Instituto Juan March
de Estudios e Investigaciones

10 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Engineering Plants Against Pests and Pathogens

Organized by

G. Bruening, F. García-Olmedo and F. Ponz

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I N D E X

PROGRAMME

ENGINEERING PLANTS AGAINST PESTS AND PATHOGENS

MONDAY, January 11th

Opening. F. Ponz

Session I
Chairperson: T. Boller

J. Dangl - *Genetic Approaches to Specific Resistance of Arabidopsis Thaliana Against Phytopathogenic Pseudomonads.*

J. Ryals - *Toward the Genetic Engineering of Fungal Resistance in Crops.*

F. Meins - *The Biology of Plants Transformed with Chimeric Class I Chitinase and β-1,3-Glucanase Genes.*

B.P.A.Cammue - *Structure and Properties of Cysteine-Rich Antifungal Proteins from Plant Seeds.*

Posters.

Session II
Chairperson: J. Ryals

T. Boller - *Vacuolar Targeting of Chitinases in Plants: Molecular Mechanism and Biological Significance.*

L. Herrera-Estrella - *Expression of a Bacterial Ornithyl Transcarbamylase in Transgenic Plants Confers Resistance to Phaseolotoxin.*

J.M. Jaynes - *Lytic Peptides and Their Potential for Enhancing Plant Disease Resistance.*

F. García-Olmedo - *Toxic Properties of Thionins and Non-Specific LTPs.*

TUESDAY, January 12th

Session III
Chairperson: M. Zaitlin

R.N. Beachy - *Coat Protein Mediated Resistance: The TMV Example.*

J.F. Bol - *The Use of Modified Viral Genes and Host Defense Genes to Obtain Virus Resistant Plants.*

G. Bruening - *A Satellite RNA of a Plant Virus Has Linear, Multimeric Forms and Circles as Likely Intermediates in Replication.*

R. de Feyter - *Transgenic Tobacco Expressing Ribozyme or Antisense Sequences Have Increased Resistance to Virus Infection.*

T. Candresse - *Pathogen Derived Protection Against Hungarian Grapevine Chrome Mosaic Nepovirus (GCMV).*

Posters.

Session IV
Chairperson: J.F. Bol

F. Ponz - *On the Mechanism of Action of a Plant Virus Recessive Resistance Gene.*

M. Zaitlin - *Induction of Resistance in Plants by Transformation with Viral Replicase Gene Sequences.*

G.P. Lomonossoff - *The Development of Replicase-Mediated Resistance as a Strategy for Producing Virus-Resistant Legumes.*

M. Russo - *Reaction to Cymbidium Ringspot Tombusvirus Infection in Transgenic Nicotiana Benthamiana Plants Expressing Secuences of the Viral Genome or Satellite RNA.*

M. Heinlein - *The Transposase of Maize Transposable Element Ac Forms Large Rod-Like Aggregates in Nuclei of Maize Endosperm and Transfected Petunia Protoplasts.*

WEDNESDAY, January 13th

Session V
Chairperson: F. García-Olmedo

J. van Rie - *Insect Control with Transgenic Plants Expressing Bacillus Thuringiensis Insecticidal Crystal Proteins.*

V.A. Hilder - *Plant Genes for Protecting Transgenic Crops from Chewing and Sap-Sucking Insect Pests.*

P. Carbonero - *Cereal Inhibitors of Insect Hydrolases (α-Amylases and Trypsin): Genetic Control, Transgenic Expression and Insect Tests.*

P. Zabel - *Towards the Map-Based Cloning of the Tomato Root-Knot Nematode Resistance Gene Mi.*

- *Introduction to Megabase Mapping and Cloning Strategies.*

J. Schell - *Molecular Plant Breeding: Plant Protection and Subsequent Goals.*

Session VI
- *General Discussion Led by G. Bruening.*
- *Closing Remarks.*

INTRODUCTION

F. Ponz

For centuries, the fight against the main biological enemies that cultivated plants have, has been a major effort in all cultures. All sorts of agronomical practices have been developed along vasts periods of time, in order to minimize the damage that insects, nematodes, or microbes cause in the plants that man uses as sources of food, fiber, chemicals or entertaiment. The second half of the 20th century was the witness of a "green revolution", that through the quantitative increase in the productivity of major crops such as wheat, maize or rice, caused a complete change in the name of the game of agriculture. Steming this revolution was the possibility of incorporating in cultivated varieties, genes for resistance to important pathogens and the massive use of chemicals, pesticides and fertilizers, to aid in the control of pests and diseases.

The scenario has been dramatically modified since then. New concerns have arised, and now important tasks are to pollute less, or not at all, to diversify productions, and to preserve and rationally exploit biodiversity. It is in this context where Plant Biotecnology has found its nest to flourish. Under the notions that resistance genes can be much more effectively used, if we isolate them and understand the way they work; that we can take advantage of other genes involved in plant defense mechanisms; or even design pathogen-based genes to incorporate them in the genome of the plant, a number of different approaches have arisen that are taking their way into becoming a new global strategy in the old fight. The field is in itself a fascinating scientific challenge. On top of the obvious practical applications, new discoveries regarding the basic mechanisms that plants use to protect themselves, or that they develop when modified to express genes that have never seen before, are already rewarding enough to justify the effort.

The workshop wants to bring together scientists involved in the engineering of plants against pests and pathogens. The organizers feel that some of the rationales that researchers are using their work related to specific pests or pathogens, merit discussion and consideration by others apparently unrelated. Obviously some of the responses of plants when challenged by another biological entity, do not differentiate the origin of

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the attack, or the strategy used to go after a specific gene involved in a defense reaction or a resistance can have many traits in common with the one followed by other people working on a very different topic. Even the designing involved in the engineering using pathogen-based approaches, could have more poinis in common among different pests and pathogens than originally suspected. It is mainly the pursuit of this fluid interchange of ideas that prompted us to organize the meeting. The wonderful support provided by the Fundación Juan March made it possible.

