Expression of Insectical Protease Inhibitors in Arizona Cotton

John C. Thomas and Hans J. Bohnert

Abstract

Insect damage impacts tremendously on the value of the Arizona cotton crop. As traditional pesticides become increasingly less useful, due to insect resistance and regulatory problems, new methods for insect control are needed. For these reasons, we engineered genes encoding protease inhibitors (PIs) from Manduca sexta (tobacco hornworm), for expression in cotton, with the hope that these inhibitors would have insecticidal activity. Transgenic plants containing PIs have been generated: 22 fertile lines of the duplicated 35S promoter anti-elastase, 4 fertile lines of the anti-chymotrypsin and 5 fertile lines of the anti-trypsin. Over 3,000 T-1 seeds have been collected and T-2 generation seeds are in production. Many crosses have been made into Delta Pine 16, 90 and 5415 respectively. No significant effect of the PIs on boll number or seed yield was observed. Insect tests have been conducted and the results indicate that plants expressing the protease inhibitors (PI's) have decreased emergence of whiteflies compared to control plants. We believe this research is a significant step towards a bio-pesticide producing Arizona cotton variety.

Introduction

In higher plants the natural repertoire of weapons available to combat insect attack is believed to include protease inhibitors present in particular plant tissues for the purpose of protection (Read and Haas, 1938; Richardson, 1977). Under certain conditions, the addition of PIs to artificial diets of insects indicated insecticidal activity (Broadway et al, 1986a, 1986b). Transfer and expression of protease inhibitors has been shown to decrease larval weight gain by 15% (Johnson et al., 1989) and insect survival by 50% (Hilder et al., 1989). However, the mechanism of protease inhibitor action as an insecticides is unclear. Generally it has been assumed that the PIs arrest trypsin, chymotrypsin, and elastase activity of insect digestion, thereby lowering the availability of essential amino acids. Recently, evidence suggests that the protease inhibitors interrupt digestive enzymes, signaling the insect gut to produce more trypsin, elastase, and chymotrypsin. As protease synthesis is strained, decreased levels of essential and rare amino acids occur, lowering their availability to the developing insect (Broadway and Duffy, 1986b; Burgess et al., 1991).

Materials and Methods

An anti-elastase gene from *Manduca sexta* was isolated by Mike Kanost (Department of Biochemistry, Univ. of AZ). The cDNA gene product was found in the circulatory system of *Manduca* and thought to protect against elastase activity leaking from the digestive tract. The cDNA had an open reading frame coding for 392-residue polypeptide of 43,500 Mr, including a 5' leader sequence which could target the protein into the fat body of the insect. This protein inhibits elastase and chymotrypsin. The cDNA sequence was modified to create an anti-chymotrypsin and anti-trypsin enzyme. All three genes were inserted into plant gene expression cassettes using the 35S promoter from cauliflower mosaic virus and a variety of poly A adding terminators. DNA constructions were mated into *Agrobacterium tumefaciens* and introduced into cotton and tobacco.

A second gene, tryptophan decarboxylase (TdC) was obtained from Dr. W. Kurtz, (RC-CRC Canada) and introduced into plants. TdC promotes tryptamine production, however it is unclear how tryptophan decarboxylase acts as a insecticide. The enzymatic product tryptamine, or a immediate product from tryptamine may be toxic to insects including white flies. Secondly, the enzyme may covert excess tryptophan to tryptamine, limiting tryptophan availability to the developing insect. Previous study has determined that tryptamine accumulates to high concentrations in transgenic tobacco (over 1000 ug/g fwt), as compared to non-transformed controls (Songstad et al., 1990).

Plant Transformation/Regeneration

Introduction of engineered genes into dicotyledonous plants makes use of *Agrobacterium tumefaciens*-mediated gene transfer. Generally, disarmed versions of the Ti plasmid such as Bin 19 and pAN 70 (An et al., 1985; Bevan, 1984) contain restriction sites for insertion of genes to be expressed in plants. These vectors also contain a kanamycin antibiotic resistance marker (NPT II), under the control of the nopaline synthase promoter. After triparental mating and confirmation of the correct disarmed plasmid in *Agrobacterium*, leaf, stem, and/or cotyledon co-cultivation in the *Agrobacterium* permits the transfer of the engineered gene(s) on the Ti plasmid into the plant cell (Horsch et al., 1985). Upon explant sub-culture to medium obtaining kanamycin, only cells producing NPT II will grow. We overcame a major obstacle by developing a reproducible procedure to transfer DNA (via *Agrobacterium*) into cotton tissues, and regenerate the resultant progeny, using Coker 312.

