Identifying Proteins with Insecticidal Activity: Use of Encoding Genes to Produce Insect-Resistant Transgenic Crops*

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Abstract: Crops resistant to insect attack offer an alternative strategy of pest control to a total reliance upon chemical pesticides. Transgenic plant technology can be a useful tool in producing resistant crops, by introducing novel resistance genes into a plant species. This technology is seen very much as forming an integral component of a crop management programme.

Several different classes of plant proteins have been shown to be insecticidal towards a range of economically important insect pests from different orders; in some cases a role in the defence of specific plant species against phytophagous insects has been demonstrated. Genes encoding insecticidal proteins have been isolated from various plant species and transferred to crops by genetic engineering. Amongst these genes are those that encode inhibitors of proteases (serine and cysteine) and α-amylase, lectins, and enzymes such as chitinases and lipoxygenases.

Examples of genetically engineered crops expressing insecticidal plant proteins from different plant species, with enhanced resistance to one or more insect pests from the orders Lepidoptera, Homoptera and Coleoptera are presented. The possibility of 'pyramiding' different resistance genes to improve the effectiveness of protection and durability is discussed and exemplified. The number of different crop species expressing such genes is very diverse and ever-increasing. The viability of this approach to crop protection is considered. © 1998 SCI.


Key words: insecticidal proteins; enzyme inhibitors; lectins; transgenic plants; insect resistance

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1 INTRODUCTION

Crop protection plays a vital and integral role in modern-day agricultural production. The ever-increasing demands on yield and the intensification of farming practice, particularly in the developed world, have increased the problem of pest damage, and hence control.

It has long been recognised that extensive cultivation of certain crops to the exclusion of other plants, i.e. monoculture, may favour drastic increases in the populations of insects that feed upon these crops, the number of herbivores exploiting a given host species increasing with the area occupied by that host.1 Not only does this situation occur in the field, but also during storage of food crops, where accumulation of large quantities of seed means that any insect which can utilise them as a food source is almost certain to undergo a population explosion, resulting in significant damage and loss. Although the build-up of pests to a specific host plant is generally reduced in diverse plant communities,2 agricultural practices such as combining crops which each act as host for a particular pest, as exemplified by cotton and maize, both of which are attacked by the corn earworm Helicoverpa zea (Boddie), can have the opposite effect, thereby exacerbating the situation.3

At present crop pest control is primarily dependent upon the use of agrochemicals and to a lesser extent upon inherent varietal resistance, with biological control methods being successfully employed only in a few specific cases. However, it is only since the second world war that an almost exclusive reliance has been placed upon insecticide use. This, in many cases, has resulted in the rapid build-up of resistance by insect pests to such compounds, as is illustrated by the rapidly developed resistance to the organochlorine insecticides by the cotton bollworm, Heliothis virescens F.4 Indeed, there are examples of resistance in a major pest being observed within the first year of field use. In some cases the indiscriminate application of pesticides has exacerbated the problem of insect herbivory, where elimination of a wide range of predatory species along with the primary pests has resulted in secondary pests becoming primary pests themselves, with even more devastating consequences.5

Despite an annual insecticide expenditure of approximately seven billion US dollars it is estimated that 37% of all crop production is lost world-wide to pests and diseases, with at least 13% lost directly to insects. There is thus a great need to provide a greater level of protection to our crops. 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 for improving pest control. Within this last category the use of genetically engineered insect-resistant crops may be included. Genetic engineering of crops can make a major contribution to the production of such inherently resistant/tolerant varieties since it opens up a virtually limitless source of germplasm variability from which to select insect control genes for introduction into elite crop varieties.

As an increasing number of crops of world-wide importance become amenable to genetic transformation, the question of where to find genes encoding desirable characteristics, such as pest resistance, becomes increasingly important. To date, two main strategies for producing such plants have been successfully used. One approach is to use the entomocidal bacterium Bacillus thuringiensis Berliner as a source of resistance genes, and the other is to identify and use the insect resistance genes present in plants themselves—i.e. exploit the plants’ own solution to the problem.

The identification of suitable insecticidal proteins present in plants, and the use of their encoding genes in the production of insect-resistant/tolerant crop plants, will be discussed in this paper.

2 NATURAL DEFENCE MECHANISMS OF PLANTS—INHERENT RESISTANCE

Plant-based defence mechanisms depend primarily on three factors, namely, temporal avoidance, physical and chemical defences.