Fernando Ponz

Session I

Genetic Approaches to Specific Resistance of *Arabidopsis thaliana* against phytopathogenic Pseudomonads

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Introduction

Many interactions between plants and microbes begin with specific recognition. The nature of this recognition, and the interpretation of subsequent signal transduction by both plant and microbe have profound impact on the outcome of the interaction. Plants have evolved effective mechanisms to recognize pathogenic microbes and halt their biotrophic or necrotrophic growth in the plant. Active plant defense mechanisms obviously force the selection of microbe variants which can evade the plant´s recognition capabilities. This evolutionary tug of war has led to a complex set of both plant and microbe genes, whose interactions lead to a successful plant resistance reaction. As well as a potentially large array of cognitive gene functions, a number of subsequent signal transduction steps may be necessary to generate a completely effective resistant phenotype.

The number and function of plant genes necessary for a resistance response is unknown. Genetic analyses in many systems over the last 50 years have demonstrated that recognititon functions are provided by dominant alleles of genes in the plant (Resistance, or *R*-genes) which interact, either directly or indirectly, with either the direct or indirect product of a single pathogen gene (avirulence, or *avr* genes) (Crute, 1985; Ellingboe, 1981, 1982, 1984; Flor, 1971; Keen, 1982, 1990; Keen and Staskawicz, 1988; Pryor, 1987). The "gene-for-gene" hypothesis is a genetic explanation for interactions between plants and all classes of pathogens: fungal, bacterial, viral, and insect. Yet despite intense research, we remain naive regarding the molecular structure of *R*-genes, and the function of their products. Moreover, although a great deal is known about the multitude of genes activated subsequent to triggering of the defense response, virtually nothing is known regarding the number or nature of downstream transduction steps which are truly necessary for a resistance reaction.

The goal of projects in our group is to use a model plant species, *Arabidopsis thaliana*, to identify genetically the plant loci necessary for a resistance reaction against phytopathogenic bacteria and fungus. One powerful application of *A. thaliana* is in the dissection of the processes by which plants recognize and

respond to microbial pathogens (reviewed by Dangl, 1993). Several groups have established screening systems to define differential responses (resistance and susceptibility) of *A. thaliana* land races (ecotypes) to various bacterial and fungal pathogens, focussing on isolates of *Pseudomonas syringae* and *Peronospora parasitica*.

Our work revolves around five themes. First, identification and isolation of recognition function genes (*R* or Resistance genes). Second, use of mutation analysis to identify loci which give rise to either loss of recognition, or constitutive induction of a resistance-like phenotype. Depending on the nature of the selection scheme, mutants generated in this way will either map to predefined *R*-gene loci, or to loci involved in either specific or general interpretation of subsequent signals. Third, we also use a generalizable genetic approach to assess whether a particular activated defense response is strictly tied to *R*-gene mediated recognititon, and to critically assay the role that gene's activation in the outcome of a plant-pathogen interaction.. Fourth, we also have isolated five potentially T-DNA tagged mutants exhibiting constitutive phenotypes reminiscent of "lesion mimic" mutants which exist in many plant species, notably maize, rice, tomato, and barley. These have potentially constitutive or "hair-trigger" activation of some step in one or more pathway(s) leading to fomation of an HR-like lesion. Fifth, we are identifying the bacterial pathogen genes which are causal to triggering of a specific plant defense response. These fall into two categories: classically defined *avr* genes, and, using Tn*phoA* as a mutagen, genes encoding membrane localized or secreted products necessary for delivery of a specific *avr* function.

Identification and molecular mapping of an *Arabidopsis* resistance gene against a *Pseudomonas syringae* pv. *maculicola* isolate, and characterization of the corresponding *avr* gene

We and others have shown that *A. thaliana* is a host for phytopathogenic isolates of *Pseudomonas syringae* pathovar *maculicola* (Psm), normally pathogenic on Brassicas and tomato (Debener et al., 1991; see also Davis et al., 1991; Dong et al., 1991; Whalen et al., 1991). All groups found that there is variability in this interaction that is dependent on both host and pathogen genotype. Thus, some Psm isolates are pathogenic on all tested *A. thaliana* genotypes, some on none, and some on only a subset. Based on this series of "differential responses", we began to ask whether or not single loci in both plant and pathogen controlled the generation of a hypersensitive resistance response (HR) as predicted by the gene-for-gene hypothesis. We used RFLP mapping to localize one resistance gene, which we named *RPM1*, to an interval of around 5 map units near the top of chromosome three.

Concurrent with this plant genetic analysis, we cloned the avirulence gene from the appropriate Psm isolate which triggers the HR in combination with the *RPM1* product. We used three different assays, formation of an HR, *in planta* bacterial growth, and diminution of symptoms, to solidly identify the *avrRpm1* gene (Debener et al., 1991). We further demonstrated that the cloned *avrRpm1*

gene was, in fact, responsible for *RPM1*-mediated recognition. We have subsequently defined *avrRpm1* to a functionally active 2.5kb fragment. Tn3spice mutagenesis further localized *avrRpm1* to a 1.0kb fragment. In collaboration with Marjorie Gibbon and Alan Vivian at Bristol Polytechnic University in Bristol, the *avrRpm1* and the highly related *avrPpiA1* from *P. s.* pv. *pisi* were both sequenced. Moreover, both avr genes are recognized by specific cultivars of pea, bean, and soybean (Dangl et al., 1992). This finding clearly suggests that *A. thaliana* has functional, and possibly molecular, homologs to resistance genes in those species. Recently, we have shown that *avrRpm1* also encodes a product required for pathogenicity on susceptible *A. thaliana* ecotypes.

