SESSION 10

GENETIC MANIPULATION OF PEST AND PATHOGEN RESISTANCE IN PLANTS

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PRODUCING VIRUS TOLERANCE IN PLANTS THROUGH GENETIC ENGINEERING

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Transgenic plants expressing the coat protein genes (CP+) from tobacco mosaic virus (TMV), alfalfa mosaic virus (AlMV), cucumber mosaic virus (CMV), and potato virus X (PVX) are protected from infection by TMV, AlMV, CMV and PVX, respectively. The CP+ plants contain reduced numbers of lesions on inoculated leaves, delay or absence of systemic symptom development and reduction in virus accumulation in both inoculated and systemic leaves. The extent of protection observed in CP+ plants depends on the level of expression of the coat protein. Plants expressing antisense RNA were protected only at low inoculum concentrations. In contrast to previous reports for plants expressing TMV or AlMV CP, inoculation of plants expressing high levels of PVX CP with PVX RNA did not overcome the protection. Although these results do not rule out that the mechanism of CP-mediated protection involves inhibition of uncoating of the challenge virus, they suggest that mechanisms of CP-mediated protection might be different for different viruses.

Under field conditions, tomato plants expressing TMV CP were protected from infection by different strains of TMV and tomato mosaic virus (ToMV). Tomato fruit yields of the control plants decreased due to virus infection, while yields of the CP+ plants were unaffected. Tomato fruit yields from CP+ plants were equal to the yields from uninoculated control plants, indicating that expression of the CP gene does not cause yield depression. The results of field experiments demonstrate the applicability of CP-mediated protection for general use in agriculture.

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INTRODUCTION OF GENES CONFERRING INSECT RESISTANCE

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ABSTRACT

Insect pests are responsible for heavy crop losses and high expenditure on insecticides throughout the world. In attempting to combat insect damage little attention has been given to the natural defence mechanisms of plants against their predators. One example of such is the cowpea protease inhibitor (CpTI). By cloning the gene that encodes this inhibitor and transferring it to tobacco plants, new transgenic plants resistant to several different types of major insect pests were obtained. These plants can be used directly or as parental material in breeding programmes, thereby reducing chemical damage to the environment and the very high chemical insecticide expenditure.

It is estimated that approximately 37 percent of all crops produced worldwide are lost to pests, with 13% lost to insects, 12% to disease and 12% to weeds and grasses. Thus one of the most important constraints on the yields of food and cash crops world-wide can be attributed to insect attack. In order to reduce these levels of crop damage enormous sums of money are spent annually on chemical pesticides, and in 1984 it was estimated that approximately 74 million acres around the world were sprayed with chemical insecticides to control moths alone, at a cost of $317 million. A summary of the insecticide expenditure for the 3 major crops grown world-wide, together with their major insect pests is given in Table 1. Thus the necessity and advantages of breeding crops resistant to insect attack are all too obvious, both in terms of reducing the levels of external application of chemicals, resulting in a reduction in damage to the environment, and also in terms of financial savings to the farmer and ultimately the consumer.

TABLE 1

Insecticide Expenditure, by Territory and Pest Order

CROP	COTTON					MAIZE			RICE			
Major Territory:	USSR,	USA,	CHINA,	INDIA,	OTHER,	USA,	EUROPE + USSR,	OTHER,	JAPAN,	CHINA,	INDIA,	OTHER,
Insecticide Expenditure +	295	260	195	155	635	220	114	156	460	128	108	344
Application Expenditure *	53	200	24	23	86	35	40	25	165	20	17	45
Total World Cost:			1925				590			1287		
Major Pest Order			Lepidoptera				Coleoptera			Homoptera		

Source: + Wood Mackensie; * AGC Ltd (estimates). 1987 expenditure, values given are x 10^6

One of the major objectives of the plant breeder and biotechnologist is to produce insect resistant plants wherein resistance is durable. This is best achieved by a multimechanistic approach and an understanding of the underlying mechanisms involved. Particular advantages genetic engineering offers over conventional plant breeding is firstly that the desired gene(s) can be transferred to the recipient plant without the co-transfer of undesirable characteristics and secondly, and perhaps of more importance, is that this technology enables the transfer of genes across species barriers. Furthermore, depending upon the site of attack within the plant externally applied insecticides may be relatively ineffective unless systemic reagents are used.

Although the techniques for introducing foreign genes into many crop plants are now becoming routine, identification of useful genes to be transferred is perhaps the most limiting factor with this approach. Examples which are of agricultural importance include herbicide resistance (Comai *et. al*, 1985; Shah *et. al*, 1986) and virus resistance (Baulcombe *et. al*, 1986; Abel *et. al*, 1986). To date, examples where genes conferring insect resistance have been transferred by this approach are limited to the *Bascillus thuringiensis* endotoxin genes (Vaeck *et. al*, 1987, Fischhoff *et. al*, 1987) and the cowpea protease inhibitor gene (Hilder *et. al*, 1987).

Several general mechanisms for plant protection against insects which confer field resistance to a wide range of pests exist in nature and our strategy has been to attempt to identify and exploit these. Although many secondary plant compounds such as alkaloids e.g. castanospermine, DMDP (Evans, Gatehouse & Fellows, 1985; Nash *et. al*, 1986), isoprenoids e.g. gossypol, and non-protein amino acids have been implicated in conferring insect resistance, these classes of compounds are not at present available to the genetic engineer, should they be considered suitable, owing to their complex biosynthetic pathways. Suitable candidates are therefore limited, in the main, to proteins, i.e. primary gene products. One such mechanism involves the trypsin inhibitors from cowpea (*Vigna unguiculata*).

