GENETIC ENGINEERING AND BREEDING OF WALNUTS FOR CONTROL OF AFLATOXIN

Ryann Muir, Abhaya Dandekar, Gale McGranahan, Patrick Vail, Charles Leslie, Sandie Uratsu and Steven Tebbets

ABSTRACT

Our strategy for reducing aflatoxin contamination has two major goals; (1) develop insect resistance to reduce insect damage and (2) identify genes that reduce \emph{A. flavus} toxin production. For developing insect resistant walnuts, we have focused on the \emph{cry}IAc gene from \emph{Bacillus thuringiensis}. We have analyzed field grown transgenic plant material and whole nuts in bioassays and have analyzed larval development relative to the control untransformed lines to investigate expression levels. Our second goal is based upon the observation that walnuts have less aflatoxin contamination as compared to other nut crops. To investigate this phenomenon, we have been investigating walnut tissues (embryo and seed coat) as potential sources of resistance genes. Our work has emphasized seed coat (pellicle) tissue. Studies conducted at the USDA facility in Albany have shown that seed coat extracts from several walnut varieties, and especially from the cultivar Tulare, contain a factor that strongly inhibits aflatoxin production by \emph{A. flavus}. Our results have suggested that this factor is gallic acid (GA), a key component of hydrolysable tannins (HTs), which are produced by all flowering plants. Estimation of free GA by staining with rhodanine and precipitation with boric acid indicates that GA is the component of HTs responsible for the observed suppression of aflatoxin synthesis in Tulare pellicle tissue. We have successfully cloned the gene responsible for GA synthesis in walnuts and other organisms. Functional complementation in bacteria has confirmed the role of the gene in metabolism/GA production. Biochemical analyses have been performed to understand and control the regulation of GA production and accumulation in walnuts and other plant species.

BACKGROUND

Mycotoxins are noxious compounds produced by a variety of fungal species. One such mycotoxin, aflatoxin, has been linked to contaminated food (corn, rice, peanuts, and tree nuts) and animal feed (Woloshuck 1998, Smela 2001). Aflatoxins are produced by specific strains of the filamentous fungus \emph{Aspergillus} (aflatoxin = \emph{Aspergillus flavus} toxin) and encompass a group of structurally related compounds (Trail 1995). One member in particular, aflatoxin B1 (AFB1), is the most toxic and the most prevalent in nature (Woloshuck 1998). In fact, up to twenty five percent of the global food supply is contaminated, annually, by AFB1 (Trail 1995, Moreno 1999). In the United States, the Food and Drug Administration (FDA) regulates the levels of aflatoxin in foods such that crops with more than 20ppb total aflatoxins cannot be imported/exported and sold (Gourama 1995, Trail 1995).

The chemical toxicity of aflatoxins induces specific point mutations in DNA. Typically, aflatoxins, like AFB1, are metabolized in the liver. There, they are converted into epoxides (AFB-8,9-epoxide) which subsequently become covalently linked to guanine bases in the liver cell DNA (Eaton 1994, Wang 2000, Smela 2001). Addition of the epoxide (usually at the N7 position) stimulates depurination of the guanine base which is then misinterpreted during subsequent DNA replication (Smela 2001). Thus, aflatoxin induces GC? TA transversions.
within the DNA. One such point mutation has been shown to readily form within the liver p53 tumor suppressor gene (G249T) and in fact, this particular transversion has been directly correlated with the occurrence of hepatocellular carcinoma (i.e. liver cancer) (Hussain 1994, Moreno 1999, Tiemersma 2001).

In recent years, strategies have been proposed to eliminate aflatoxins from food and feed. In the field, application of fungicide has prevented fungal infection and subsequently, mycotoxin contamination. Aflatoxin detection via chromatography and \textit{uv} luminescence coupled with post-harvest removal techniques have also been utilized (Kathuria 1993). These current elimination strategies, however, have been costly, ineffective, and/or environmentally unsound (Trail 1995). Thus, there is a need for simpler, less expensive ways of limiting or preventing aflatoxin contamination.

**OBJECTIVES**

1. Bioassay of nuts from walnut trees engineered for resistance to insect pests.
2. Identification of genes in Tulare responsible for the natural resistance to \textit{A. flavus} growth and toxin production.

**PROCEDURES**

Bioassay of nuts from walnut trees engineered for resistance to insect pests: Nuts harvested from the transgenic trees and kernel pieces derived from these nuts were introduced into insect feeding cups and exposed to 1 or 2 neonate insect larvae. 15 to 30 insects were evaluated per transgenic line. Mortality was evaluated after 7 to 14 days of insect feeding. Kernels from untransformed nuts of the same cultivar were used as a comparison.