We isolated several YAC (yeast artificial chromosome) clones containing large *A. thaliana* DNA inserts which hybridize to the RFLP markers genetically closest to *RPM1*. One of them, with a 270kb insert, was isolated for us by Joe Ecker and must contain *RPM1*. Progress in map-based cloning of this locus will be detailed. We have also recently learned, via collaboration with Roger Innes at Indiana University and Brian Staskawicz at UC Berkeley, that *RPM1* also determines resistance to another, wholly unrelated avr gene, *avrB* from *P. syringae* pv. *glycinea*. Whether or not the same, or a very closely linked, gene product determines resistance to both *avr* gene products awaits cloning of the *RPM1* locus.

Isolation and characterization of *A. thaliana* mutants with altered resistance responses

We have two current activities in this area. First, we isolated and are characterizing an EMS mutant of ecotype Col-0 which now exhibits chlorosis after inoculation with a *P.s.* pv. *tabaci* isolate that makes no lesion on any tested wild type *A. thaliana* ecotype, even at very high inoculum density. The mutant becomes very chlorotic, and supports around 30-50 fold higher levels of bacterial growth when re-challenged with the selecting bacteria. F1 individuals from an outcross to ecotype Nd-0 shows an intermediate phenotype, which subsequently segregates as a single dominant locus in F2. We are analysing this mutant further and are beginning RFLP mapping

With the cloned *avrRpm1* gene characterised, we are also screening for *A. thaliana* mutants unable to recognize its signal. These mutants should either be in the *RPM1* gene, and their analysis will further understanding of the critical recognition event mediated by its product, or they will be in loci necessary to transduce the *RPM1* signal. We use a "null" bacterial strain, a *P.s.* pv *phaseolicola* which is non-pathogenic and generates no macroscopic reaction on *A. thaliana*, but which is capable of specifically delivering the *avrRpm1* signal, to isolate "loss of recognition" mutants. We screened around 120,000 seedlings from fast neutron irradiated seed from over 40 independent seed lots, around 3000 bulked EMS seed, and the available T-DNA insertion mutant population from Ken Feldmann. At least three confirmed mutants have been isolated, and we expect many more.

Assessing the requirement for induced gene expression during specific resistance reactions: The *ELI3* example

Imre Somssich and colleagues cloned 19 different parsley cDNAs, encoding predicted proteins of known and unknown function, which were transcriptionally activated in cultured cells by fungal elicitor (Somssich et al., 1989). We are collaborating with him to analyze pathogen activated expression of recently cloned *A. thaliana* homologs after infiltration of *A. thaliana* leaves with various Psm isolates. Of the many clones tested, only the *ELI3* homolog is of immediate interest (Kiedrowski et al., 1992). Its mRNA accumulates rapidly (3-5 hours p.i.) to extremely high levels in incompatible interactions involving Psm isolate m2, while the accumulation is slower, and the amount of mRNA lower, in compatible interactions involving m2. The virulent Psm isolate m4 also induces *ELI3* mRNA to high levels, but much later in the interaction. A Psm m4 transconjugant harboring *avrRpm1* generates the rapid *ELI3* induction kinetic idiosyncratic of the Psm m2 interaction with Col-0, suggesting, but by no means proving, that gene-for-gene recognition mediates early *ELI3* activation. We have now shown convincingly that the early activation of *ELI3* during m2 incompatible interactions is stricly governed by the presence of a dominant allele at *RPM1*. We used the homozygous F3 families from the Col-0 x Nd-0 cross described above to prove this point. In RNA blot experiments, 15 *RPM1/RPM1* families had very high levels of *ELI3* mRNA, as did the resistant Col-0 parent; 15 *rpm1/rpm1* families had very low levels, as did the susceptible Nd-0 parent. These families were essentially "genotype selected" (Michelmore et al., 1991) at *RPM1*, and are expected to be freely segregating at all other loci. This random assortment virtually eliminates any possible effects of genetic background on the phenotype being analysed. We are now constructing anti-sense and sense constructs in order to make phenocopy mutants of *ELI3* and ask whether the expected mutants have an altered phenotype after infiltration with Psm isolate m2 and other incompatible pathogens.

***Arabidopsis* mutants showing HR-like lesions in the absence of pathogen: Cell-cell communication triggers**

We isolated five mutants from Ken Feldmann´s T-DNA insertion mutagenesis population which dispalyed varying degrees of "spontaneous" lesion formation. They were first characterized with respect to how the observed lesions are related to "defense response" and are not a consequence of simple senescence. Subsequently one mutant, caused by a single recessive mutation (which may be T-DNA tagged) has been analyzed in some detail. This mutant is characterized by formation of rapidly spreading lesions which form on all tissues (but most noticably on leaves) after shifting plants from short to long days. Lesion formation occurs under sterile conditions, as well. Prior to lesion formation, infection of normal appearing leaves with any live bacteria or a virulent fungus triggers lesion formation and spreading tissue collapse. As well, these plants have a heightened sensitivity to Salicylic Acid, but not to SA

analogs inactive in triggering systemic acquired resistance. Lesions spread unchecked to cells not initially infected, suggesting that the mutation is in a gene whose product normally limits or "dampens" transfer of a local signal required for SAR or limits the formation of a bordered HR.