The cowpea is one of the principal grain legumes of West Africa and the north-east of South America where it provides a major source of dietary protein (FAO, 1970). Loss during storage due to the bruchid beetle *Callosobruchus maculatus* F. is unacceptably high, with up to 100% seed damage after 5 months storage (Singh, 1978). In view of these serious losses a breeding programme was established at the International Institute of Tropical Agriculture (IITA) in Nigeria to select for resistance against this pest. Out of 5000 accessions only one, TVu 2027, showed significant levels of resistance towards the larvae of this pest (Redden, Dobie & Gatehouse, 1983). This resistant variety therefore provided a potential source of insect resistance genes. After establishing that resistance did not have a physical basis, seeds of the resistant variety TVu 2027 and seeds of several different susceptible varieties were screened for a range of secondary compounds including endopeptidase inhibitors (trypsin and chymotrypsin inhibitors), exopeptidase inhibitors, lectins (phytohaemagglutinins), saponins, alkaloids and the major non-protein amino acids. Of all these antimetabolic secondary compounds screened for, only inhibitory activity against trypsin and, to a much lesser extent chymotrypsin, could be detected. The resistant variety of cowpea contained significantly higher levels of inhibitors, at least as twice as much as any other variety and in the order of 3 to 4 times more than the majority of the varieties tested (Table 2).

TABLE 2

Performance of developing *Callosobruchus maculatus* larvae, and the physiological concentration of trypsin inhibitors in seeds of different varieties of cowpea (*Vigna unguiculata*).

Variety	% Adult emergence ± 1 S.D	Trypsin inhibitor content (% w/w)	TIU mg per seed $x10^3$
TVu 2027	0.0 ± 0.0b	0.92	22.7
TVu 4557	95.1 ± 6.2a	0.44	11.0
TVu 76	90.0 ± 2.5a	0.34	8.3
TVu 3629	90.6 ± 9.5a	0.30	7.3
TVu 37	86.6 ± 4.3a	0.26	6.3
TVu 57	91.7 ± 6.7a	0.25	6.2
TVu 1190E	89.0 ± 6.7a	0.23	5.6
TVu 1502-1D	92.0 ± 6.0a	0.19	4.6

All figures followed by the same letter are not significantly different at the 5% level of significance (Duncan's Multiple Range Test).

Electrophoresis on polyacrylamide gels and isoelectric focussing of the purified inhibitors from the above cowpea varieties showed essentially the same banding patterns and thus it was concluded that differences in the inhibitor content of the resistant variety were qualitative and not quantitative.

The antimetabolic properties of the purified cowpea trypsin inhibitor was demonstrated in feeding trials with the larvae of *C. maculatus* (Gatehouse *et. al*, 1979). The basal diet was autoclaved cowpea meal from a susceptible variety, and this was supplemented with various protein fractions. Initially, a protein extract of cowpea TVu 2027 was fractionated into albumin and globulin fractions, the former containing about 98% of the trypsin inhibitory activity. Addition of 10% albumin fraction (equivalent to approx. 0.85% trypsin inhibitor) to the basic meal caused considerably reduced larval survival, whereas the globulin fraction at the same concentration had no effect. Removal of the trypsin inhibitor from the albumin fraction by affinity chromatography resulted in a loss of toxicity. Further feeding trials were carried out by adding the purified inhibitor at a range of concentrations. At a level of 0.1% the inhibitor had no deleterious effects upon larval development, however at 0.5% survival was reduced by approx 75%; at a level of 0.8%, which is marginally lower than the physiological concentration found in the resistant seeds, no larvae survived (Table 3). These results confirm that the trypsin inhibitors play a major role in conferring seed resistance in this particular example of 'field' resistance.

Of prime importance to the biotechnologist before exploiting this form of insect resistance is the question of how broad a spectrum of insects will this protein be effective against, and will it be toxic to those pests the breeders want to protect the crop from? The purified protein was therefore tested in artificial diets against a wide range of both field and storage insect pests, including members of the lepidoptera such as *Heliothis*

and *Spodoptera*, and coleoptera such as *Diabrotica* (Fig. 1) and *Anthonomus*, all of which cause crop losses of major economic importance. In all cases the cowpea protease inhibitor (CpTI) was found to be an effective insecticide. As one of the prime metabolic targets of the inhibitor is the catalytic site of an enzyme, the ability of the insect to evolve a resistance mechanism based on mutation at this site should be minimal. A list of insect pests against which CpTI was found to be toxic is given in Table 4. Thus CpTI was found to be toxic against those pests of major economic importance, i.e. against pests of cereals and cotton where insecticides account for the majority of the world insecticide expenditure. Despite its insecticidal properties, there is no suggestion that cowpea seeds are toxic to humans, and although normally cooked, they can be eaten raw (Peterson, 1984). Recent feeding trials showed that the inhibitor in untreated cowpea seed meal did not adversely affect the growth of rats (unpublished data).

