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Approaches to insect resistance using transgenic plants

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SUMMARY

Crops resistant to insect attack offer a different strategy of pest control to indiscriminate pesticide usage, which has undesirable effects on both the environment and humans. Transgenic plant technology can be a useful tool in producing resistant crops, by introducing entirely novel resistance genes into a plant species. Although most work in this area has focused on the use of genes encoding insecticidal *Bacillus thuringiensis* δ-endotoxins in transgenic plants, an alternative approach is to use plant genes which encode proteins with insecticidal properties.

Protease inhibitors are involved in endogenous plant defence against insects. Over-expression of several inhibitors from constitutive promoters has been shown to afford protection in transgenic tobacco plants against attack by lepidopteran larvae. However, the degree of protection is not sufficiently high, and shows species- and inhibitor-specific effects. By assaying the interactions of protease inhibitors with insect gut proteases *in vitro*, the most effective inhibitor can be selected for a particular insect species. Data from bioassays of insects using artificial diets, and with transgenic plants, suggest that the *in vitro* assay of relative inhibitor effectiveness is consistent with the effects of different inhibitors on insect development and survival *in vivo*. Development of this techniology is considered.

A different approach must be taken with sucking insect pests, as they do not rely on proteolysis for nutrition, and as *Bt* toxins effective against homopterans have not been reported to date. Bioassay in artificial diet was used to identify plant proteins with insecticidal effects on the rice brown planthopper (a model homopteran). The lectin from snowdrop (GNA) was found to be the most effective of the proteins tested. GNA was shown to be present in the phloem sap of a transgenic tobacco plant transformed with a chimeric gene construct, containing the rice sucrose synthase-1 gene promoter and the GNA coding sequence, by immunoassay of honeydew produced by aphids feeding on it. GNA is also insecticidal to the aphid *Myzus persicae*, which will feed on tobacco, and thus a bioassay of transgenic tobacco, to 'prove' the technology, can be carried out.

The effects of combining different resistance genes in the same transgenic plant to improve the effectiveness of protection are discussed, and exemplified.

1. INTRODUCTION

Crop protection plays a vital and integral role in modern-day agricultural production. The ever-increasing demands on yield and the projected shortfall in production relative to demand has led to an intensification of farming practice worldwide; this in itself has increased the potential for pest damage, and hence the requirements for control. At present, crop protection in such agricultural systems relies almost exclusively on the use of agrochemicals, although a few specific cases do exist where inherent varietal resistance and biological control have been successfully employed. Not only does the exclusive use of chemical pesticides result in the rapid build-up of resistance to such compounds (Metcalf 1986), but their non-selectivity also affects the balance between pests and natural predators, in favour of the pest (which develops resistance quicker). For example, two outbreaks of rice brown planthopper infestations in Thailand, in the late 1970s and late 1980s, were each preceded by an increase in the use of pesticides (Hadfield 1993). A decrease in pesticide usage has been shown in certain cases to have beneficial results; following the introduction of an integrated pest management (IPM) programme in Indonesia in the 1980s, pesticide application fell by more than 50%, and this was accompanied by a decrease in planthopper infestations and an increase of rice yields by 12% (Hadfield 1993).

The 'blanket' approach to pesticide application employed hitherto, at present estimated to cost approximately \$7.5 billion per annum worldwide, has not only reduced the effective life-span of a given

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compound, but has also led to serious environmental consequences and concerns for human health (Dirham 1993). However, there still remains the need to provide a greater and more efficient level of protection to our crops, because an estimated 37% of all crop production is lost world-wide to pests and diseases, with at least 13% lost directly to insects. An integrated pest control programme, comprising a combination of practices including the judicious use of pesticides, crop rotation, field sanitation, but above all exploiting inherently resistant plant varieties would appear to provide the best option (Meiners & Elden 1978). Within this last category the use of genetically engineered insect-resistant crops may be included.

2. USE OF PLANT PROTEASE INHIBITOR GENES

The presence of antimetabolic proteins which interfere with the processes of digestion in insects is a strategy for defence that plants have used extensively. Such proteins can occur constitutively, in tissues that are particularly vulnerable to attack, such as seeds, or can be induced by mechanical wounding in tissues attacked by chewing insect pests, such as leaves (Green & Ryan 1972). Although the ubiquity of occurrence of protease inhibitors in plants initially obscured their function in protecting the plant from insect attack, abundant evidence now exists for the defensive role of these proteins, which has been summarized in earlier reviews (e.g. Gatehouse *et al.* 1991, 1992).