Identification of bacterial membrane proteins necessary for delivery of *avr* gene function

We are interested in probing the bacterial membrane and periplasm for proteins involved in the HR. This project is based on the observation that, although many bacterial *avr* genes have been cloned, none encodes an obviously membrane bound product. This prompts the question of how the avr signal reaches the plant. It has also been recently shown that *hrp* cluster encoded proteins can be members of two-component regulatory systems. One component of these systems is always a membrane bound "sensor". We utilize the specialized transposon mutagen Tn*phoA* to generate the desired mutants. We mutagenized Psm m2 with Tn*phoA* and generated over 60,000 Km^r inserts, of which around 400 were $phoA^+$. These were screened for loss or attenuation of *avrRpm1* signal delivery to resistant Col-0 plants. Several mutants have been found which are either HR^- or HR attenuated. They can be divided into at least three phenotypic classes, one of which still retains the ability to generate an HR on tobacco, and is thus not a classic *hrp* mutant. We will cure the *avrRpm1* containing cosmid from these mutants, introduce either of the other two *avr* genes recognized by *A. thaliana*, and ask whether loss of *avr* signal delivery is specific to *avrRpm1*, or a general phenomenon. Molecular characteization of these mutants will lead to furthered understanding of mechanisms controlling *avr* gene signal delivery.

Summary

A great deal of progress has been made, by several groups, in the exploitation of *A. thaliana* as a model in the molecular analysis of plant-pathogen interactions in the last few years (Dangl, 1993). This trend will only continue as further *R*.gene loci are defined and mapped, and as loss of recognition and altered recognition mutants are isolated using a variety of strategies discussed by others in this volume. We hope to gain a basic understanding of *R* gene structure and function through molecular isolation of *RPM1*, and its potential homologs in a variety of crop species. Moreover, via utilization of either natural or induced variation at *RPM1*, we hope to dissect signals reaching the plant cell nucleus, and effector pathways involved in establishing and maintaining the resistant state. Finally, we hope to integrate this knowledge with definition of specific *avr* genes, and the genes encoding passage of *avr* signals through the bacterial membrane. This presentation has outlined our current progress in these areas.

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Toward the Genetic Engineering of Fungal Resistance in Crops

John Ryals[1], Eric Ward[1], Scott Uknes[1], Thomas Gaffney[1], Leslie Friedrich[1], Danny Alexander[2] and Manuela Gut Rella[2].

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Introduction

Improving crops in their ability to resist disease has been a goal of plant breeders for many years. In wheat, disease resistance is the top priority among breeders while in corn it is secondary to insect resistance. Most wheat and corn breeders are turning away from breeding single-gene resistance and moving toward improvements of generalized plant health. At CIBA-GEIGY, we began examing ways to genetically engineer improvements in plant health several years ago. At that time there were not many sources for "generalized health genes". However, one possible source was in the genes responsible for the phenomenon of systemic acquired resistance.

Many plant species can be "immunized" by an initial inoculation with a necrotizing pathogen. This "acquired resistance" was first documented in the first part of the century and is thought to "play an important role in the preservation of plants in nature" (1). A particularly well characterized example of systemic acquired resistance (SAR) is the response of tobacco to tobacco mosaic virus (2). In this case inoculation by TMV results in systemic protection against subsequent infections by a number of agronomically important bacterial, fungal and viral pathogens that can last for weeks to months. This phenomenon is not restricted to tobacco but is also observed in many other plants, notably cucumber (3) and arabidopsis (4). Thus, acquired resistance might provide a source for genes that confer broad spectrum disease control.

Our initial experimental approach to understand SAR at a molecular level was based on a working model that could explain the basic steps leading to the establishment of a resistant state. To induce resistance there must first be a necrotic reaction to a pathogen. This requirement for a biological inducer is strict since salt, acid or mechanical damage will not induce SAR. The necrosis triggers the release of a signal molecule that translocates to various parts of the plant which is perceived, with the final result being the expression of a set of genes responsible for the maintenance of the resistant state. We distinguished the events leading to the onset of resistance (initiation) from events involved in maintaining the resistance (maintenance), although these are probably not completely distinct processes. Our research objectives have been to catalogue the genes involved in maintenance, to identify the signal compound(s) responsible for induction and, as a long term objective, to identify genes involved in resistance initiation. The goal of this project is to understand SAR at a molecular level and to use this information to genetically engineer plants with improved health in the field.

Maintenance of Resistance

Over the past few years we have identified and characterized a number of cDNA's that are expressed to high levels in systemically resistant tissue. Nine of these "SAR gene families" were coordinately expressed in tobacco leaves after treatment with either tobacco mosaic virus (TMV) or 2,6-dichloroisonicotinic acid (INA), a synthetic plant immunomodulator (5, 6). The timing and the amount of gene expression correlated well with the onset and the degree of resistance to further TMV infection (5). The role of each of the genes in mediating disease resistance is currently being evaluated using transgenic expression of the sense and the antisense of the cDNA of each gene.

In order to determine what role a particular gene plays in the maintenance of resistance, each of the SAR cDNA's were subcloned into a high-level expression cassette, based on a double 35S promoter (7). The expression cassette containing the cDNA in either a sense or antisense direction was then subcloned into an agrobacterium vector (8) and leaf disk transformation (9) of Xanthi.nc tobacco was carried out. Many independent transformants were selected in each transformation experiment and these were screened for high levels of the protein encoded by the cDNA. The transformants were allowed to self twice and homozygotic lines were established. In each case it was possible to develop genetically stable lines expressing the transgenic protein at levels between 1 and 0.1% of the total protein. The lines were then evaluated for resistance against a number of fungal and bacterial diseases. This evaluation is still in progress but has already yielded interesting results. So far, there are indications of resistance with single genes against the following three diseases: 1) post-emergent damping off (*Rhizoctonia solani*) in transgenic tobacco expressing high levels of either the basic class I chitinase, the acidic class III chitinase or the cucumber class III chitinase; 2) black shank (*Phytophthora parasitica*) in transgenic tobacco expressing high levels of the protein SAR8.2, a tobacco SAR protein with unknown structural or enzymatic function; 3) bluemold (*Peronospora tabacina*) and black shank resistance in transgenic tobacco expressing high levels of pathogenesis related protein 1. The indication of resistance to bluemold and black shank by PR-1 and the resistance to black shank with SAR8.2 have been demonstrated to be statistically significant in multiple independent lines.