Since one of the most important classes of natural defence mechanisms is chemical defence, and since it is pertinent to this paper, this aspect of natural defence will be considered in some detail. Plants have a vast metabolic capability and produce many secondary chemicals which are toxic, anti-nutritional, or aversive to species which might otherwise be potential predators.6 Examples include the pyrethrins from chrysanthemums and alkaloids like nicotine from tobacco. Other classes of plant secondary compounds which have been implicated in protection from insect attack include the terpenoids, steroids, flavanoids, phenolics, glucosinolates, cyanogenic glycosides, rotenoids, saponins and nonprotein amino acids.7 Production of some of these compounds imposes a demonstrable metabolic cost on the plant, indicated by a reduced fitness in the absence of predation; this suggests that their production in the plant is a selective response to insect feeding.8

As secondary compounds are the products of multi-enzyme pathways which involve the interaction of many gene products, such defence systems are in most cases too complex for the ‘state of the art’ of plant genetic engineering, although work is in hand towards exploiting them.9,10 A few plant defence mechanisms, however, are based on proteins themselves, the product of a single gene. Because proteins are non-volatile, they must be ingested to have an effect, and the target site of many is the insect digestive system. For example, resistance of
certain wild accessions of the common bean (*Phaseolus vulgaris* L.) to the Mexican bean beetle (*Zabrotes subfasciatus* (Boh.)) is due, in part, to a protein, arcelin-4, which largely replaces the conventional bean storage protein, phaseolin. Arcelin-4 is not digestible by this insect, and the larvae therefore starve to death on these resistant beans.\(^\text{11}\)

Proteinaceous inhibitors of insect digestive enzymes have a similar effect\(^\text{1,2}\) and certain lectins bind to, and disrupt, cells of the insect gut epithelium.\(^\text{13}\) Ribosome-inactivating proteins (RIPs) such as ricin from castor beans have also been shown to be highly toxic to certain insects.\(^\text{14}\) Most of these types of single gene characters are eminently suitable for gene transfer using current technology. Being of plant origin, they have the added advantage that they are likely to have a high degree of compatibility with the metabolic system of the transgenic host plants.

### 3 USE OF PLANT-DERIVED INSECTICIDAL GENES

#### 3.1 Plant genetic modification for insect pest resistance

Recent advances in molecular techniques have led to the development of methods for the genetic transformation of a wide range of plants. A major advantage that genetic manipulation offers over conventional plant breeding is the ability to improve specific characteristics, including resistance to pests, without risking the loss of existing desirable traits such as palatability, nutritional quality and yield. Since this technique enables the transfer of genes between unrelated taxa, it therefore greatly increases the pool from which desirable agronomic characteristics may be selected.

Progress in the development of transgenic insect/pest-resistant plants is limited by the speed of development of efficient methods for the transformation and regeneration of commercially favoured cultivars of the selected species of plant. Additionally, effort is required in the identification and purification of potential candidate proteins, insect bioassay to select those with the desired insecticidal properties, isolation of the encoding gene(s), and incorporation of these into vector constructs which express the protein adequately within the host plant, and in the appropriate plant tissues.

#### 3.2 Protease inhibitors

Although interest in the effects of protease inhibitors on insect development was aroused as early as 1947,\(^\text{15}\) it was not until 1972 that a protective role for these proteins was proposed when Ryan and co-workers demonstrated that damage to the leaves of certain solanaceous plants, either by insect feeding or mechanical wounding, induced the synthesis of protease inhibitors.\(^\text{16}\) This induction occurs not only in the attacked leaf but throughout the plant. Production of these inhibitors was shown to be as a result of a wound hormone, protease inhibitor-inducing factor (PIIF) which is released from the damaged leaves and transported to other leaves where it initiates synthesis and accumulation of inhibitors.\(^\text{17–19}\)

Evidence for this protective role in the ‘field’ was first provided by Gatehouse *et al.*\(^\text{12}\) when they demonstrated that elevated levels of these inhibitors in one variety of cowpea (*Vigna sinensis* (L) Savi) were involved in the observed resistance of the seeds to a major storage pest of this crop, the bruchid *Callosobruchus maculatus* (F.). This particular trait was later exploited by conventional plant breeding, whereby resistance was transferred to an agronomically improved background of cowpea.\(^\text{20}\) This demonstration supported the hypothesis put forward by Ryan and colleagues and opened the way to the subsequent exploitation of such compounds in crop protection *via* genetic manipulation.