TABLE 3

Survival of *Callosobruchus maculatus* larvae on different treatments of cowpea meal

Treatment	% Survival to adult
Susceptible cowpea meal (Control)	78.1+
Cowpea meal + 10% albumins	32.8
Cowpea meal + 10% globulins	75.8+
Cowpea meal + 10% albumin minus T.I.	74.9+
Cowpea meal + 0.1% T.I.	80.0+
Cowpea meal + 0.5% T.I.	20.1
Cowpea meal + 0.8% T.I.	0

Cowpea meal in all treatments was autoclaved to remove endogenous trypsin inhibitory activity. T.I. = trypsin inhibitor. Figures marked + are not significantly different at the 5% level of significance (Duncan's Multiple Range Test); other results for survival are significantly different from those marked + at a level of significance of 5% or less.

Fig. 1.

Survival of *D. undecimpunctata* larvae on artificial diet.
—●— control; —○— + 5% CpTI

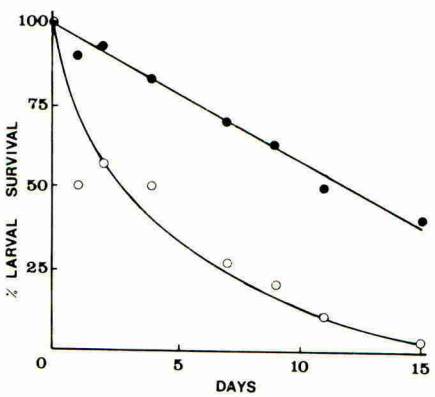

TABLE 4

Summary of insect-bioassays on artificial diets and on CpTI transgenic tobacco plants against a range of insect pests

	Insects killed by CpTI in artificial diets	CpTI transgenic tobacco plants resistant to following insects:
Lepidoptera:	*Heliothis virescens* (tobacco budworm)	*H. virescens*
	H. zea (corn earworm)	*H. zea*
	Spodoptera littoralis (army worm)	*Spodoptera littoralis*
	Chilo partellus (stem borer)	*
	Manduca sexta (tomato hornworm)	*Manduca sexta*
Coleoptera	*Anthonomus grandis* (boll weevil)	*
	Diabrotica undecimpunctata (corn rootworm)	*
	Tribolium confusum (confused flour beetle)	*
	Callosobruchus maculatus (cowpea seed weevil)	*

* insects unable to attack control tobacco plants.

Chemical studies showed the cowpea trypsin inhibitors (CpTIs) to be small polypeptides of around 80 amino acids belonging to the Bowman-Birk type of double-headed serine protease inhibitors and to be products of a repetitive gene family (Gatehouse *et. al*, 1980). This, together with the finding that they are effective against a broad spectrum of insect pests make the CpTIs ideal candidates for genetic transformation. In the present study the crop plant chosen for transformation was tobacco (*Nicotiana tabacum* c.v. Samsun N.N.). The CpTI gene which was used was derived from plasmid p USSRc3/2, a member of a complementary DNA library prepared from cowpea cotyledon polyadenylated RNA (Fig. 2). A 550-base-pair (bp) long *Alu* 1 - *Sca* 1 - restriction fragment containing the entire coding sequence for the mature protein, a long leader sequence and the majority of the 3'-non-translated sequence was transferred to the *Sma* 1 site of *Agrobacterium tumefaciens* Ti plasmid binary vector, pROK 2 (Baulcombe *et. al*, 1986). This placed the cowpea sequence under the control of a strong constitutive promoter derived from cauliflower mosaic virus (Guilley *et. al*, 1982) and the nopaline synthase gene-transcription termination sequence (Bevan *et. al*, 1983). Constructs were identified which contained an insert in the correct orientation relative to the CaMV promoter to produce CpTI (pROK/CpTI + 5) and in the 'reverse' orientation (pROK/CpTI-2), which has six short open reading frames with no identifiable features. This 'reversed' construct was used to produce control transformants. These constructs were then mobilised into *A. tumefaciens* (Bevan, 1984) and used to transform leaf discs of *N. tabacum*. The transformants were selected by their antibiotic resistance to kanamycin, and the transformed plants were regenerated from shootlets by transfer to a root-inducing, kanamycin-containing agar medium (Horsch, 1985). Rooted plants were grown on in soil-based compost.

These transformed plants showed no phenotypic abnormalities.

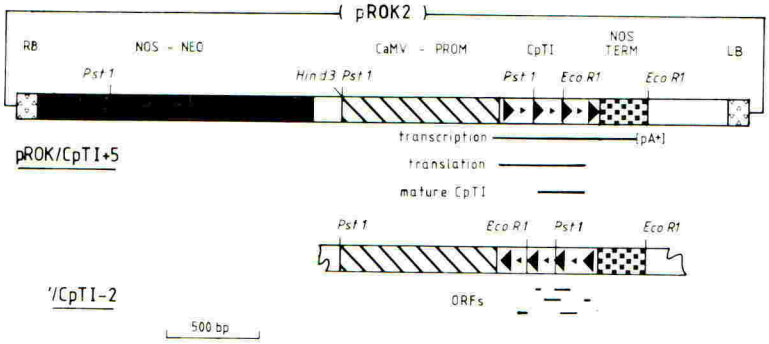

Fig. 2. Structure of the CpTI gene transfer/expression constructs.

