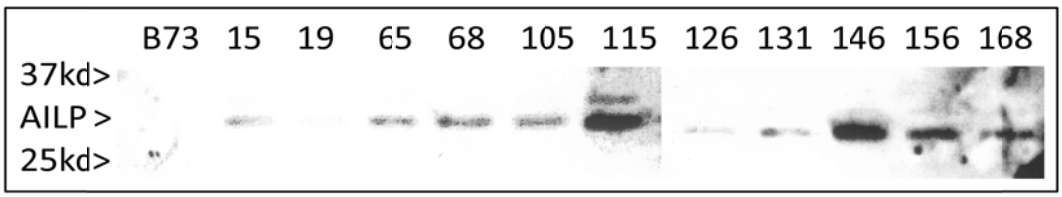

Figure 1. Western blot of transgenic maize lines expressing the 30 kd AILP B01. B73 represents the parental untransformed control lines, 15 through 168 are transformed lines. Line 115 and 146 have the greatest expression of B01 α-amylase inhibitor.

Analysis of antifungal activity. After lines with relatively high expression of the hyacinth bean inhibitor were identified, the biological activity of these transgenic lines needed to be analyzed. In order to test the level of antifungal activity in these lines, a spore germination assay was developed. Total proteins were extracted from the leaves of these lines and incubated with starch medium and 2000 spores of *A. flavus*. Data from two transgenic lines with α-amylase inhibitor activity, confirmed by western blot analysis (data not shown), showed a reduction in growth in two individual plants compared to segregated controls that lost the transgene (**Figure 2**). These results suggest that the α-amylase inhibitor is expressed at concentrations sufficient to inhibit the growth of *A. flavus*.

Figure 2. Bioassay displaying spore tube length between transgenic corn lines with the α-amylase inhibitor (+) and without (-) after 24 hour incubation. Two thousand spores were incubated in 20 ul of starch medium and 20 ul of crude protein from leaf tissue that was normalized for protein concentration with dH_2O. Plant number is in parentheses.

α-Amylase Inhibitor from Western Soapberry. The first indication that an α-amylase inhibitor may exist in Western Soapberry came from a tube of dried protein, extracted years earlier at Purdue University. To confirm the presence of an inhibitor in this desert tree, total proteins were extracted from the seeds. A commercial preparation of the α-amylase from Apsergillus required additional purification before use in subsequent assays. The purified α-amylase and the extracted Soapberry proteins were premixed and then incubated with the the enzyme substrate. Results from this initial experiment suggest that an inhibitor of α-amylase from Western Soapberry may exist (Figure 3). When the volume of Soapberry proteins was doubled from 16 to 32 ul, the inhibition of α-amylase was also roughly doubled for all three samples tested (Figure 3). Further testing of these proteins is ongoing.

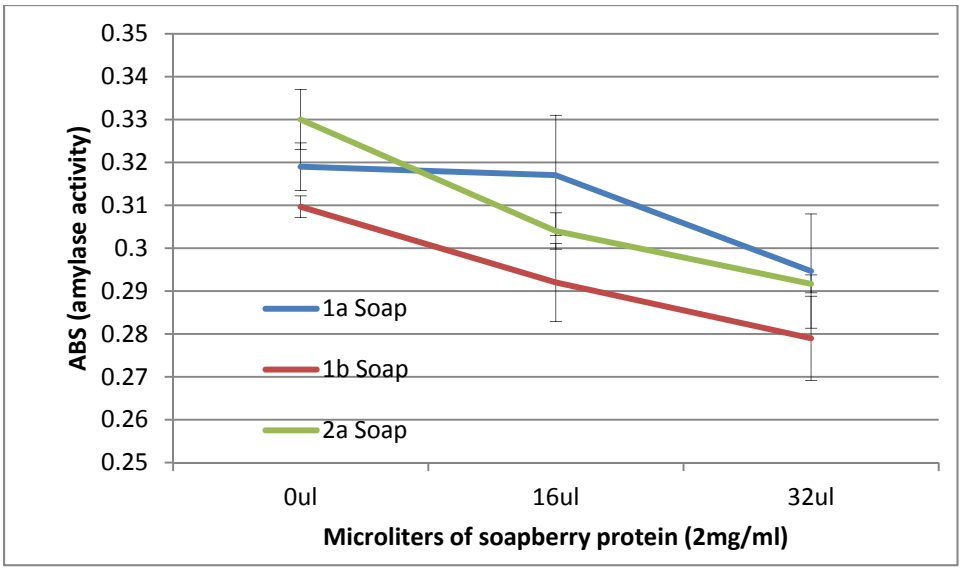

Figure 3. Activity of α-amylase with 0, 16 and 32 microliters of total protein from Western Soapberry seeds. Samples 1a and 1b are extracts from the same accession of Western Soapberry but stored under different conditions, while sample 2a is a separate accession of Western Soapberry.

Leveraging. The PI is currently working to recruit to international students that would come with their own support 20K per student per year. The PI has established collaborations with USDA scientist specializing in transgenic approaches to aflatoxin control in cotton using antifungal peptides and is following up on initial discussions for support. The PI has and will continue to apply for competitive funding for this area of research.

Currently. The PI is developing inbred lines homozygous for the hyacinth bean α-amylase inhibitor in the greenhouse so that these lines can be bulked and field tested. The PI is also pursuing experiments to confirm and isolate α-amylase inhibitors from Western Soapberry. The PI is also pursuing funding for peptide technology shown to reduce aflatoxins in transgenic cotton.