#### 3.2.1 Identification of suitable protease inhibitors, and their encoding genes, for transfer

There have been many examples of protease inhibitors being active against different species of insect, both *in vitro*, in assays against insect gut proteases and *in vivo* in artificial diet bioassays.\(^\text{12,21–27}\) Such studies have been useful in the identification of potential inhibitors which could be used in crop protection. To this end, a systematic study of a wide range of different inhibitors has been made in an attempt to find a parameter which could be useful in predicting the potential of any inhibitor to act as a resistance factor to a given insect pest; the dissociation constant of the inhibitor : protease complex was suggested as such a parameter.\(^\text{28}\) This strategy may well be a valuable tool in choosing the most appropriate enzyme inhibitor to control specific insect pests, although recent work suggests that this approach should be adopted with caution, since some insects are able to overcome the deleterious effects of protease inhibitors by synthesising different proteases which are insensitive to inhibition by these particular inhibitors.\(^\text{29,30}\) Despite certain limitations with this approach, there have been several successful examples based on this strategy.

#### 3.2.2 Insect-resistant transgenic plants expressing serine protease inhibitors

The first gene of plant origin to be transferred successfully to another plant species resulting in enhanced insect resistance was that isolated from cowpea encoding a double-headed trypsin inhibitor (CpTI).\(^\text{31}\) In this instance a full-length cDNA clone encoding a trypsin/trypsin inhibitor from cowpea was produced and the coding sequence was placed under the control of a CaMV 35S promoter in the final construct produced for
transfer to plants. The construct employed the Agrobacterium tumefaciens Conn. (Smith & Towns) Ti plasmid binary vector pROK2; a terminator from the nopaline synthetase gene was placed 3′ to the coding sequence, and the construct also contained a nos-neo gene to allow transformants to be selected on the basis of kanamycin resistance.

Although a range of expression levels was obtained in the primary transformants, varying from undetectable to approximately 1% of the total soluble protein, bioassays were only carried out on those plants expressing CptI at the higher levels. Initially bioassays were carried out using first-instar larvae of the tobacco budworm (H. 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 only minor damage compared to the control plants. Although the larvae began to feed on the CptI-expressing plants, causing some limited damage, they usually died or failed to develop as they would on control plants. This protection afforded by CptI has subsequently been demonstrated for other lepidopteran pests including H. zea, Spodoptera littoralis (Boisd.), and Manduca sexta (Joh.). Statistical analysis of the bioassay in terms of plant damage by leaf area, and insect survival and biomass confirmed the highly significant protection afforded by CptI. Subsequent trials carried out in California showed that expression of CptI in tobacco afforded significant protection in the field against H. zea; results from these trials closely resembled those obtained previously in trials carried out under controlled environmental conditions in growth chambers.

Following these earlier studies whereby tobacco was used as a model system for expression of CptI, its encoding gene has now been engineered into many different crops including potato, oil seed rape, rice, and soft fruits such as strawberry. Very recent trials with CptI transgenic strawberry plants suggested that these plants were highly resistant to the vine weevil.33 Furthermore, field trials with transgenic rice demonstrated that CptI expression conferred enhanced levels of resistance to rice stem borers.34

Despite CptI being insecticidal to a wide spectrum of insect pests, mammalian feeding trials incorporating the purified protein at levels of 10% of the total protein have failed to demonstrate toxicity; this may well reflect differences in the organisation of the insect and mammalian digestive systems.

Not only have the genes encoding protease inhibitors isolated from cowpea been shown to confer resistance when expressed in transgenic crop plants, but the tomato inhibitor II gene, PI-II (which encodes a trypsin inhibitor with some chymotrypsin inhibitory activity), when expressed in tobacco, was also shown to confer insect resistance.35 Johnson et al. showed that the decrease in larval weight was roughly proportional to the level of PI-II being expressed.36 Several of the transgenic plants were shown to contain inhibitor levels over 200 μg g⁻¹ tissue; these levels are within the range that is routinely induced by wounding leaves of either tomato or potato plants.37 However, tobacco plants expressing tomato inhibitor I at similar levels had no deleterious effects upon larval development. More recent results obtained by McManus et al.38 showed that growth of the noctuid lepidopteran Chrysodeixis eriosoma (green looper) was adversely affected when they were fed leaf tissue from transgenic tobacco plants expressing the potato proteinase inhibitor II gene, POT-II. Larvae fed on transgenic leaf tissue expressing this inhibitor grew significantly more slowly than those fed either control non-transformed tissue, or transformed leaves with no detectable POT-II accumulation.