The presence and levels of CpTI production in the original transformants was measured by dot-immunobinding assays (Jahn *et. al*, 1984) using polyclonal antibodies raised in rabbits against total CpTI. The level of expression in young leaves from different individual pROK/CpTI + 5 transformants ranged from below the limit of detection to ~ 1% of total soluble protein; using the CaMV 35S gene promoter this range in levels of expression is expected. No CpTI expression could be detected in the pROK/CpTI-2 transformants i.e. when the gene was inserted in the incorrect orientation no inhibitor was produced (Hilder *et. al*, 1987). Western blotting of soluble leaf proteins from CpTI expressing transformants showed that polypeptides produced and processed in the transformants corresponded to one of the isoinhibitors present in the cowpea seed; no corresponding polypeptides were produced in the control transformants. The functional integrity of the CpTI produced in these transformed tobacco plants was demonstrated by *in vitro* trypsin inhibitor activity assay. Thus the transformed tobacco plants were able to express the foreign CpTI gene and produce an active trypsin inhibitor whose levels of expression in the highest expressing plants were similar to that present in the mature seeds of the resistant variety of cowpea, TVu 2027.

The critical test on these CpTI expressing transformed tobacco plants was the bioassay to test their respective levels of insect resistance or tolerance. This was carried out by infesting the young plants with newly emerged larvae of the lepidopteran *Heliothis virescens*. The infested plants were sealed into individual plantaria and kept under controlled light and temperature regimes within a growth cabinet. *H. virescens*, the tobacco budworm, was tested in the first instance as it is classified as a serious economic pest, one of whose primary hosts is tobacco. After a trial period of seven days all larvae (both dead and surviving) were removed, their size recorded and the extent of leaf damage measured by computer aided image analysis. The results clearly showed that those CpTI transformants which expressed the foreign protein at approximately 1% were relatively resistant to attack compared to control plants. Some of the transformants showing enhanced levels of insect resistance and some of the control plants were replicated as stem cuttings (Baulcombe *et. al*, 1986) to

provide sets of genetically identical plants on which statistically sound insect feeding trials could be run. These further trials provided convincing evidence that the CpTI-producing plants were much more resistant to insect attack. Control plants were devasted by this level of infestation; in trials which we ran beyond seven days, i.e. to 'termination' these control plants were reduced to a stalk. However, on the CpTI-producing plants, although the larvae begin to feed and do some very limited damage to the leaves, they either die or fail to develop as they would on control plants (Fig. 3). This observation is consistent with the mechanism of CpTI toxicity proposed by Gatehouse and Boulter (1983) relying upon a finely controlled balance within the host plant which has to make sufficient nutrients for itself but insufficient to maintain predation, thus the larvae die at a very early stage by starvation.

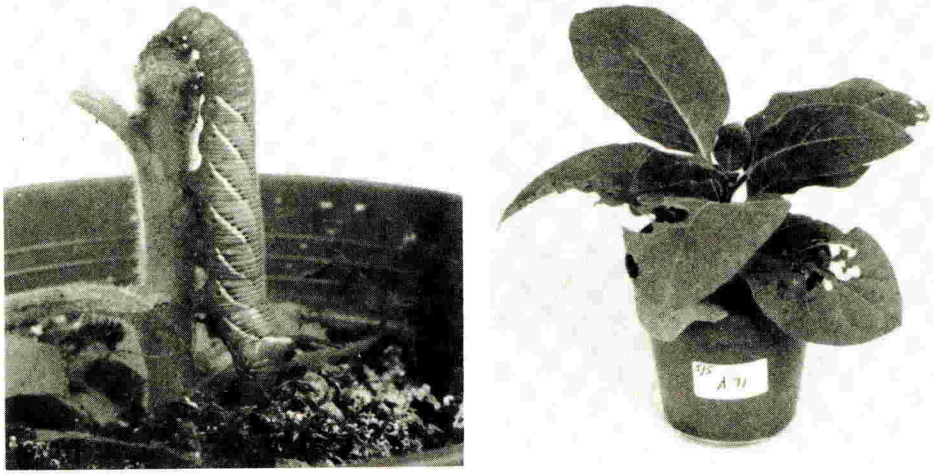

Fig. 3. Effect of *M. sexta* larvae on *N. tabacum* transformed with CpTI + 5 and CpTI-2 constructs. The plant on the left is a clonal replicate of a CpTI-2 line; on the right, a CpTI-expressing CpTI + 5 line.

The CpTI gene was shown to be stably inherited through subsequent generations, and these plants at each generation were also screened for insect resistance. As with the original clonal CpTI transformants, these seed derived plants were resistant to insect attack.

Although the initial bio-assays of the transformed plants were carried out using *H. virescens*, trials were subsequently carried out using *H. zea* (corn earworm) *Spodoptera littoralis* (armyworm) and *Manduca sexta* (tomato and tobacco hornworm). In all cases the CpTI transformants were resistant to attack compared to control plants; routinely ~75% larvae die within 2-3 days. Unfortunately, it was not possible to carry out screening trials on these plants using any members of the coleoptera which we are interested in, since they would not attack the control plants; in these instances the only information available on the toxicity of CpTI is from artificial diets.

A question of prime importance which has to be addressed when introducing foreign gene(s) into plants is will they have any adverse effects upon the plant itself? We are currently attempting to address this question systematically, primarily by analysis of yield etc. of a large number of transformed plants, both non and high expressers of CpTI, and also in non-transformed controls under a range of regulated growth conditions. Our preliminary conclusions are that transformation may have marginal effects on yield but these should easily be outweighed by the benefits of the transformed character; this is supported by the results on virus resistant transgenic tomatoes (Nelson *et. al*, 1988).