(*a*) *Transgenic plants containing the CpTI gene*

The possibility of using genes encoding protease inhibitors to engineer insect resistance in transgenic plants was explored in the production of tobacco plants expressing the cowpea protease inhibitor (CpTI) gene (Hilder *et al.* 1987). This protein was considered to be a particularly suitable candidate for transfer to other plant species via genetic engineering, as it had been shown to be an effective antimetabolite against a range of field and storage pests including members of the Lepidoptera, Coleoptera and Orthoptera. Also, there was no evidence that it had toxic or deleterious effects on mammals; this was subsequently confirmed, at least in the short term, by rat feeding trials (Pusztai *et al.* 1992). CpTI is a small polypeptide of about 80 amino acids, belonging to the Bowman-Birk inhibitor family (Gatehouse *et al.* 1980); homologous sequences are encoded by a moderately repetitive gene family in the cowpea genome (Hilder *et al.* 1989).

A full-length cDNA clone encoding a trypsin inhibitor from cowpea was produced, and the coding sequence was placed under the control of a CaMV 35*S* promoter in the final construct produced for transfer to plants (Hilder *et al.* 1987). After standard transformation and selection procedures, the transformed plants were shown to be expressing CpTI in the leaves by a dot blot immunoassay. CpTI accumulated to levels ranging from undetectable to nearly 1% of total soluble protein; this range of values has subsequently been found to be fairly typical for plant genes driven from the CaMV promoter. A further advantage of using CpTI was demonstrated by showing that the protein was expressed in a fully functional form, as shown by assay with bovine trypsin, and was correctly processed. In general, expression of plant-derived genes in other plants can be achieved without the problems of very low expression levels due to codon usage, mRNA stability, protein processing, etc., that have been observed when attempts have been made to express proteins derived from non-plant sources (e.g. *Bt* toxins).

Bioassay of clones of selected CpTI transformants was carried out using first instar larvae of the cotton budworm (*Heliothis virescens*); this insect was chosen as it is a serious pest of tobacco, cotton and maize and thus represents a pest of major economic importance. With these clonal plants, and subsequent generations derived from their self-set seed, the CpTI expressing plants showed significantly less damage than control plants (figure 1). Although the larvae begin to feed on the CpTI-expressing plants, causing some limited damage, they either die or fail to develop as they would on control plants, leading to reduced survival and lower total insect biomass (Hilder *et al.* 1987). These observations are consistent with CpTI acting to decrease amino acid availability, and thus leading to a failure to take up enough of the critical amino acids to ensure normal development, as proposed by earlier authors (Gatehouse & Boulter 1983).

Recent trials carried out in California showed that expression of CpTI in tobacco afforded significant protection in the field against *Helicoverpa zea* (Hoffman *et al.* 1992). This field trial ran assays of plants expressing *Bt* toxin (*cryIA*(*c*) gene, from *Bacillus thuringiensis* HD-73) and CpTI in parallel. The results clearly demonstrated the efficacy of the *Bt* gene, but also showed that the CpTI gene has a negative effect on both larval survival and plant damage (figure 2). However, this effect was not always significant, and results with the CpTI transgenic plants showed variability from one trial to another, suggesting that the degree of protection afforded by the CpTI gene (and, to a lesser extent, *Bt*) in transgenic plants can be affected by factors such as plant age, environmental conditions and heterogeneity of insect populations. Further development work would be necessary to make the CpTI gene as effective as *Bt* toxins.

(*b*) *Other protease inhibitor genes*

The tomato protease inhibitor II gene (PI-II), which encodes a trypsin inhibitor (with some chymotrypsin inhibitory activity), has been shown to confer resistance against the larvae of the lepidopteran *Manduca sexta* (tobacco hornworm) when expressed in tobacco (Johnson *et al.* 1989). The decrease in larval mass was roughly proportional to the level of protease inhibitor II being expressed; at levels over 100 μg of the foreign protein per g of tissue, larval growth was severely retarded, whereas at lower levels (*ca.* 50 $\mu g\ g^{-1}$ tissue) growth was retarded to a lesser degree. However, tobacco plants expressing tomato inhibitor I (specific for chymotrypsin) at levels of