Since these initial examples of insect resistance of transgenic plants expressing protease inhibitors (PIs), there have been many other examples where the range of PIs used and the range of crops transformed has been increased. Examples of these are summarised in Table 1.

### 3.2.3 Insect-resistant transgenic plants expressing cysteine protease inhibitors

Not only have inhibitors of serine proteases been engineered into crops for enhanced resistance to insect pests, but genes encoding cysteine protease inhibitors are now also being used to this end, particularly for control of coleopteran insects. Although there have been several studies carried out demonstrating in-vitro inhibition of insect digestive proteases by cysteine protease inhibitors,39–45 with a few examples demonstrating their deleterious effects against insects when incorporated into artificial diets,42,44,45 as yet there are few published examples describing their insecticidal effects in planta. However, one such example is the gene encoding oryzacystatin which has been engineered into poplar trees for resistance towards Chrysomela tremulae.46

#### 3.3 α-Amylase inhibitors

Unlike protease inhibitors, induced synthesis of amylase inhibitors by insect attack has not been observed, and therefore the precise physiological role of these proteins remains open to speculation. However, those purified from wheat and Phaseolus vulgaris have been shown to be insecticidal to non-pest species when tested in artificial diet.47–49 A very specific α-amylase inhibitor has been isolated from a particular wild line of P. vulgaris (G12953) which is resistant to attack by a major storage pest of P. vulgaris, Zabrottes subfasciatus.11 Gatehouse et al.50 demonstrated that its presence was correlated with seed resistance to this particular pest species and that in vitro it was a potent inhibitor. Very recently it has
Use of encoding genes to produce insect-resistant transgenic crops

TABLE 1

Transgenic Plants Expressing Insecticidal Plant Genes

<table>
<thead>
<tr>
<th>Plant</th>
<th>Genes^a</th>
<th>Insect</th>
<th>Reference^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>CpTI</td>
<td><em>Heliothis virescens</em></td>
<td>31</td>
</tr>
<tr>
<td>Pot PI II</td>
<td></td>
<td>Lepidoptera</td>
<td>30, 36, 38</td>
</tr>
<tr>
<td>GNA</td>
<td></td>
<td><em>H. virescens</em></td>
<td>a</td>
</tr>
<tr>
<td>α-AI</td>
<td></td>
<td><em>Agrotis ipsilon</em></td>
<td>b</td>
</tr>
<tr>
<td>p-lec</td>
<td></td>
<td><em>H. virescens</em></td>
<td>71</td>
</tr>
<tr>
<td>CpTI + p-lec</td>
<td></td>
<td><em>H. virescens</em></td>
<td>71</td>
</tr>
<tr>
<td>Na PI</td>
<td></td>
<td><em>Helicoverpa punctigera</em></td>
<td>82</td>
</tr>
<tr>
<td>Potato</td>
<td>CpTI</td>
<td><em>Lacanobia oleracea</em></td>
<td>74</td>
</tr>
<tr>
<td>GNA</td>
<td></td>
<td><em>L. oleracea</em></td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. persicace</em></td>
<td>76</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aulacorthum solani</em></td>
<td>75</td>
</tr>
<tr>
<td>BCH</td>
<td></td>
<td><em>A. solani</em></td>
<td>c</td>
</tr>
<tr>
<td>GNA + BCH</td>
<td></td>
<td><em>M. persicace</em></td>
<td>76</td>
</tr>
<tr>
<td>Tomato</td>
<td>Pot PI I</td>
<td><em>Helicoverpa armigera</em></td>
<td>d</td>
</tr>
<tr>
<td>Pot PI II</td>
<td></td>
<td><em>Teleogryllus commodus</em></td>
<td>e</td>
</tr>
<tr>
<td>CpTI</td>
<td></td>
<td><em>H. armigera</em></td>
<td>d</td>
</tr>
<tr>
<td>GNA</td>
<td></td>
<td><em>T. commodus</em></td>
<td>e</td>
</tr>
<tr>
<td>Rice</td>
<td>Pot PI II</td>
<td><em>Sesamia inferens</em></td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Chilo suppressalis</em></td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>CpTI</td>
<td><em>S. inferens</em></td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. suppressalis</em></td>
<td>34</td>
</tr>
<tr>
<td>Strawberry</td>
<td>CpTI</td>
<td><em>Otiorynchus sulcatus</em></td>
<td>33</td>
</tr>
<tr>
<td>Lettuce</td>
<td>Pot PI II</td>
<td><em>T. commodus</em></td>
<td>g</td>
</tr>
<tr>
<td>Oilseed rape</td>
<td>OC-I</td>
<td>Coleoptera</td>
<td>h</td>
</tr>
<tr>
<td>CII</td>
<td></td>
<td>Lepidoptera</td>
<td>h</td>
</tr>
<tr>
<td>Pea</td>
<td>α-AI</td>
<td><em>Zabrotes subfaciatus</em></td>
<td>54</td>
</tr>
<tr>
<td>Azuki bean</td>
<td>α-AI</td>
<td><em>Callosobruchus chinensis</em></td>
<td>84</td>
</tr>
<tr>
<td>Apple</td>
<td>CpTI</td>
<td><em>Cydia pomenella</em></td>
<td>i</td>
</tr>
<tr>
<td>Poplar</td>
<td>OC-I</td>
<td><em>Chrysomela tremulæ</em></td>
<td>46</td>
</tr>
<tr>
<td>CH</td>
<td></td>
<td>Lepidoptera</td>
<td>h</td>
</tr>
</tbody>
</table>