To date, the only other work relating to the introduction of foreign genes to confer insect resistance by genetic engineering has been to use a gene of bacterial origin. During sporulation *Bacillus thuringiensis* produces intracellular crystals which, under the alkaline conditions of some insects' midguts are hydrolysed so releasing proteins of 65,000 - 160,000 M_r; these proteins are then proteolytically processed to yield smaller toxic fragments. For more than 20 years various formulations of the *B. thuringiensis* (Bt) toxins have been used to spray crops (Dulmage, 1980) but their commercial use is limited by high production costs and the instability of the crystal proteins when exposed in the field. Recently Vaeck *et. al*, (1987) transformed tobacco plants with a Bt gene which was selected on the basis that its products were toxic to the lepidopteran *Manduca sexta*. Interestingly, none of these plants produced insecticidal activity above levels obtained in transformants possessing the antibiotic marker gene on its own. However, when a modified truncated form of the gene (Bt 884) was introduced, clear insecticidal activity was detected in most plants expressing the gene, of which two-thirds induced more than 75% larval mortality (Vaeck *et. al*, 1987). Shortly afterwards Fischhoff *et. al*, (1987) successfully transformed tomato plants with a lepidopteran specific protein gene from *B. thuringiensis*. In this study the authors found that on the best performing transformants (pMON9711) all larvae of *M. sexta* were killed within 72 hours, with little leaf damage to the plant. They subsequently showed some of their plants to be resistant to attack by *Heliothis virescens* larvae and one plant tested was shown to be tolerant, though to a lesser extent, to *H. zea*. In addition to damaging the foliage of tomato plants, *Heliothis* species can do considerable damage to the tomato fruit, making it unmarketable. Newly hatched larvae typically feed on the foliage before feeding on the fruit, so Bt expression in the leaves might be sufficient to reduce or eliminate fruit damage. However, Fischhoff *et. al*, (1987) claim to have preliminary evidence that some Bt activity is detectable in the fruit from their transgenic plants; furthermore, *H. virescens* larvae fed on these particular fruits gained weight at only half the rate of larvae fed fruit from non transformed plants. Whether such fruits would be commercially acceptable remains to be seen.

One of the major drawbacks of using Bt genes to confer insect resistance is their specificity. In order for this technology to be exploited to give a broad spectrum of field protection to crops, strains of *B. thuringiensis* active against all insects to be controlled have to be identified. At present commercial Bt insecticides are effective against more than 50 lepidopteran pest species (Wilcox *et. al*, 1986). Unfortunately considerably less have been identified to give protection against coleoptera (Herrnstadt *et. al*, 1986), a family of insect who are responsible for the majority of the insecticide expenditure of maize. Comprehensive protection using this approach may have to rely on a large number of genes being

transferred to the required crops. In this respect mechanisms of resistance derived from plants which appear to be rather less specific in their action, such as CpTI (Table 4), might have advantages where a crop has, or potentially has, more than a single insect pest.

In conclusion, we have demonstrated that expression of a CpTI gene in tobacco plants leads to enhanced resistance to several major economic insect pests, at least in laboratory trials. Incorporation of this gene into other agronomically important crops, such as cotton, maize and rice should soon be technologically feasible (Umbeck *et. al*, 1987; Graves & Goldman, 1987), although the ultimate effectiveness of this method can only be determined by testing for resistance in the field.

The strategy we have adopted in Durham is to identify, and exploit the plants' own natural defence mechanisms. Plants and herbivorous insects have coevolved for around 400 million years. During this time plants have evolved multiple, effective mechanisms for limiting the damage done by the herbivores they are exposed to (and from which they have no other means of escape) to a tolerable level, whilst insect pests have evolved sophisticated mechanisms for overcoming them. Genetic engineering allows us to transfer such resistance mechanisms across huge physical and evolutionary distances. We have identified several such genes which affect the metabolism and physiology of the pest in very different ways. These will be transformed in a 'package' into the desired crop to provide a multimechanistic basis for resistance.

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APPROACHES TO MANIPULATING FUNGAL RESISTANCE IN PLANTS

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ABSTRACT

Transformation of plants with genes coding for antifungal proteins is a promising approach to manipulating fungal resistance. Plant genes involved in active defense against pathogens may be used in such an approach; genes involved in pathogen recognition (resistance genes) as well as genes coding for antifungal defense functions (defense genes) are of interest. Microbes or animals could also provide potential resistance genes. A better understanding of the biochemical functions of proteins with an antifungal potential as well as of intracellular protein targeting is required to make good use of such genes.

INTRODUCTION

It has always been an important goal of plant breeding programs to incorporate genetically based resistance against pathogenic fungi into crop plants. Breeders have identified, by genetic analysis, many different "resistance genes" in a variety of plant species. Most frequently, these resistance genes are dominant and highly specific, conferring resistance only against specific races of one specific pathogen. These resistance genes appear to be responsible for "recognition" of a race of a pathogen in a similar way as antibodies recognize foreign organisms in animals (Vanderplank 1978, Ellingboe 1981).

"Recognition" is followed by a dramatic change of gene expression in the plant cells. Many of the activated genes appear to be involved in defense against pathogens and have therefore been termed "defense genes" (Chappell & Hahlbrock 1984). Among them are enzymes involved in the production of phytoalexins, in lignin biosynthesis, and in the degradation of fungal cell walls; they are elements of the active defense response that inhibits or destroys an invading fungus (Collinge & Slusarenko 1987).