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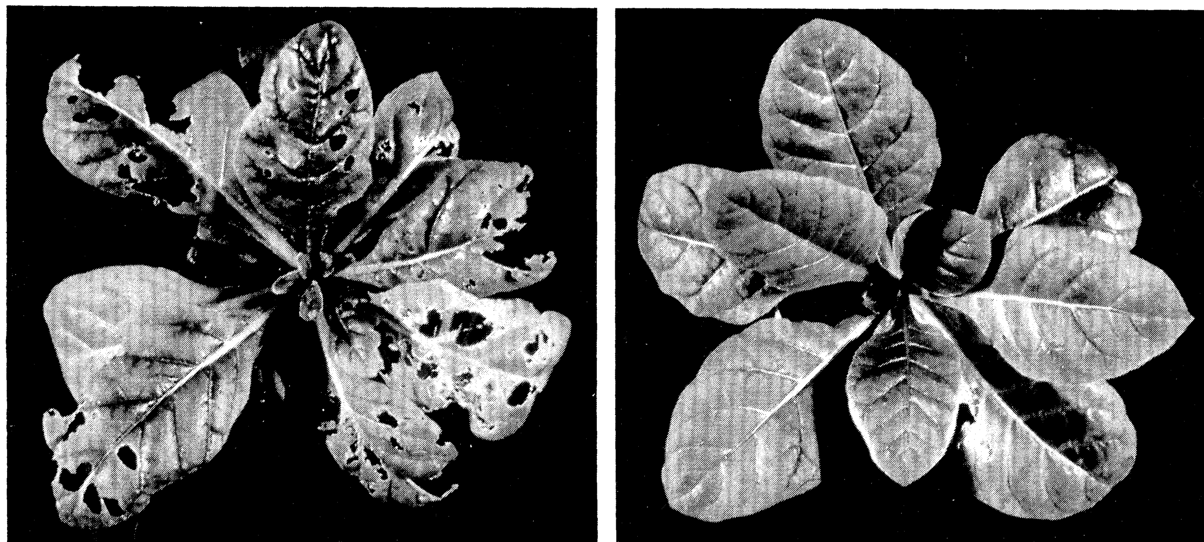

Figure 1. Effect of protease inhibitor expression on insect damage to transgenic plants. Sample results from bioassay (controlled environment cabinet conditions) of control (left) and transgenic CpTI-expressing (right) tobacco plants against first instar larvae of *Heliothis virescens*. Eight larvae per plant were applied, and the assay was run for eight days.

130 $\mu g\ g^{-1}$ had no deleterious effects upon larval development. Interestingly, when PI-II was expressed from a wound-inducible promoter, the resulting transgenic plants did not show significantly enhanced levels of resistance to insect attack.

More recent results obtained by McManus *et al.* (1993) showed that growth of larvae of the noctuid lepidopteran *Chrysodeixis eriosoma* (green looper) was adversely affected when they were fed leaf tissue from transgenic tobacco plants expressing potato proteinase inhibitor II (PPI-II), an inhibitor most active against chymotrypsin-like proteases. Larvae fed on transgenic leaf tissue expressing PPI-II grew significantly slower than those fed either control non-transformed tissue, or transformed leaves with no detectable PPI-II accumulation.

(*c*) *Problems with the use of protease inhibitor genes*

While these results have established the possibility of using protease inhibitor genes in transgenic plants as a protective mechanism, there are a number of problems that have militated against a widespread adoption of this method. First, with a given insect species, some inhibitors are found to be effective antimetabolites, whereas others are not. Secondly, a given inhibitor can show varying degrees of effectiveness as an antimetabolite against different insect species. Thirdly, no inhibitor tested is as effective against any insect tested as are chemical insecticides, or as a *Bacillus thuringiensis* toxin specific to the target organism. How can these drawbacks be addressed?

(*d*) *Insect digestive biochemistry*

The conditions under which digestive processes take place differ considerably between insects and mammals, and between different orders of insects. Most mammals have an acidic foregut and a neutral midgut, which is the major site of digestion of proteins; the mammalian endoproteases thus function at *ca.* pH 7–8. The midgut in lepidopteran larvae, in contrast, is highly alkaline, and the digestive proteases have optimal activity at pH 10–11 (Johnston *et al.* 1991). Larvae of phytophagous coleopterans have acidic conditions in their midgut regions, with pH optima for digestive enzymes typically in the region 4–5. Although enzymes with apparently similar properties to mammalian trypsin and chymotrypsin can be found in these and other orders of insects, the assumption that any inhibitor of the mammalian enzymes will also be effective against the digestive proteases of any insect species, and thus be antimetabolic, is not valid. Adaptations in the type of enzyme used for digestion are also found; seed weevils feeding on plant tissues rich in inhibitors of serine proteases have replaced these digestive enzymes with a thiol protease (Gatehouse *et al.* 1985; Liang *et al.* 1991). The ultimate adaptation is found in many sucking pests that do not appear to have any digestive protease activity, relying on free amino acids in the phloem as a nitrogen source.