^a α-AI = α-amylase inhibitor; BCH = beam chitinase, CII = double headed serine protease inhibitor from soybean; CpTI = cowpea trypsin inhibitor; GNA = snowdrop lectin; Na PI = *Nicotiana alata* protease inhibitor; OC-I = rice cysteine inhibitor; Pot PI I = Potato proteinase inhibitor I; Pot PI II = Potato proteinase inhibitor II; p-lec = pea lectin

^b Personal communications: a = Gatehouse & Newell; b = Carbonero; c = Down; d = Ryan & Markwick; e = Ryan & Burgess; f = Edwards & Newell; g = Appleby & Burgess; h = INRA/CNRS; i = James.

been shown to be toxic to *Z. subfasciatus* when tested in artificial seeds.

Studies have shown that the different types of α-amylase inhibitor present in wheat endosperm are differentially active against various lepidopteran pest species.\(^51\)

3.3.1 Insect-resistant transgenic plants expressing α-amylase inhibitors

Strong evidence has been presented to suggest that an α-amylase inhibitor present in the seeds of *P. vulgaris* is encoded by an already identified lectin gene whose product is referred to as LLP.\(^52\) A chimeric gene, consisting of the coding sequence of the lectin gene that encodes LLP and the 5' and 3' flanking sequences of the lectin gene that encode PHA-2, has been constructed and expressed in tobacco.\(^53\) Not only did seeds from these transgenic plants show antigenicity to bean α-amylase inhibitor, but they also expressed a series of polypeptides of molecular weight corresponding to these particular inhibitors. Furthermore, seed extracts were found to be active against both porcine pancreatic
α-amylase and the α-amylase present in the midgut of mealworm, *Tenebrio molitor* L. This last observation led to the suggestion that introduction of this lectin gene (ai) into other leguminous plants may be a strategy to protect the seeds from seed-eating larvae of Coleoptera. Recently, expression of bean α-amylase inhibitor in pea plants has been found to confer significant levels of resistance to bruchid beetles.54

Other workers have expressed a gene encoding an α-amylase inhibitor from wheat in tobacco, resulting in increased protection against the army worm (*Spodoptera* spp.) and greasy cut worm (*Agrotis* spp.) (Carbonero, pers. comm.).

### 3.4 Lectins

A role for lectins as defensive proteins in plants against insect predators was first proposed by Janzen *et al.*65 who suggested that the common bean (*P. vulgaris*) lectin was responsible for the resistance of these seeds to attack by coleopteran storage pests. Subsequent studies have confirmed that many lectins are insecticidal; these have been identified mainly through the screening of purified lectins against insect pests of economically important crop plants in artificial diet bioassays.56 Expression of lectin genes in transgenic plants has been advanced both as a means of crop protection, and as a method of investigating lectin function.

#### 3.4.1 Identification of suitable lectins with insecticidal activity

Work carried out to date which has identified insecticidal lectins, will be discussed by insect order.