The development of transformation systems for many crop plants has opened the way to introduce new genes for pathogen resistance. The new genes could come from other plants as well as from microorganisms or animals. Here, both the prospects of introducing resistance genes or defense genes from one plant species into another and the possibilities to incorporate potentially antifungal non-plant genes are discussed. The discussion is necessarily somewhat speculative since, according to the available literature, successful transformation of a plant to increased fungal resistance has not yet been achieved.

PLANT GENES INVOLVED IN PATHOGEN RESISTANCE

Resistance genes

Typically, a resistance gene is identified by breeders as a dominant Mendelian trait conferring resistance against a specific race of a specific pathogen. If genetic analysis on the side of the pathogen is possible, it is generally observed that avirulence of these pathogen races is a single dominant Mendelian trait as well. In well-studied plant-pathogen interactions, there may be dozens of different resistance genes in the plant, with corresponding dozens of different avirulence genes in the pathogen; this pattern is called a gene-for-gene relationship (see Vanderplank 1978, Ellingboe 1981). When a plant has no resistance gene matching any of the avirulence genes of a pathogen, then it is susceptible to that pathogen. The interaction is compatible, i.e. it leads to disease. However, when the resistance gene of a plant matches the avirulence gene of a pathogen, the resulting interaction is incompatible: the plant displays an active defense response and eliminates the pathogen. Frequently, this defense response, the so called hypersensitive response, is at least superficially identical for all the different matching resistance-avirulence interactions. In the hypersensitive response, a large number of defense genes are activated; it culminates in the death of a small group of plant cells around the invading pathogen. A simple physiological model of a gene-for-gene system is that the product of a given avirulence gene of a pathogen is specifically recognized by the product of the corresponding resistance gene in the plant (see De Wit 1987). In this model, the avirulence gene product can be called a specific elicitor of the defense reaction, the corresponding resistance gene product a specific receptor of the elicitor. Elicitor-receptor interaction subsequently leads to the activation of a number of defense genes.

Although there has been much progress in the identification and characterization of specific avirulence genes of pathogens, particularly of bacterial pathogens, none of the resistance genes has been cloned so far. Therefore, it has not been possible to substantiate or disprove the elicitor-receptor model. Nevertheless, one might ask to what extent such a resistance gene could be transferred to other plants to make them more resistant, if it had been cloned.

Predictably, a resistance gene that is presently incorporated into new lines of a crop by conventional breeding could be introduced also by genetic engineering. However, there will be the same kinds of limitations that also arise in conventional breeding (Vanderplank 1978):

- Resistance genes are highly specific in that they usually act only against one specific pathogen species, and even there only against specific races of the pathogen.

- Resistance in gene-for-gene systems breaks down easily, due to the evolution of new pathogen strains lacking the corresponding avirulence gene.

- Resistance genes directed against one pathogen may cause susceptibility to a different pathogen. For example, the Pc-2 gene developed in oats against crown rust (Puccinina coronata) protects the plant against races of P. coronata but at the same time makes the plants susceptible to Helminthosporium victoriae (see Scheffer & Livingston 1984).

It is difficult to predict the effect of transferring resistance genes across breeding barriers from one species to another. Resistance genes are most frequently studied in diseases caused by highly host-specific pathogens. Since these resistance genes operate only against a single biotype of a single pathogen species within its host, one might expect that they are ineffective against all the different pathogens of a different plant species. However, it is well possible that a given avirulence gene of a host-specific forma specialis of a pathogen is also present in the related formae speciales that are pathogenic for other plants. Considering the evolution of pathogens, each avirulence gene most probably has a positive function for the pathogen in colonization of host plants lacking the corresponding resistance genes; otherwise, one should expect that avirulence genes are rapidly eliminated from a pathogen population interacting with a plant population in which the corresponding resistance gene occurs (Vanderplank 1978).

Defense genes

As mentioned above, plants display an active defense response after recognition of a pathogen. While recognition is highly specific, the active defense response is not; recognition of many different pathogens, fungi, bacteria, and viruses, elicits the same type of response (Collinge & Slusarenko 1987). One part of this defense response is the activation of a large number of genes, the so-called defense genes. As many of these genes and their products are now being characterized, it is interesting to evaluate their potential use for plant protection.

Genes of the enzymes for phytoalexin biosynthesis.

Among the "defense genes", the genes coding for enzymes of phytoalexin biosynthesis have received particular attention (Ebel 1986). Phytoalexins are antibiotic substances that accumulate in plants in the course of the active defense response. In legumes, flavonoids are the most common phytoalexins. Analysis of the biosynthetic pathway of flavonoid phytoalexins is well under way. For phenylalanine ammonia-lyase and chalcone synthase, two key enzymes of the pathway, cDNA clones and genomic clones have been characterized.

A problem in the use of such genes is the complicated biosynthetic pathway of many phytoalexins. In the case of the glyceollins, the phytoalexin of soybeans, for example, five enzymes are necessary to produce the precursor 4-coumaroyl-CoA from phenylalanine, and seven more enzymes are needed to convert this compound into the glyceollins (Ebel 1986). All these enzymes appear to be co-ordinately induced in the defense response of soybeans. The complexity of this and many other biosynthetic pathways for phytoalexins makes a complete transfer of a phytoalexin pathway from one plant to another unrealistic at the present time.