F1 crosses of each homozygotic line to a different tobacco line have also been made and these lines are currently being evaluated. There are several indications of resistance when two genes are expressed in a line that were not observed in lines expressing either single gene. Currently, pairs of SAR genes are being reengineered in order to develop homozygotic lines expressing two genes at once. These will then be crossed to give three and four genes expressed at high levels in each line.

We have also taken the approach of expressing high levels of the antisense RNA to each SAR gene. Homozygotic transgenic lines were developed for each of the genes and it has been shown that the protein target is dramatically reduced in each case. Two further vectors have been constructed, one that contains anti-PR-1, anti PR-2 and anti PR-4 and one that contains anti-PR-3 and anti-PR-5. Homozygotic lines are being developed and these will be crossed to give an F1 in which all of the ten classic PR-proteins (10) have been functionally mutated. This hybrid will be used to determine the role of the PR-proteins in the maintenance of SAR.

The results of these experiments has led us to a working model for the activity of the genes expressed in SAR. Several of these genes may target one particular pathogen and a different, overlapping set of genes may affect a different pathogen. It will require a systematic and detailed study of the interactions of the different genes to determine which set of genes to use to protect against a particular disease.

The signal for SAR

In previous work we demonstrated the appearance of a fluorescent compound in phloem exudate of cucumber plants that had been induced to resistance by either tobacco necrosis virus or *Colletotrichum lagenarium*. The compound was purified to homogeneity and determined to be salicylic acid (SA) using gas chromatography/mass spectral analysis (11). SA accumulated to high levels prior to the onset of induced resistance to *C. lagenarium* making it a candidate for the signal molecule. In independent experiments, Raskin and Klessig (12) demonstrated that SA was induced in tobacco that had been induced with TMV and Hammerschmidt's group demonstrated the induction of SA in cucumber preceding the development of resistance induced by *Pseudomonas syringae* (13). If SA were the signal molecule for SAR in tobacco then exogenously applied SA should coordinately induce the same SAR genes as are found in the biologically induced resistance. Ward et al (5) demonstrated that SA coordinately induced the same nine gene families as TMV in tobacco and that the timing and extent of expression correlated with the onset and degree of protection against further TMV infection (5). Therefore, there is considerable evidence implicating SA as a signal molecule involved in triggering resistance.

When the biosynthetic pathway of SA is considered the finding of SA as a putative signal becomes particularly interesting. Of the two proposed pathways for SA biosynthesis in higher plants, both branch from the phenylpropanoid pathway after the synthesis of *trans*-cinnamic acid. It has been known for a long time that the phenylpropanoid pathway is induced following pathogen attack and by products such as isoflavonoid phytoalexins and lignin precursors are used in disease resistance. It is clear that SA production could be a further consequence of inducing this pathway and this could provide an answer as to why a biological inducer is required for signal production.

As appealing as this model may be there are two results that cast some doubt on the role of SA as the signal molecule. First, it is clear from our studies and from work of others that exogenously applied SA does not translocate in the plant. Of course it can be argued that the application of a compound to a leaf surface is not tantamount to the production and secretion of the compound in vivo. A second interesting result came from Hammerschmidt's studies with cucumber (13). He found that cucumber leaves infiltrated with a suspension of *Pseudomonas syringae* do not accumulate high levels of SA within four hours after infiltration. However, removal of the leaves at four hours post infiltration does not prevent the accumulation of high levels of SA or the onset of resistance in other parts of the plant (13). It can be argued that SA induces its own biosynthesis, which is indeed similar to the biosynthesis of ethylene (14) or that the level of detection is not sensitive enough. Therefore, it is still not clear that SA is the translocated signal that induces SAR.

In order to investigate the role of SA as a signal molecule for SAR we are attempting to engineer plants that degrade salicylate. It is known that certain strains of *Pseudomonas putida* are capable of degrading naphthalene through salicylate to acetaldehyde and pyruvate. One step of this pathway is the conversion of salicylate to catechol, which we have shown does not induce SAR. This conversion is carried out by salicylate hydroxylase an enzyme encoded by the *NahG* gene. Therefore, we have cloned the *NahG* coding sequence into different plant expression vectors and developed transgenic tobacco expressing high levels of the corresponding mRNA. In preliminary experiments we have found that plants that express high levels of the mRNA are not capable of inducing SAR, while plants expressing moderate levels give moderate levels of resistance and transformants with undetectable levels of the mRNA respond normally. Thus the data support the role of SA as a signal molecule that somehow induces SAR.

Summary

The molecular events that take place during the initiation and maintenance of SAR are starting to be resolved. Based on the results presented in this report, our current working model is that the infection by a pathogen induces the synthesis of salicylic acid which translocates throughout the plant and is recognized by a specific receptor. The bound/activated receptor transduces the signal with the result being the coordinate expression of a set of SAR genes. The encoded SAR proteins accumulate to high levels systemically. Disease control is provided by certain groups of proteins acting on particular pathogens.

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The Biology of Plants Transformed with Chimeric Class I Chitinase and β-1,3-Glucanase Genes

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β-1,3-Glucanases and chitinases are abundant, basic proteins localized in the cell vacuole. They exhibit complex hormonal and developmental regulation and are induced in parts of plants infected by viral, bacterial, and fungal pathogens. Combinations of class I β-1,3-glucanase and chitinases are potent fungicides *in vitro* suggesting that the enzymes may be important in the defense of plants against fungal infection. We are using sense and antisense transformation to test this hypothesis and to identify other physiological functions of these proteins.