##### 3.4.1.1 Lectins active against Coleoptera

Janzen *et al.*55 showed that lectin from common bean, *P. vulgaris* (PHA), was toxic to the cowpea bruchid, *C. maculatus*, by bioassay in artificial diet. Although the PHA preparation used was possibly contaminated with *P. vulgaris* α-amylase inhibitor, which is highly active as an insecticidal protein against this coleopteran,49 bioassays using highly purified PHA lectins, free of α-amylase inhibitor, also showed an insecticidal effect.13 Certain lines of *P. vulgaris* contain a related insecticidal protein, termed arcelin, which largely replaces the normal seed storage protein; arcelin shows a high level of sequence homology with PHA, but, unlike PHA, it does not possess agglutination activity. Arcelin is only insecticidal when present at high levels (> 5%) in the diet, and appears to prevent insect development by being resistant to digestion by susceptible insects.11 It is worthy of comment that *P. vulgaris* contains three different types of insecticidal protein (α-amylase inhibitor, lectin and arcelin), which are homologous in sequence, and are products of the same gene family.

Further lectins toxic to *C. maculatus*, a major storage pest of cowpeas in many parts of the developing world, have been identified in subsequent experiments. Murdoch and co-workers screened 17 commercially available plant lectins for insecticidal activity against this pest.56 Five lectins were found to cause a significant delay in larval development at dietary levels of 0-2% and 1-0% (w/w) and could be classified as those with specificity for N-acetylglucosamine/galactose (GalNAc) and those with specificity for N-acetylgalactosamine (GlcNAc). The winged bean (*Psophocarpus tetragonolobus* DC) lectin (GlcNAc-specific) was also shown to be toxic to *C. maculatus*.57 In contrast, one lectin from elderberry with specificity for GalNAc (SNA-I) was ineffective against *C. maculatus* while the other (SNA-II), with specificity for 2,6-neuraminyl-gal/GalNAc, was extremely potent.58 The rationale for using lectins specific for N-acetylgalactosamine is based on the fact that the insect midgut contains chitin, a polymer of N-acetylgalactosamine, in the peritrophic membrane.59 Of all the lectins tested against *C. maculatus* by Murdoch *et al.*, wheat germ agglutinin (WGA) was found to be the most potent.56 The same workers later identified rice and stinging nettle lectins (UDA) as being toxic to *C. maculatus*, exhibiting similar levels of toxicity to WGA; like WGA they are specific for GlcNAc.60

Czapla and Lang took a similar approach when they screened a range of lectins for activity against the Southern corn rootworm (*Diabrotica undecimpunctata howardi* Barb.), a major economic pest of corn.61 Of the lectins tested, three, from castor bean, pokeweed and green marine algae, were found to be toxic to the neonate larvae when applied topically (2%) to the artificial diet. Several others, including WGA, were found to inhibit larval growth by at least 40% when compared with larvae fed on control diet. All those lectins with insecticidal activity against corn rootworm were either specific for GalNAc or GlcNAc. The lectin from snowdrop (GNA; which is mannose-specific) was also shown to be insecticidal to this pest.62

Unfortunately, the specificity of a given lectin is not necessarily a good indicator of its potential insecticidal properties, and thus it is still necessary to test each lectin against a target pest on a case-by-case basis. For example, the toxic effects of mannose/glucose-specific lectins from different sources towards *C. maculatus* differ considerably; the lectin from garden pea has little or no toxic effect, whereas lectins from *Dioclea* spp. are significantly toxic, as is GNA from snowdrop (Gatehouse, unpublished).

##### 3.4.1.2 Lectins active against Lepidoptera

Comparatively few lectins have been tested by bioassay in artificial diet and found to be toxic to Lepidoptera. Czapla and Lang tested a range of lectins for insecticidal activity against the European corn borer, *Ostrinia nubilalis* (Hbn.).63 The lectins from castor bean (*Ricinus communis* L.), camel’s foot tree (*Bauhinia purpurea*) and...
wheatgerm (WGA), specific for GalNAc, GalNAc and GlcNAc respectively, were found to give 100% mortality after seven days when administered to neonate larvae as a 2% topical application; WGA and the lectin from R. communis were also found to inhibit larval weight gain by >50% at 0.1% topical applications. Czalpa and Lang also reported that the soya bean lectin actually increased larval weights of O. nubilalis by >25% compared with control larvae, in contrast to earlier reports where addition of this particular lectin at the 1% level was found to be detrimental to the larval growth of M. sexta, the tomato hornworm.53