(*e*) *The effectiveness of different inhibitors against insect proteases*

The most common attempted use for protease inhibitors has been as protection against larvae of phytophagous lepidopterans. These insects contain enzymes analogous to mammalian endoproteases, although differences in pH optima (see above) and substrate specificity have been noted. Work carried out in Durham (Johnston *et al.* 1994) has sought to determine the relative abilities of different protein protease inhibitors to inhibit the major gut proteases of a representative lepidopteran larva, *Heliothis virescens*, and to prevent proteolytic degradation of

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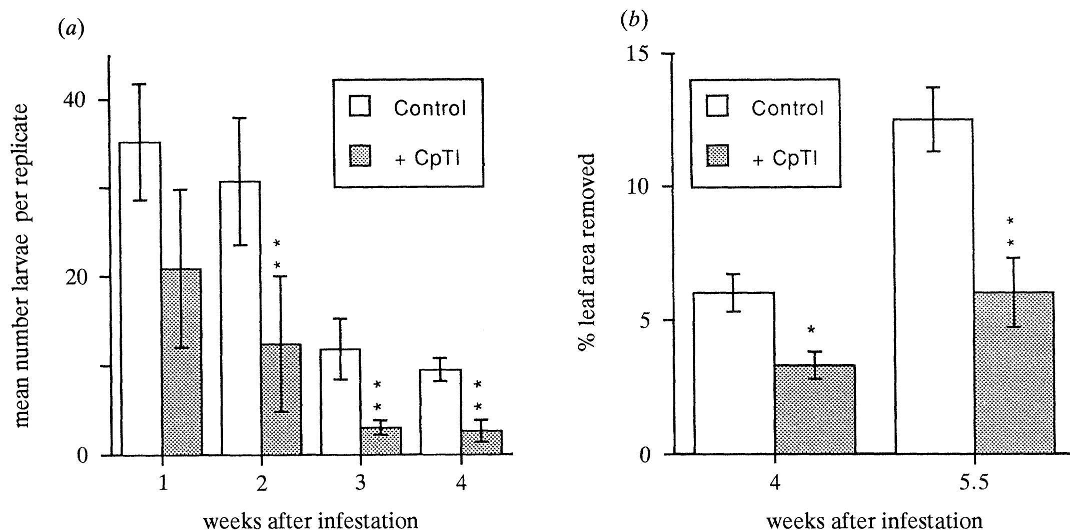

Figure 2. Field trial data for CpTI-expressing transgenic tobacco plants. The trial was carried out near Woodland, California, U.S.A. in the Autumn of 1989. (*a*) Insect survival: data for the mean number (± s.e.m.) of *Heliothis zea* larvae per replicate (three plants) on control and CpTI-expressing plants at various times after infestation (*ca.* 200 eggs per plant). (*b*) Plant damage: data for percent of leaf area removed (± s.e.m.) from leaves (four replicates of 12 plants) sampled from control and CpTI-expressing plants at two intervals after infestation (as above). Levels of significance for difference between control and transgenic plants are indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$. Adapted and redrawn from Hoffmann *et al.* (1992).

ingested proteins. In this species, two major digestive proteolytic enzyme activities were found to be present; a trypsin-like enzyme strongly inhibited by *N*-p-tosyl-lysine chloroketone (TLCK) but not by chymostatin, and a chymotrypsin-like enzyme strongly inhibited by chymostatin, but not TLCK. Low levels of elastase-like activity were also detected. Overall proteolytic activity was almost equally divided between these two proteases, with approximately 55% being due to the chymotrypsin-like enzyme, and approximately 45% due to the trypsin-like enzyme. Three plant protein protease inhibitors (soya bean Kunitz trypsin inhibitor, SBTI, soya bean Bowman-Birk trypsin/chymotrypsin inhibitor, SBBI, and cowpea protease inhibitor, CpTI) were assayed for their effect on the enzymes *in vitro*, using synthetic substrates specific for each activity. In terms of concentration required for 50% inhibition, SBBI was the most effective against the chymotrypsin-like activity, SBTI against the trypsin-like activity. CpTI was the least effective against the former activity, and less effective than SBTI against the latter. Studies carried out on digestion of protein substrates showed marked differences between the inhibitors in their effectiveness at inhibiting overall proteolysis by an insect gut extract; SBTI was approximately twice as effective as SBBI (in terms of concentration required to inhibit proteolysis by 50%), and 20 times as effective as CpTI (figure 3).