Nicotiana sylvestris plants were transformed with binary Ti-plasmid vectors containing the coding sequence of the tobacco chitinase gene CHN48 or the tobacco β-1,3-glucanase gene GLA regulated by cauliflower mosaic virus 35S RNA expression signals. The *NPTII* gene with *Nos* expression signals was included to provide kanamycin resistance as a plant selectable marker. Chitinase sense transformants accumulated extremely high levels of chitinase- up to 120-fold that of comparable tissues in non-transformed plants. The transgene-encoded protein is enzymatically active, correctly processed, and correctly targeted to the cell vacuole. β-1,3-Glucanase antisense transformants containing ≈580 bp of coding sequence in reverse orientation showed a marked ≈20-fold reduction of class I β-1,3-glucanase accumulation. Induction of the class I isoform by ethylene treatment and infection with the fungal pathogen, *Cercospora nicotianae* was also effectively blocked.

Antisense β-1,3-glucanase transformants and sense chitinase transformants grown under greenhouse conditions were fertile and developed normally. Their susceptibility to infection by *C. nicotianae*, which contains chitin and β-1,3-glucans as cell wall components, did not consistently differ from non-transformed plants. Apparently, accumulation of class I chitinases and β-1,3-glucanases is not a limiting factor in the defense of plants against this pathogen.

Interpretation of these results was complicated by the finding that the expression of homologous host genes can be dramatically altered by transformation. For example, some homozygous sense transformants showed a marked inhibition in the expression of the transgene and homologous host gene. This effect, called silencing, results from a *trans*-inactivation at the mRNA level. It depends on the dose of the transgene and is developmentally regulated. The incidence of silencing is also influenced by environmental factors such as the conditions used to germinate the seedlings. Applications of gene technology for crop improvement depend on the reliable expression of transgenes under field conditions. Silencing of chitinase and β-1,3-glucanase gene expression provides an experimental system well suited for studying environmental effects on the stability of transgene expression.

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Structure and properties of cysteine-rich antifungal proteins from plant seeds

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Based on an extensive screening of about 600 plant species, we have recently isolated 3 novel types of highly basic and cysteine-rich seed peptides with pronounced antifungal activity. The first class was isolated from seeds of *Mirabilis jalapa*, which contain two nearly identical antimicrobial proteins (*Mj*-AMP1 and *Mj*-AMP2), showing amino acid sequence homology to insect neurotoxins (1). Another type of antimicrobial peptides was purified from seed extracts of *Amaranthus caudatus* (*Ac*-AMP1 and *Ac*-AMP2) and are structurally related to the chitin-binding domain of N-acetylglucosamine-specific lectins and class I chitinases (2). Both *Mj*-AMPs and *Ac*-AMPs are also active against yeast and Gram-positive bacteria, and their antimicrobial activity is reduced in the presence of cations. The latter properties are not shared by the third novel class of antifungal proteins, present in *Raphanus sativus* seeds (*Rs*-AFPs), whose antibiotic activity is specific for filamentous fungi and is comparatively less sensitive to cation antagonism (3). Homologous seed proteins with comparable antifungal activity could also be demonstrated in other Brassicaceae-species (4). They all revealed amino acid sequence homology with wheat and barley -thionins, sorghum α-amylase inhibitors, and with the derived primary structures of two pea cDNAs that are specifically induced by fungal attack.

Protectant activity against *Cercospora beticola* was demonstrated for *Mj*-AMPs and *Rs*-AFPs in spray tests on artificially inoculated sugar beet plants, and found to be comparable to that of commercial fungicides when expressed on a molar base.

cDNA clones of all three types of AFPs were isolated and the nucleotide sequence determined. The relatively small open reading frames consist in all cases of a N-terminal signal peptide and a region coding for the mature protein. In addition, the cDNAs of the *Ac*-AMPs are extended with a small C-terminal propeptide with a putative glycosylation site. The coding regions of *Mj*-AMP2 and *Rs*-AFP2 were subcloned into a CaMV35S promoter-driven expression vector and introduced into tobacco by *Agrobacterium*-mediated transformation. Expression of the antifungal proteins in the transgenic plants, as demonstrated by Western blotting and chromatographic analysis, resulted in an increased antifungal activity in leaf extracts.

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Session II

Vacuolar targeting of chitinases in plants: molecular mechanism and biological significance.

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Many plant species accumulate chitinases and β-1,3-glucanases in response to plant pathogens and to the plant stress hormone, ethylene (Boller, 1991; Meins et al., 1992). These hydrolases are thought to be involved in the defense against fungal pathogens since their substrates, chitin and β-1,3-glucan, are the main components of the cell walls of most higher fungi, and since they inhibit many saprophytic and potentially pathogenic fungi on agar plates or in liquid medium, particularly when applied in combination (Schlumbaum et al., 1986; Mauch et al., 1988; Ludwig and Boller, 1990). It has recently been found that transgenic plants constitutively expressing chitinase show an increased resistance against the root pathogen *Rhizoctonia solani* (Broglie et al., 1991).

Interestingly, some isoforms of chitinases and β-1,3-glucanases are localized in the vacuole, while others are secreted into the intercellular space. In tobacco, the deduced amino acid sequences of the vacuolar isoforms differ from the homologous acidic isoforms by the presence of a short C-terminal extension. We have studied the localization of chitinase in transgenic *Nicotiana silvestris* plants constitutively expressing various forms of chitinase. In plants expressing the unmodified form of the vacuolar tobacco chitinase, the enzyme was correctly targeted to the vacuole. In contrast, in plants expressing a mutant form lacking the C-terminal extension, the enzyme was absent from the protoplasts and present in the intercellular space. Likewise, a structurally unrelated, extracellular cucumber chitinase was secreted into the intercellular space when constitutively expressed in *Nicotiana silvestris* plants. However, in plants expressing the same cucumber chitinase with the C-terminal extension from the vacuolar tobacco chitinase, most of the enzyme was present in the vacuoles. Thus, the C-terminal extension is necessary and sufficient to target chitinases to the vacuole, indicating that it comprises a targeting signal for plant vacuoles (Neuhaus et al., 1991).