Identification of genes in Tulare responsible for the natural resistance to \textit{A. flavus} growth and toxin production: Several recent experiments point to the chemical nature of the component in the Tulare pellicle responsible for the inhibition of aflatoxin synthesis. We observed that extraction of Tulare pellicles using PVP and borate resulted in a marked precipitate not observed in the seed coat extracts from a control (i.e. \textit{Juglans regalia} cv. Chandler). This suggested the presence of tri-hydroxyl compounds typically found in HTs. Our collaborators, natural chemists at the USDA-Albany, performed GC-MS analysis on the pellicle phenolic compounds of these two walnut cultivars. Their analysis suggested the presence of the compound methylgallate or another closely related molecule. We hypothesized that gallic acid, the key component of HTs, is responsible for the aflatoxin resistance phenotype. After staining Tulare and Chandler seed coats with rhodanine to visualize free gallic acid, we determined that Tulare exhibited a four-fold increase in gallic acid accumulation relative to the Chandler control. In addition, gallic acid was able to reproduce the aflatoxin resistance phenotype in feeding studies with \textit{A. flavus} (Mahoney, 2004).
RESULTS AND CONCLUSIONS

In the current field trial we are testing transgenic walnut trees derived from two vectors. We have been investigating the tissue level expression of the cry1Ac gene regulated by either the Ubi3 promoter (pDU96.3113) or the CaMV35S promoter (pDU92.710). Results of bioassays obtained from field grown nuts harvested in 2002 and 2003 suggest that nuts obtained from either Ubi3 or 35S lines exhibited significantly high levels of mortality with codling moth larvae. In all cases, larval mortality was higher with hull tissues relative to meat (embryo) tissues, presumably due to the presence of inhibitory compounds.

One novel observation regarding fungal infection has been the occurrence of a unique resistance mechanism in a specific walnut cultivar (*Juglans regia* cv. Tulare). Tulare seed coat tissue is able to inhibit the production of a toxic fungal compound, aflatoxin, produced by the fungus *Aspergillus*. Interestingly, the Tulare seed coat has minimal effect on fungal growth and reproduction. Results have indicated that a secondary metabolite, gallic acid, is directly responsible for the resistance to aflatoxin contamination.

Gallic acid is a primary structural component of hydrolysable tannins (HTs), one of the two major classes of tannins in plants. HTs are composed of gallic acid groups covalently linked to a central sugar moiety (most commonly glucose) (Bhat 1998, Niemetz 1998). Plants synthesize large and complex HTs; up to twelve gallic acid units have been observed within a single hydrolysable tannin molecule (Niemetz 1998). The structural variation of HTs within and between plant species is achieved by oxidative coupling of gallic acid units and/or oxidation of their aromatic rings (Grunhofer 2000, Harvey 2001).

HTs have the ability to bind to and precipitate surrounding proteins. It is this very characteristic that distinguishes tannins from other types (i.e. classes) of phenolics (Bhat 1998, Salminen 2001). During protein precipitation, hydrogen bonds are formed between the tannin hydroxyl, and protein amino groups (Donovan 2001). The steric environment and number of phenolic moieties per tannin molecule (i.e., gallic acid groups per HT) affect the precipitating ability of the compound. An increased number of hydroxyl groups, and thus increased hydrogen bonding ability, has been correlated with increased protein precipitation (Donovan 2001).

Gallic acid is synthesized from an early intermediate (i.e. 5-dehydroshikimate or DHS) of the shikimate pathway. We have identified two potential enzymes responsible for controlling the levels of DHS conversion to GA. One enzyme, DAHP synthase, regulates carbon flow towards the synthesis of DHS and therefore, towards the synthesis of GA. We successfully cloned DAHP synthase from Tulare walnuts and detected an inverse relationship between transcript levels and GA accumulation. We have therefore proposed that levels of carbon flow towards DHS do not directly affect levels of GA biosynthesis. One possible explanation of this observation is that sufficient levels of DHS are constitutively present in plants; endogenous DHS concentration does not appear to limit GA production.

A second enzyme, DSDG, is responsible for the direct conversion of DHS to GA. We have recently cloned DSDG from Tulare walnuts and have been investigating its’ role in GA biosynthesis. We tested several plant species for DSDG activity and found this enzyme to exhibit considerable structural variation between species.
DSDG from Tulare synthesizes and accumulates elevated levels of GA in planta relative to other species tested. To manipulate and increase the levels of GA in plants, we transformed somatic embryos from *J. regia* cv. Chandler with the Tulare gene. We are currently testing our transgenic lines for *in vitro* enzyme activity and GA production. To date, we have identified four individual lines producing elevated levels of GA relative to the non-transformed control. These results indicate that levels of GA in walnuts can be controlled through genetic engineering and/or breeding. Increasing the levels of GA in seed coats could potentially eliminate aflatoxin production and its' contamination by *A. flavus*.

**REFERENCES**