(*f*) *Comparison of results* in vitro *and* in vivo

The above results were extended into feeding trials on artificial diets. Third instar larvae of *H. virescens* were transferred to diet containing fixed levels of SBTI, SBBI or CpTI, and the effects of the antimetabolites were assayed by measuring the total insect biomass relative to controls. Both SBTI and SBBI caused a marked loss in weight relative to controls over the first 8 days of the trial whereas CpTI did not (figure 3). The decrease in weight did not differ significantly between SBTI and SBBI. These data are in broad agreement with predictions from the *in vitro* proteolysis experiments, and support a mechanism of toxicity based on inhibition of proteolysis. However, as the trial was continued, no further weight loss occurred with SBTI or SBBI, but weight loss did occur with CpTI, due to increased larval mortality.

Experiments are underway to demonstrate that the results obtained from *in vitro* assays and artificial diet bioassays can be extended to transgenic plants. Plants expressing SBTI have been produced, in which the SBTI coding sequence is driven by the CaMV 35*S* promoter, in constructs analogous to those employed for CpTI. Comparison of bioassay data from CpTI-expressing, and SBTI-expressing plants have shown, in preliminary trials, that SBTI-expressing plants were significantly more resistant to insect attack in terms of leaf damage, insect survival and total insect biomass than CpTI-expressing plants, although the latter were significantly more resistant to attack than control plants (figure 3). These data are not perfect, because the expression levels of CpTI and SBTI have not been exactly matched, a high expresser from the SBTI transformation having been compared to the highest-expressing CpTI transgenic line; however, on the basis of previous results, the expression levels are not likely to differ by more than a factor of twofold.

Similar strategies have been followed by other groups. McManus *et al.* (1993) showed that the chymotrypsin inhibitor, PPI-II, which was effective in protecting transgenic plants from attack by *Chrysodeixis eriosoma* (see above), was not effective against *Spodoptera litura* or *Thysanoplusia orichalcea*. When this inhibitor was purified from the transformed plants, it

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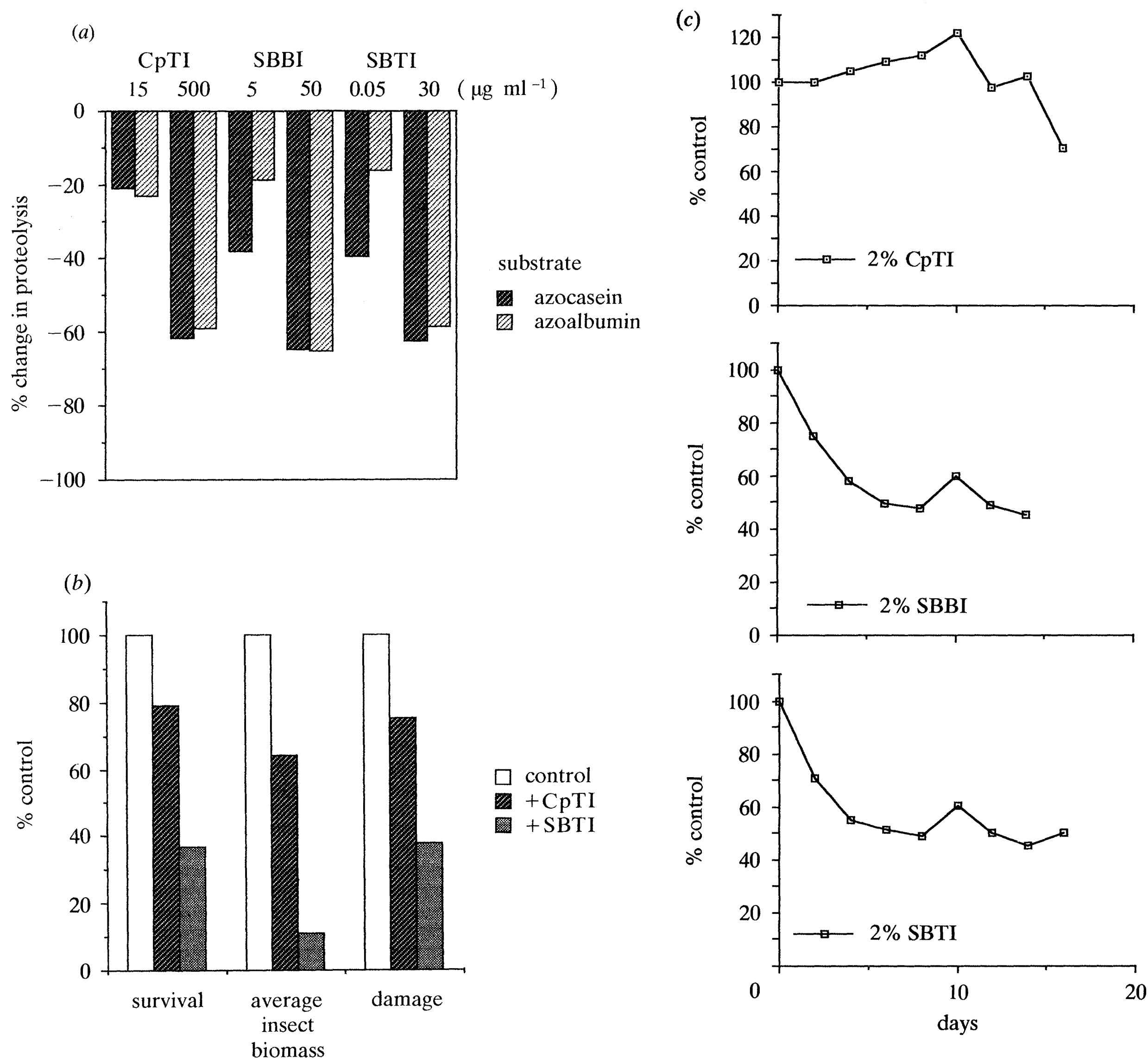