At first glance, since fungal invaders generally come from the outside, the antifungal potential of chitinase appears to be hampered by a vacuolar localization, and an extracellular localization of chitinase would seem more adequate for a defense function. This view may be too simplistic, however.

Microscopic studies indicate that the enzymes attack primarily the hyphal tip. Studies with fungi on artificial media show that growing hyphae are highly sensitive when suddenly brought into contact with the antifungal hydrolases. However, fungi appear to have a potential to adapt and become resistant when germinated and grown in the continuous presence of the enzymes. This suggests that antifungal hydrolases may be most effective in defense when initially compartmentalized and brought into contact with invading fungi only after a time, e.g. by release from the vacuole during a hypersensitive response.

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Expression of a bacterial ornithyl transcarbamylase in transgenic plants confers resistance to phaseolotoxin.

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Chemicals with phytotoxic activities are produced by many plant pathogenic fungi and bacteria. Nonhost-specific toxins are generally acknowledged to be an important virulence element, and this is especially evident among the pathovars of *Pseudomonas syringae* . However, considerable debate has focused on whether toxin production has a primary role in virulence since phytotoxic symptoms occur long after lesion formation.

Phaseolotoxin is a tripeptide toxin [Nδ-(N'-sulpho-diaminophosphinyl)ornithylalanylhomoarginine] produced by *Pseudomonas syringae pv. phaseolicola*, the causal agent of the halo blight disease of common bean (*Phaseolus vulgaris* L.). Phaseolotoxin inhibits the enzyme Ornithine Carbamoyltransferase (OCTase) . This enzyme converts ornithine and carbamoyl phosphate to citrulline, reaction involved in the biosynthesis of arginine and interconversion of the glutamate family of aminoacids, and is located in plastids of plant cells. "In vitro" phaseolotoxin is a reversible inhibitor of OCTase, but "in planta" the toxin is cleaved by plant peptidases to produce the irreversible inhibitor Nδ-(N-sulpho-diaminophosphinyl)-L-Ornithine (Octicidin).

Phaseolotoxin is a not host-specific toxin and it causes similar chlorotic symptoms and ornithine accumulation in other plants that resist infection by *P. s. pv. phaseolicola* , and it also inhibits the growth of cultivated plant cells and bacteria. It has been shown that *P. s. pv. phaseolicola* is able to produce two detectable OCTase activities, one sensitive and one resistant to phaseolotoxin. The *P. s. pv. phaseolicola* gene encoding the phaseolotoxin-resistant OCTase (arg K) has been cloned and sequenced in our laboratory[1].

Using the pathogen-derived resistance strategy [2], we aimed our research at producing transgenic plants that express the *P. s. pv. phaseolicola* phaseolotoxin-insensitive OCTase. Using this system the role of phaseolotoxin in the virulence of *P. s. pv. phaseolicola* was investigated. It was found that transgenic plants expressing the bacterial OCTase are no longer

suceptible to the effect of phaseolotoxin and respond with a hypersensitive response to infection by *P. s. pv. phaseolicola*. It was also determine that these plants have an increased level of arginine and other biochemically related aminoacids.

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Lytic Peptides and Their Potential for Enhancing Plant Disease Resistance

by

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Summmary:

It has been said that plant disease is the exception rather than the rule [1]. In effect, only the interplay between a virulent pathogen and a susceptible cultivar leads to disease. Despite this delicate interaction between host and pathogen, plant disease is one of the leading causes of crop loss in the world. Its economic import can be especially significant in the developing world where up to forty percent of crop destruction can be directly attributed to plant disease [2]. Also, their cultivation of just a few plant species within a localized area accelerates the spread of disease, exacerbating the problem.

.../...

Plants under microbial attack elaborate several inducible defensive responses of a structural and biochemical nature. Among them are the synthesis of hydrolytic enzymes, such as chitinase and ß-glucanase, and proteinase inhibitors; the modification of the plant cell wall by lignification and accumulation of callose, a ß-1,3 glucan, and hydroxyproline-rich glycoproteins (HRGPs); and the synthesis of phytoalexins, low molecular weight compounds with antimicrobial activity [3].

Recent advances in genetic engineering have made it possible to develop plants with new predictable phenotypes. The expression of the TMV coat protein in transgenic plants was shown to cause a delay in the development of symptoms upon infection by the virus in concordance with the idea of cross-protection [4]. Also, expression of an insecticidal protein, the BT protein from *Bacillus thuringiensis*, has rendered plants resistant to insect attack [5]. Therefore, appropriate small modifications in the biochemistry of a plant may significantly augment the resistance of this plant to the action of a pest or pathogen. Jaynes et. al. [6, 7] and more recently, Casteels et. al. [8] have proposed the idea of using the genes for proteins with antimicrobial activity, found in insects, to enhance bacterial disease resistance in plants.

Lytic peptides are small proteins that appear to be major components of the antimicrobial defense systems of a number of animal species including insects, molluscs, amphibians, and at least one mammal [9]. They consist of 23-39 amino acid sesquences, which have potential for forming amphipathic α-helices. An amphipathic α-helix may be depicted as a cylinder with one curved face composed primarily of nonpolar amino acid side chains and the other face composed of amino acids containing polar side chains (the property of amphipathy can best be visualized by casting the amino acid sequence on a helical wheel projection [10]). The presence of lytic peptides in other higher organisms is under intensive investigation and it has been proposed that they will be found in all mammals, including humans [9]. Defensins, a family of small molecular weight antimicrobial peptides found in mammals and more recently in insects, are a separate kind of lytic peptide, because their action is intracellularly localized following phagocytosis and their *in vivo* conformation seems to be a ß-pleated sheet [11].