Transfer of the three protease inhibitors was affected in cotton and tobacco. TdC transformation of tobacco was successful, however cotton plants were not recovered. One reason may be that TdC may influence indole acetic acid levels (an important plant growth regulator). Tobacco regenerates 5-10 times faster than cotton providing a model from which to conduct insect tests. Both cotton and tobacco tissues were analyzed for PI expression using SDS-polyacrylamide gel electrophoresis and Western blot analysis with rabbit antibodies directed against the anti-elastase from *Manduca*. Results indicated a protein was produced in both cotton and tobacco with the correct MW of the anti-trypsin, anti-chymotrypsin and anti-elastase. Furthermore the protein(s) were not expressed in a uniform fashion, but rather varied depending on the tissue analyzed.

Crossing of the PI expressing Coker 312 plants was conducted using Delta Pine 16, 90 and 5415 as females. No deleterious effects were associated with the introduction of the PI genes into Arizona-grown varieties. Initial analysis indicated that the PI genes can pass into Arizona-grown cotton according to Mendelian laws. Crosses into *G. barbadense* have been limited because normal chromosomal breakdown results from such an interspecies (*G. hirsutum* X *G. barbadense*) cross, resulting in large variation from plant to plant, sterility problems, and complications that were avoided in lieu of the Delta Pine varieties.

Insect Tests

Insect tests on PI expressing plants has taken time. Any positive result in an insect test indicates that some protection can be afforded by the use of the PI, provided side-by-side comparisons are conducted. We anticipate different pests will be affected by the PIs differently, however a protective effect due to the expression of one transgene against a particular insect is expected to be protective against that same insect, when the gene is expressed in a different plant species.

Insect tests against *Manduca sexta* (tobacco horn worm) and *Bemisia tabaci* (whitefly) have been completed in transgenic tobacco. Cotton tests are continuing, with results that parallel the tobacco findings. The method of insect damage assessment varies depending on the pest being tested. Tobacco horn worm predation was only

partially effected by the presence of anti-trypsin, anti-chymotrypsin, and anti-elastase (data not shown).

Tests with whiteflies using clip-cages were more promising. Much of this study was aided by Dr. Judith K. Brown, (Plant Sciences, Univ. of AZ). Ten whitefly pairs were placed on transgenic cotton, tobacco, or tomato and allowed to incubate for 2-3 days, presumably allowing egg deposition. Adults were removed and the eggs allowed to develop. Significantly decreased whitefly emergence was routinely observed on some anti-trypsin and anti-chymotrypsin accumulating tobacco (Fig 1). These plants produced high levels of protein as well. Other plants, with greater emergence of whiteflies contained little PI expression, or seemed to accumulate PIs in only some leaves. We have obtained evidence that the growth conditions of the plant often dictate the levels of PI expression and subsequent insect emergence results. TdC containing plants also showed decreased emergence of whiteflies on repeated occasions (see Figure 1). While unknown, we speculate that tryptamine is entering the phloem of these plants, where the whiteflies feed. Either as an anti-feedant or as a toxin, protease inhibitors and tryptamine may discourage insect feeding.

Conclusion

We have engineered and expressed *Manduca sexta* PIs and TdC in tobacco and cotton. The expression of these genes has little effect on *Manduca sexta*, however whitefly (*Bemisia tabaci*) emergence is decreased as much as 90% on plants expressing these transgenes. Crosses into DP 5415 are successful and introgression into popular cotton seed varieties will distribute this technology to the Arizona Cotton Growers.