Figure 3. Effects of different plant protein protease inhibitors on *Heliothis virescens in vitro* and *in vivo*, and in transgenic plants. (*a*) *In vitro* inhibition of proteolysis: comparative effects of cowpea trypsin inhibitor (CpTI), soya bean Bowman–Birk trypsin/chymotrypsin inhibitor (SBBI) and soya bean Kunitz trypsin inhibitor (SBTI) on proteolysis of two protein substrates by larval gut extract. Inhibitor concentrations in the assays have been adjusted to give approximately the same reductions in levels of proteolysis, corresponding to 50% inhibition of one of the two major gut proteolytic activities, negligible inhibition of the other (lower levels of inhibitor), and more than 90% inhibition of one protease activity, approximately 50% inhibition of the other (higher levels of inhibitor). (*b*) Transgenic plant bioassay: relative insect survival, insect biomass, and plant damage (assessed by leaf area eaten; Hilder *et al.* 1987) after an 8-day growth cabinet bioassay in which first instar *H. virescens* larvae (eight per plant) were placed on transgenic and control tobacco plants. (*c*) Artificial diet bioassay: effect of incorporation of CpTI, SBBI and SBTI into an artificial diet for *H. virescens*. Third instar larvae (ten per treatment) were placed on this diet, and the total live larval biomass is plotted as a percentage of the control (diet with no added antimetabolite) over time.

was tested by *in vitro* proteolysis assays against gut extracts from two of the three pests. As expected, the inhibitor effectively retarded proteolysis by *C. eriosoma* gut extracts, but not by *S. litura* gut extract, illustrating a correspondence of results obtained *in vivo* with those of enzyme assays *in vitro*. The type of digestive protease activity present in a target insect species has been used in the design of transgenic plants by Jouanin and coworkers. The cysteine protease inhibitor from rice (oryzacystatin) has been expressed in poplar trees to give resistance to the insect pests *Chrysomela populi* and *Chrysomela tremula*, with encouraging results in preliminary trials (L. Jouanin and J. C. Leple, personal communication), and has also been expressed in oilseed rape with the intention of conferring resistance to the seed weevil *Ceuthorhyncus* (L. Jouanin and M. Bonade-Bottino, personal communication).

(*g*) *Developing the protease inhibitor approach*

On the basis of these results, optimization of the interaction between a protease inhibitor and its target protease will lead to increased protection for transge-

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nic plants expressing that inhibitor. However, achieving this aim is not straightforward. One approach is to screen as many naturally occurring inhibitors as possible, by *in vitro* enzyme assays with a gut extract from the target pest. A gene encoding the inhibitor with the strongest interaction would then be used for transgenic plant production. Alternatively, because the interactions of protease inhibitors with mammalian proteases have been studied in some detail at the structural level, and functional residues on the inhibitors are fairly well characterized, protein engineering of inhibitors could be undertaken to increase the strength of interaction between the inhibitor and its target. The drawback of this approach is the absence of structural, or even amino acid sequence information for digestive proteases of insect pests.

A further requirement for optimization of protease inhibitor-based protective systems may be to use more than one foreign inhibitor in a transgenic plant to affect different digestive proteases in the pest (or pests). The use of gene combinations in transgenic plants is considered later in this article. There may be inherent limitations to this technology, in that even if the major digestive proteases of the insect pest are very effectively inhibited, the insect may still be able to survive, utilizing free amino acids or other nitrogen sources; however, evidence does suggest that very high levels of control could be achieved.