Assuming nevertheless for a moment that it would be possible to isolate, characterize and transfer the whole set of genes needed for biosynthesis of a given phytoalexin, it is interesting to discuss how these genes could be employed. Studies of phytoalexin accumulation in various plant-pathogen interactions have frequently shown that phytoalexins reach high local levels earlier in incompatible than in compatible interactions (see Ebel 1986). This has been taken as an indication that phytoalexins are effective in defense only if they reach toxic levels before the pathogen can establish itself in the plant. At first sight, then, constitutive synthesis of phytoalexins would appear to be valuable for defense. Unfortunately,

however, phytoalexins are toxic not only for microorganisms, but also for animals and even for the plant itself. This renders constitutive synthesis of phytoalexins unrealistic from the point of view of producers and consumers. A more refined strategy would be to aim at acceleration of phytoalexin accumulation by constitutive expression of the kinetically limiting enzyme or enzymes. This would require information about levels of substrates and intermediates and about the flux through the phytoalexin pathway, information that is presently unavailable. A better understanding of the biochemistry of phytoalexin accumulation is therefore essential to make optimal use of the genes involved in phytoalexin biosynthesis.

Typically, phytoalexins are specific for a plant species. Pathogens of a given plant species frequently possess enzymes capable of detoxifying the phytoalexins of that species (Van Etten et al. 1982). It is conceptually attractive to change or extend the phytoalexin pattern of a given plant to confer resistance against the pathogens that are adapted to its native repertoire of phytoalexins. It might be possible to achieve this by incorporating just one or two genes coding for the last enzymes of the biosynthetic pathway of a phytoalexin from a related plant that shares the initial part of the pathway. Since the substrate for these late enzymes would not normally be present, it should be possible to express them constitutively.

Genes of proteins with a direct antifungal potential.

Among the defense genes activated in response to a pathogen attack are chitinases and ß-1,3-glucanases (see Boller 1987, 1988). It has been shown that plant chitinase has a potential to inhibit fungal growth, using Trichoderma viride, a saprophytic fungus, as a test organism (Schlumbaum et al. 1986). However, most fungi are insensitive to chitinase alone; in contrast, many fungi are inhibited by combinations of chitinase and ß-1,3-glucanase (Mauch et al. 1988). In pea pods and bean leaves, we found chitinase and ß-1,3-glucanase to be the principal antifungal activities in protein extracts (Mauch et al. 1988). A combined transfer of both chitinase and ß-1,3-glucanase genes to a plant should therefore be most promising. Both enzymes have been purified from various sources, and several cDNA clones and genomic clones are available (see Boller 1988). How could chitinase and ß-1,3-glucanase genes be employed optimally to confer resistance to a susceptible plant?

Many successful pathogens are not "recognized" by the plant and therefore do not induce the defense response. In these cases, constitutive expression of the two enzymes might make a plant resistant. Constitutive expression would appear to be harmless for consumers since chitinase and ß-1,3-glucanase are expected to be non-toxic to vertebrates. A possible toxicitity of chitinase to insects might be an additional benefit. However, the possibility that constitutive expression of chitinase and/or ß-1,3-glucanase is interfering with the metabolism of the plant itself should be of concern. Chitin is not present in plants; therefore, a priori, there is no reason to expect problems with chitinase. However, ß-1,3-glucans occur in plants, and constitutive expression of ß-1,3-glucanase might alter plant growth and development. For example, pollen tubes cell walls consist primarily of ß-1,3-glucans; constitutive expression of ß-1,3-glucanase in the male gametophyte or in the style tissue might alter pollen tube growth and prevent fertilization.

Redirection of the subcellular distribution of chitinase and β-1,3-glucanase is another promising approach. Chitinase has been found to be present in two different cellular locations. In cucumber leaves, the predominant chitinase is localized in the extracellular space (Boller & Métraux 1988). There, it is in a position to interact directly with invading fungi. In bean leaves, in contrast, the predominant chitinase is present in the vacuoles (Boller & Vögeli 1984). This enzyme does not come into contact with fungi that enter the tissue through the intercellular spaces. Vacuolar enzymes can interfere with fungi only when the cell disintegrates and releases its contents, a process that occurs in the course of the hypersensitive response for example. The advantage of such a deployment of chitinase might be that, in general, a fungus has less possibilities to adapt to the sudden massive increase in chitinase activity occurring during the hypersensitive response than to the gradual increase of activity of extracellular chitinase. It will be interesting to investigate the effect of switching the localization of antifungal hydrolases in a given plant. A better understanding of protein traffic in the plant cell is highly important to target these proteins for export to the vacuole or to the cell wall.

Many pathogens elicit an active defense response and thereby cause accumulation of chitinase and β-1,3-glucanase as well as of phytoalexins but nevertheless colonize their host plant. They obviously have the capability to neutralize the plant's defenses. Enzymes detoxifying phytoalexins have received much attention; less is known about neutralization of antifungal hydrolases. In one instance, a pathogen has been found to possess proteinaceous inhibitors of plant β-1,3-glucanase (Albersheim & Valent 1974). If such inhibitors prove to be of general occurrence, they might be of similarly high specificity as are the well-studied protease inhibitors; pathogens might possess inhibitors for the hydrolases of their host plant, but these might be inactive against the same activities from different plants. In view of this, it is interesting that cucumber chitinase has been found to be very different from bean chitinase with regard to its physical, biochemical, and immunological properties, although the catalytic activity is the same (Métraux & Boller 1986). Exchanging the chitinase genes between these plants might be a promising approach to confer resistance, since bean pathogens might be unable to cope with cucumber chitinase and vice versa.