3.4.1.3 Lectins active against Homoptera. The insecticidal activity of lectins against homopteran pests is now receiving much attention. Powell et al. used an artificial diet bioassay system to test a series of lectins against the rice brown planthopper (Nilaparvata lugens (Stalo)), an important pest of rice in S.E. Asia, and although some lectins (e.g. those from garden pea or potato) had no effect on insect survival, other lectins decreased insect survival significantly.54 The two most effective proteins tested were GNA (mannose-specific) and WGA (GlcNAc-specific), each of which gave approximately 80% mortality at a concentration of 0.1% w/v in the diet. The LC50 value for GNA against brown planthopper was found to be 0.02% w/v, or approximately 6 nm.65 GNA was also found to be toxic to another sucking pest of rice, the rice green leafhopper, Nephrotettix cincticeps Ish.

Habibi et al. carried out similar bioassays in order to identify lectins which may be suitable in the control of the potato leafhopper (Empoasca fabae Harr); the lectins tested were specific for glucose/mannose, GlcNAc or GalNAc.66 Of those tested, six were found to cause a significant reduction in insect survival at dietary levels of 0.2% to 1.5% (w/w). Those found to be effective were from jackfruit, pea, lentil and horse gram and also PHA and WGA. Rabhi and Febvay demonstrated that the lectin from Canavalia ensiformis (L.) DC (Con A) was a potent toxin of the pea aphid Acyrthosiphon pisum (Harr.), having a significant effect upon both survival and growth; in comparison, WGA was relatively ineffective.67 Subsequent experiments have shown that GNA is also inhibitory to aphid development. Not only did GNA cause a significant reduction in growth of the peach-potato aphid Myzus persicae (Sulz), but it also significantly reduced female fecundity.68 This effect would be significant in preventing the build-up of an insect population.

3.4.1.4 Lectins active against Diptera. To date, most reports on the effects of plant lectins on insects have concerned phytophagous insects. Recently, the effects of plant lectins on larvae of the blowfly, Lucilia cuprina (Weid.), have been assayed, in an attempt to identify possible control strategies for this pest,59 the larvae of which feed on tissue and tissue fluids of susceptible sheep. This study demonstrated that both WGA (GlcNAc-specific) and Con A (glucose/mannose-specific) caused a concentration-dependent inhibition of larval growth, and significant mortality. Of the two lectins, WGA was the most potent, resulting in 50% inhibition of larval growth at a concentration of 2 mm, and 100% mortality at 25 mm. Deleterious effects caused by the lectins could be prevented by the presence of the appropriate inhibitory sugars, suggesting a highly specific interaction.

3.4.2 Insect-resistant transgenic plants expressing lectins

The aim in producing transgenic plants expressing foreign lectin genes has been primarily that of crop protection, i.e. to exploit the insecticidal action (where demonstrated) of lectins. These experiments have provided direct evidence that lectins can play a protective role in plants against insect predators (which could not be done by artificial diet studies), and offer the possibility of being able to investigate how lectins function in concert with other defensive compounds present in plants.

A gene encoding the pea lectin (P-Lec) has been expressed in transgenic tobacco plants using the constitutive CaMV 35S promoter.70 Plants expressing the pea lectin at up to 1-0% of total protein were then tested in bioassay using H. virescens (tobacco budworm). Both larval biomass and leaf damage, as determined by computer-aided image analysis, were significantly reduced on the transgenic plants.71

A gene encoding the snowdrop lectin (GNA) has been engineered into transgenic plants; a cDNA clone described by van Damme was used in the constructs.72 Initial experiments placed the GNA coding sequence under control of the CaMV 35S promoter, and looked at expression in transgenic tobacco plants. Constructs containing a complete coding sequence for the pre-protein gave rise to levels of GNA up to 1% of total protein in leaf tissue of primary transformants, as determined by quantitative dot-blot immunoassay. Higher levels of GNA (up to 1.5%) were observed in progeny plants produced by selfing the primary transformants. 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. These plants were shown to be resistant to aphids.73

A similar construct was used to produce transgenic potato plants, which were vegetatively propagated to produce clonal replicates for bioassay. The resulting plants expressed GNA at similar levels to the transgenic tobacco, and were significantly protected against attack by larvae of the tomato moth, Lycanobia oleracea L.; although reduction of larval survival was less than 25%
in the transgenic plants, highly significant reductions in larval biomass (>50%) and in leaf damage to the plants (>70%) were observed. Similar results were obtained in growth cabinet and large-scale glasshouse trials. The GNA-expressing potato plants also reduced the fecundity of the glasshouse potato aphid, *Aulacorthum solani* Kalt., by up to 80% in laboratory assays, and significantly reduced its population build-up in a glasshouse experiment. Similar effects have been observed with the peach-potato aphid, *M. persicae*, in growthroom trials. GNA has also been specifically expressed in the phloem of transgenic plants in order to target phloem-feeding homopteran pests.