3. APPROACHES TO THE CONTROL OF SUCKING INSECTS

Sucking insect pests, of the order Homoptera, cause serious crop damage, both directly and by acting as vectors for plant pathogens. Furthermore, they are difficult to deal with using conventional pesticide régimes due to their rapid adaptation, resulting in insecticide-resistant phenotypes, and despite some success with biological control, there is a need for improving the endogenous resistance of plants.

Most work on resistance of plants to sucking pests has concentrated on the role of semiochemicals, and plant secondary metabolites as feeding deterrents. The feasibility of engineering transgenic plants to confer the ability to produce secondary metabolites has yet to be demonstrated, and the ability to do this on a routine basis for given secondary compounds is some way in the future, due to the complexity and species-specificity of the biochemical pathways involved, although this approach is now being addressed (Hallahan *et al.* 1992). To attempt to tackle the problem of producing transgenic plants with resistance to sucking pests, it was therefore necessary to go back to insect bioassays, using products of genes that could be obtained reasonably easily, and which could be expressed in transgenic plants using existing technology. Rice brown planthopper (*Nilaparvata lugens*) was chosen as a test organism, as it is an economically important pest in Southeast Asia.

(*a*) *Bioassay of potential insecticidal proteins*

The bioassay system developed for rice brown planthopper used a liquid diet, which was fed to the insects under pressure through a parafilm membrane, and has been described elsewhere (Powell *et al.* 1993). As expected, the inhibitors of digestive enzymes which were tested (cowpea protease inhibitor and wheat α-amylase inhibitor), had no effect on planthoppers, which have been reported not to possess digestive endoproteinases, and do not digest starch. However, when a series of lectins were tested, although some (for example pea lectin, potato lectin) had no significant effect on insect survival, others (for example, concanavalin A, wheat germ lectin) did. The most effective protein tested was the lectin from snowdrop (*Galanthus nivalis*; GNA), which gave approximately 80% corrected mortality at a concentration of 1 g l^{-1} in the diet, when used in assays with first and third instar nymphs. GNA was also toxic to another sucking pest tested, the rice green leafhopper (Powell *et al.* 1993). Insecticidal effects due to this protein have been observed previously with some coleopteran and lepidopteran species (Hilder *et al.* 1992).

(*b*) *Use of a gene encoding* Galanthus nivalis *lectin (GNA)*

On the basis of the bioassays, a gene encoding GNA could be identified as having potential in engineering plants for resistance to sucking insects. A 'proving' experiment would, if successful, provide a basis from which to develop the system, when information on the mechanism of action of the protein became available. To carry out such an experiment, three requirements had to be met: (i) a sequence encoding GNA had to be obtained; (ii) a suitable promoter for expressing the protein in phloem sap had to be constructed; and (iii) a suitable insect–plant bioassay system for the effects of GNA had to be devised, in the absence of a rapid and straightforward transformation system for rice.

A cDNA encoding GNA was obtained from the laboratory of W. Peumans and E. van Damme (van Damme *et al.* 1991). GNA is a heterogeneous protein as isolated from snowdrop bulbs, and is apparently encoded by a large gene family; however, the use of a cDNA clone ensured that the sequence used was expressed in the source plant. Initial experiments placed the GNA coding sequence under control of the CaMV 35*S* promoter, and looked at expression in transgenic tobacco plants. As was the case for CpTI, achieving reasonable levels of expression of GNA proved to be straightforward. The complete coding sequence gave rise to levels of GNA protein up to 1% of total protein in leaf tissue of primary transformants, as determined by quantitative dot-blot immunoassay using anti-GNA primary antibodies. The functional integrity of GNA expressed in the transgenic tobacco was demonstrated by haemagglutination assay; in this assay the highest dilution to agglutinate erythrocytes was consistent with the level of GNA expression determined for the tissue, and with the known haemagglutination activity of pure GNA.

(*c*) *Phloem-specific expression*

Although immunolocalization of GNA in transgenic tobacco plants expressing the CaMV 35*S* promoter-

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GNA construct showed that GNA was present in phloem cells, as well as in all other tissues, a phloem-specific promoter would possibly lead to higher levels of the protein in phloem tissue, and might be advantageous under some circumstances in confining expression to the site of insect attack. The maize sucrose synthase gene (*Sh-1*) has been shown to be specifically expressed in phloem (and in developing grains), and the promoter from this gene has been characterized by reporter gene assays in transgenic tobacco (Yang & Russell 1990). The promoter from the corresponding rice gene was therefore chosen as optimal for the ultimate aim of engineering resistance to sucking pests into rice. The rice sucrose synthase gene *RSs1* has been isolated, characterized and fully sequenced (Wang *et al.* 1992), and its promoter (1.9 kb of 5′ flanking sequence, plus the transcription start, first exon and first intron of the gene) has been tested in transgenic tobacco by means of a suitable reporter gene (*gus*) fusion. The *RSs1-gus* chimeric gene directs expression of GUS protein in the phloem tissue of leaves, stems, petioles and roots of transgenic plants with no detectable expression in other tissues, as required (Shi *et al.* 1993).