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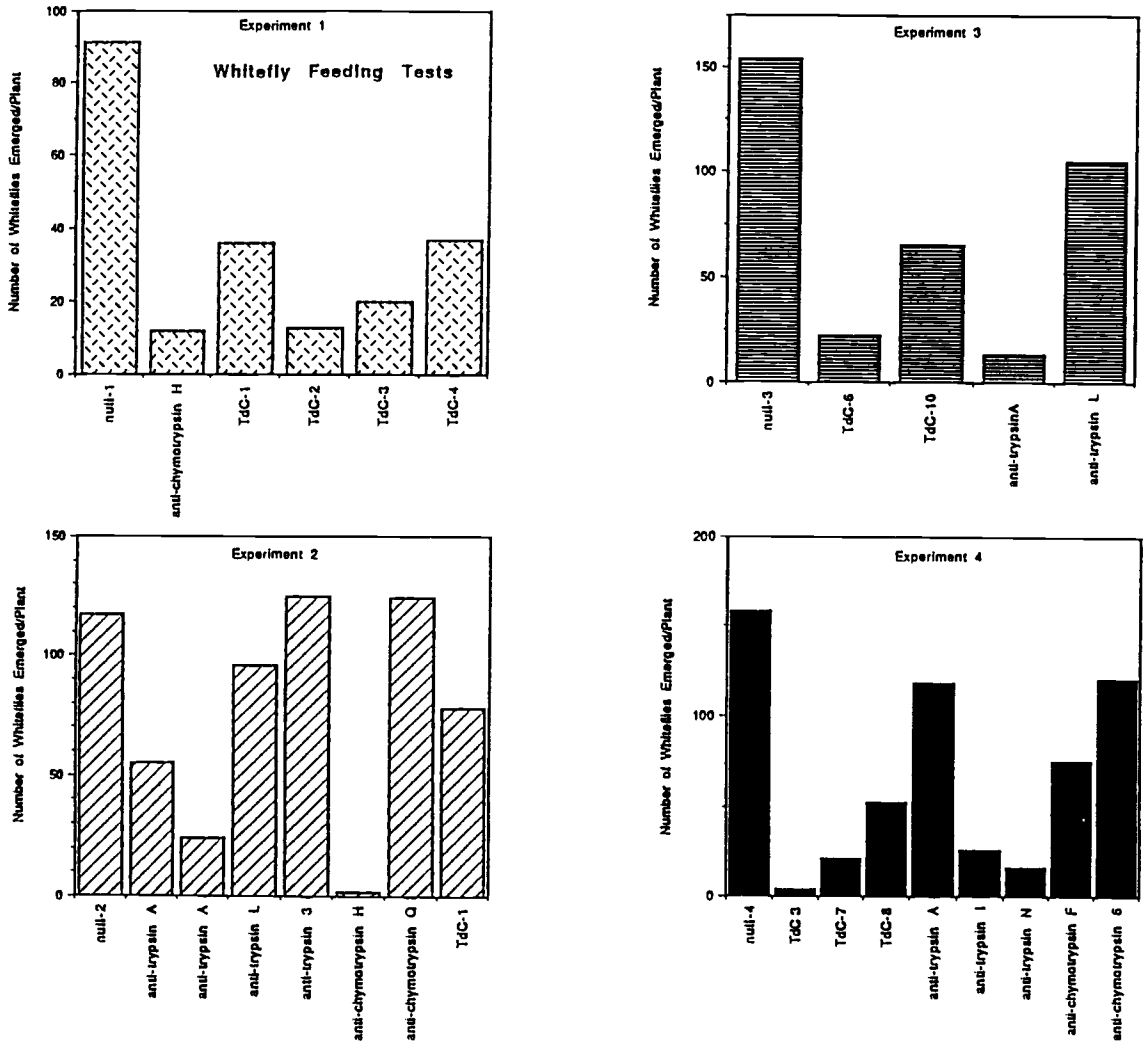

Figure 1: Feeding of Whiteflies on transgenic tobacco expressing the tryptophan decarboxylase (TdC) or protease inhibitors (anti-trypsin or anti-chymotrypsin). Letters or numbers indicate individual transgenic plants. Results are from 4 independent experiments. Data represents the number of emerged pupal cases after a 30 d incubation.