### 3.5 Transgenic plants expressing chitinases

Although mainly studied for their anti-fungal properties, chitinases are also of interest with respect to protecting crops against certain species of insect, and in particular, Homoptera. Transgenic potato plants expressing a gene encoding bean chitinase (BCH), under control of the constitutive CaMV 35S promoter, were found to reduce fecundity of the glasshouse potato aphid *A. solani*, although this reduction was not statistically significant. However, nymphs produced on these BCH-expressing plants were significantly smaller compared to those on control, non-transformed plants (Down, R. E., pers. comm.)

### 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. In order to increase the protective efficacy, spectrum of activity and durability of resistance, it is envisaged that 'packages' of different genes will be introduced into crops. The components of such packages should each act on a different target within the insect, thus mimicking the multi-mechanistic resistance which occurs in nature. Protease inhibitors should be particularly valuable in this respect since, 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. Although the insecticidal effects of the two genes were not synergistic, they were additive, with insect biomass on the double expressors being reduced by nearly 90% compared to those from control plants and 50% to those from plants expressing either CpTI or P-Lec alone. Leaf damage was also least on the double expressing plants.

It is also interesting to note that trypsin inhibitors were reported to have a marked potentiating effect on *B. thuringiensis* toxins.

Not only have protease inhibitors been used in the pyramiding of genes, but so have other insect-resistance genes. Recently, transgenic potato plants have been produced which express both snowdrop lectin and bean chitinase. The rate of population build-up of *A. solani* on these plants was significantly lower than on those plants expressing either GNA or BCH alone. The results obtained showed that pyramiding the genes encoding these particular proteins had a synergistic effect (Down, R. E., pers. comm.)

### 5 SAFETY OF INSECTICIDAL PROTEINS TO BENEFICIAL INSECTS

Before genetically modified organisms are released into the field, it is essential that their safety to the environment is fully established. Proposals to use any of the above insecticidal proteins as resistance factors in transgenic plants, as part of an integrated pest management plan, must take into account effects on beneficial insects. Such insects include parasitoids and predators which contribute to the suppression of pest populations, as well as pollinators such as bees. Several laboratories, including Durham, are now actively addressing this aspect.

### 6 CONCLUSION

There have been several clear examples that certain proteins present in plants, e.g. enzyme inhibitors and lectins, do have a protective role, particularly within storage tissues such as seeds. In addition to these, many have also been shown to be insecticidal to economically important insect pests both in artificial diet and, in a few examples, in transgenic plants. 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 world and developing parts of the world, and thus could make a considerable impact on agricultural systems in the future. A strategy that has been suggested to maximise the utility of this technology is not only to use single genes, but also to use gene combinations whose products are targeted to different biochemical and physiological processes within the insect. In this way, it is hoped to provide a multi-mechanistic form of resistance which can be tailored to the different crops and prevailing insect pests at a given time.
Engineering crops for pest resistance using plant genes provides an alternative strategy to that using genes encoding *B. thuringiensis* (Bt) endotoxins. At present, foreign plant genes do not routinely provide the same levels of protection as has been achieved in transgenic plants expressing Bt towards susceptible insects, although examples where protection is effective have been reported. Thus further basic research into improving the effectiveness of plant defensive genes will be required before insect-resistant transgenic plants expressing foreign plant genes reach the market place. Several crops expressing Bt endotoxins have recently been introduced as commercial products. Use of Bt endotoxins has been thoroughly reviewed by Peferoen, and the reader is referred to this source for further information. One potential advantage of using plant genes is their broad spectrum of activity across many different insect orders, including Homoptera. Since genes can be selected which have very different targets within the insect, use of a combination of such genes will provide multi-mechanistic protection; Bt-encoding genes, on the other hand, provide a form of protection based upon a single mechanism. However, these two approaches are not mutually exclusive, and work is being carried out to engineer crops which will express both types of insecticidal gene, to give protection against a range of pest species.

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**REFERENCES**

22. Broadway, R. M. & Duffey, S. S., Plant proteinase inhibitors: mechanisms of action and effect on the growth and


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