A similar *RSs1*-promoter fusion to the GNA coding sequence was used to direct the phloem-specific expression of GNA. Transgenic tobacco plants expressing this construct were shown to be expressing GNA in phloem cells by immunolocalization, and a novel method was used to demonstrate that the foreign protein was present in the phloem sap (Shi *et al.* 1993). Peach-potato aphids (*Myzus persicae*) were allowed to feed on the phloem-specific GNA-expressing transgenic tobacco plants, and the excreted honeydew from the insects (in amounts of less than 1 μl) was collected on nitrocellulose membranes. The dots of honeydew were then tested by blot-immunoassay, using anti-GNA antibodies, for the presence of GNA. Honeydew from aphids fed on transgenic plants gave a positive reaction, whereas that from control plants was negative. This experiment proved that GNA must have been present in the phloem sap ingested by the aphids, and proves the effectiveness of the promoter-coding sequence fusion used for the transformation.

(*d*) *'Proving' the technology*

The final requirement for the 'proving' experiment of GNA-mediated engineered resistance is a suitable insect bioassay system for transgenic plants. Fortunately, such a system could be developed. As indicated above, peach-potato aphids (*Myzus persicae*) feed readily on tobacco, and develop well on this host. The effects of GNA on these homopteran pests was assayed in an artificial diet bioassay, which showed a significant negative effect of the added protein on aphid survival and development (Hilder 1993), sufficient to allow the aphid to be used as a test organism on transgenic plants. Experiments are in progress to evaluate the prediction that GNA-expressing tobacco plants will show significant resistance to *Myzus persicae*.

The data obtained from feeding trials, and the work carried out on gene constructs, suggest that the 'proving' experiment will be successful, and that appropriate plant genes, if expressed in the phloem tissue of transgenic crops, will confer significant protection against sucking insect pests.

4. PYRAMIDING GENES

One of the goals of the plant breeder is to 'pyramid' genes expressing agriculturally desirable characteristics. This strategy has also been adopted by the biotechnologist. To increase the protective efficacy, spectrum of activity and durability of resistance, it is envisaged that 'packages' of different genes could be introduced into crops. The components of such packages should each act on different targets within the insect, thus mimicking the multiple mechanisms of resistance which occur in nature. Protease inhibitors should be particularly valuable in this respect because, apart from their inherent insecticidal effects, they would protect other introduced gene products from premature digestion in the insect gut.

The first demonstration of such an approach has been the introduction of both CpTI and pea lectin into tobacco; these plants were obtained by cross-breeding plants derived from the two primary transformed lines (Boulter *et al.* 1990). Although the insecticidal effects of the two genes were not synergistic, they were additive, with insect biomass on the double expressers being only 11% compared to those from control plants and 50% of those from plants expressing either CpTI or pea lectin alone. Leaf damage was also the least on the double expressing plants. However, other gene product combinations assayed in artificial diet bioassays have not shown such promising results, with the protection afforded by two gene products in combination being little different to that given by one or other product alone. The design of gene combinations, even more than the choice of single genes encoding insecticidal proteins, needs to have input from studies on the mechanism of action of the toxic or antimetabolic gene products, to reduce the number of potential experiments to a manageable level. For example, combining two gene products where one has feeding deterrence as its major effect is unlikely to give great increases in protection.

5. CONCLUSION

Engineering transgenic plants to express foreign insecticidal proteins is a means of producing crops with enhanced levels of insect resistance, which, if adopted, could complement other forms of crop protection. The technology has the potential to move farming closer to ecologically sustainable practices, both in the Developed and Developing parts of the world, and thus could make a considerable impact on agricultural systems in the future. Although the use of plant genes for conferring insect resistance is not as well developed as the use of genes encoding *Bacillus thuringiensis* toxins, the technology has the potential to give resistance to a wide range of chewing and sucking pests, and with

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development could lead to products that are of value both to subsistence and commercial growers.

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Figure 1. Effect of protease inhibitor expression on insect damage to transgenic plants. Sample results from bioassay (controlled environment cabinet conditions) of control (left) and transgenic CpTI-expressing (right) tobacco plants against first instar larvae of *Heliothis virescens*. Eight larvae per plant were applied, and the assay was run for eight days.