It should be noted that a large group of very successful pathogens appears to be out of reach of chitinase: the Oomycetes have cell walls with cellulose instead of chitin as a main structural element (see Wessels & Sietsma 1981). Obviously, enzymes capable of degrading the cell walls of Oomycetes will be of interest; however, if cellulase is needed to lyse these fungi, as should be expected, it might be difficult to make use of cellulase genes for defense without interfering with the stability of the cell walls of the plant itself.

GENES WITH A POTENTIAL FOR ANTIFUNGAL ACTION FROM OTHER SOURCES

Antifungal hydrolases

Chitinase and β-1,3-glucanase genes from microbial sources might be as useful as the corresponding plant genes for use in genetic engineering. In fact, a priori, microbial genes appear to be particularly promising since plant pathogens are expected to be less well adapted to the microbial than to the plant enzymes. Recently, a chitinase gene from Serratia marcescens has been introduced into tobacco plants, using a promoter of the small

subunit of ribulose bisphosphate carboxylase (Jones et al. 1988). The bacterial chitinase was expressed constitutively in the plant, reaching levels as high as 0.25 % of the total protein. Since the tobacco plant used had already high levels of endogenous chitinase in the leaves, bacterial chitinase contributed only an additional activity of about 30 % of the endogenous chitinase. It remains to be seen if this bacterial chitinase can contribute to resistance against fungi. It has been shown that Serratia marcescens mutants lacking chitinase have less antagonistic potential against fungi than do wild type strains (Jones et al. 1986). However, a recent direct comparison of different chitinases with regard to their antifungal activity has shown that bacterial chitinases, including that of Serratia marcescens, are at least 50 times less effective than plant chitinases (Roberts & Selitrennikoff 1988). This may be related to the different enzymatic properties and ultimately to the different functions of these chitinases. Plant chitinases probably function only in defense; they are endochitinases, attacking the substrate from within. Bacterial chitinases probably function mainly in nutrition; they are exochitinases, releasing chitobiose from the nonreducing end of chitin. Therefore, bacterial chitinase genes might be of little use as genes for resistance (Roberts & Selitrennikoff 1988). A more promising source for hydrolase genes might be the fungi themselves. Fungi need cell wall degrading enzymes in the course of their own growth and development, for example in hyphal tip extension, branching and spore formation. They may possess little protection against a sudden confrontation with excessive levels of their own cell wall degrading hydrolases.

Immunoglobulins

Animals use immunoglobulins for recognition of pathogenic microorganisms. Immunoglobulins directed against vital extracellular activities of a pathogen, expressed at the right place in the plant, could act as highly specific and non-toxic resistance factors. An example may serve to illustrate the potential and the problems of this approach. Many pathogenic fungi invade the plant through the cuticle; they need a specific cutinase to invade a plant. Fungal mutants lacking cutinase are non-pathogenic, except if they are allowed to enter through wounds (Kolattukudy 1985). Specific antibodies have been raised against the cutinase from Fusarium solani f.sp. pisi. It has been demonstrated that these antibodies, when brought on the surface of pea plants, prevent infection by F. solani f.sp. pisi (Maiti & Kolattukudy 1979). Thus, a gene for an immunoglobulin directed against cutinase might act as a resistance gene, provided the gene product could be directed to the surface of the cuticle and maintained there in an active form. The obvious advantage of an immunoglobulin gene as a resistance gene would be its high specificity; a priori, there should be no problems with unwanted side effects. An equally obvious problem would be the required specific localization of the immunoglobulin. Even assuming that an immunoglobulin could be maintained in a stable form on the plant surface, it is presently unknown how a protein could be targeted to this location. This highlights a general problem with many approaches to specific deployment of potential resistance genes, as has already been mentioned: Basic knowledge about protein traffic in plants is urgently needed, so that the gene products can be targeted to the correct cellular and subcellular locations in the plant.

CONCLUSION

Scientists interested in introducing novel genes for insect resistance had an obvious gene product of choice for their experiments, the toxin of Bacillus thuringiensis. This is a protein with a well-known, direct insecticidal action; it kills various insects but is harmless to vertebrates and plants; in addition, plants possess no related proteins, and parasites are consequently not adapted to its occurrence in plants. Therefore, work has concentrated on this protein and has already yielded transgenic plants with increased insect resistance.

There are no known non-plant proteins with a similarly potent direct action against fungi. Thus, one cannot expect a similarly rapid success in the area of fungal resistance. Nevertheless, there are approaches that might be successful in the longer run. One strategy is to make use of the plants' own repertoire of defenses against fungi. Here, the resistance genes are of great interest; they do not provide general resistance to pathogens but may be valuable in individual, specific disease problems. Defense genes are also promising; however, it may frequently be necessary to transfer genes for more than one protein from one plant to another to be successful. Constitutive expression of defense genes is probably unsuitable in many cases because the defense gene products may interfere with the plant's own metabolism; therefore, the inducible strong promoters of some of the defense genes will be highly valuable in such projects. Novel resistance genes could also come from microbes or animals. However, before such genes can be exploited, much more basic knowledge is needed in two general areas. First, there should be a better knowledge of the function and mechanism of proteins with a potential in fungal resistance. For example, bacterial chitinase, which has received much attention, appears to be poorly suited as an antifungal enzyme since it has a function in nutrition rather than in defense. Second, there should be a thorough understanding of intracellular transport and targeting of proteins. To be effective against fungi, proteins with an antifungal potential, as for example immunoglobulins directed against specific vital enzymes of a pathogen, should be placed in precisely defined locations.

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