The lytic peptides that have been described in the literature seem to fall into one of three different classes based on the arrangement of amphipathy and high positive

charge density within the molecule: cecropins (35 amino acids in length and derived from the Giant Silk Moth), N-terminal half amphipathic while the C-terminal half mostly hydrophobic [12]; magainins (23 amino acids in length and derived from the African Clawed Frog), amphipathic the full-length of the molecule [13]; and melittin (26 amino acids in length and derived from the Honeybee), C-terminal half amphipathic with the N-terminal half primarily hydrophobic [14]. The conservation of these physical properties is requisite for activity, but the requirements seem to be somewhat nonspecific in terms of amino acid sequence. For example, we have synthesized highly sequence divergent analogs for each of the peptide classes and have found some of them to be more active than their natural counterparts [15, 16].

For the past few years, my laboratory has been investigating the ability of designed synthetic lytic peptides and proteins to control plant disease. We have found that these agents possess a remarkably broad spectrum of bioactivities, with some of the newer peptides able to kill all bacterial, fungal, and nematode pathogens tested, at concentrations not harmful to plant and animal cells. These findings fortify our belief that very soon a number of different plant species will be "armed" with some of these defense components. The results to be presented in this paper indicate that this notion can have practical application and suggest a different approach to the development of new plant cultivars with increased microbial disease resistance.

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TOXIC PROPERTIES OF THIONINS AND NON-SPECIFIC LTPs

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Thionins are cysteine-rich, 5 kDa polypeptides which are active against plant pathogens (1-3). Thionin genetic variants of type I, from the endosperms of wheat (Wα1), Wα2, Wβ) and barley (Bα, Bβ), and of type II, from barley leaves (BLa, BLb, BLc) have been purified to homogeneity. For a given pathogen EC-50 values obtained with the different thionins varied over a less than fivefold range. The ranking of the variants according to their activity differed among different pathogens, but certain variants, such as Wα1, Wβ or Bβ, tended to be more active than the others. Strains of some bacterial species, such as *Clavibacter michiganensis* subsp. sepedonicus or *Pseudomonas solanacearum* were sensitive in the 10^{-7}M concentration range, whereas the most sensitive fungal pathogens, wuch as *Rosellinia necatrix* ,*Colletotrichum lagenarium* and *Fusarium solani*, had EC-50 values in the 10^{-6}M range. Thionins, which were not particularly efective in liquid medium against *Phytophthora infestans* (EC-50 3.9 x 10^{-5}M) were more effective than the fungicide Ridomil on a molar basis in a drop-application assay on leaf discs from potato.

Genes encoding thionins have been cloned and expressed transgenically in tobaco under the Cam35S promoter. The transgenic plants were challenged with the pathogenic bacteria *Pseudomonas syringae pv. tabaci* and *P. syringae pv. syringae* and found to have enhanced resistance to the pathogens (4).

Four homogeneous proteins (Cw-18,-20,-21,-22) which were active against bacterial and fungal pathogens have been isolated from barley leaves (5,6). These proteins were found to be homologous to the so-called non-specific lipid transfer proteins. Their activity in vitro was about tenfold higher than that of thionins against *C.michiganensis* and about fivefold lower against *P.infestans*. Besides this **complementarity**, the Cw-proteins showed strong **synergism** with thionins in the case of *P.infestans*. Three of the corresponding genes have been cloned and found to be expressed in leaves and stems. Transgenic tobacco plants carrying these genes have been obtained and their reaction to pahtogens is being ivestigated.

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Session III

COAT PROTEIN MEDIATED RESISTANCE: THE TMV EXAMPLE

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Since the initial report of coat protein mediated resistance (CP-MR) against tobacco mosaic virus (TMV) in 1986 (Powell-Abel et al) the list of viruses and transgenic plants to which this approach has been successfully applied has continued to grow (For a more complete review of CP-MR and other types or non-classical forms of virus resistance the reader is encouraged to see the volume on this topic in 'Seminars in Virology', R.N. Beachy, Ed., 1993.). Furthermore, plants that exhibit CP-MR have been tested under a variety of field trial situations, and based upon the successes of such trials it is likely that plant varieties with CP-MR will be commercialized within the near future.

Although there have been notable successes in CP-MR, there are also examples in which the expression of genes that encode CP have resulted in low or insignificant levels of resistance. There are other examples in which the RNA sequence rather that the CP confers resistance (van der Vlugt et al, 1992; Goldbach et al, 1992). Because of these and other research findings it is increasingly important that researchers design experiments that conclusively identify that either or both RNA and protein is/are responsible for conferring resistance to virus infection in transgenic plants.

In this laboratory we have concentrated our research efforts in CP-MR on several tobamoviruses, potyviruses, and geminiviruses. In some cases we designed experimental approaches to regulate the levels of gene expression and protein accumulation by using specific promoter sequences. In other cases gene sequences were altered so as to produce proteins that are modified in sequence and structure. The combined approaches have enabled us to further explore the mechanisms of CP-MR, particularly as it relates to TMV.

Previous work carried out in this laboratory and in collaboration with other research laboratories convincingly demonstrated that CP-MR against TMV (in tobacco) resulted in large part because one or more early events in virus infection were blocked (see the recent review by Register and Nelson, 1992). Register and Beachy (1988) demonstrated that virus pretreated at pH 8.0 before inoculating transgenic protoplasts that contain

TMV-CP overcame coat protein mediated resistance, presumably because the virus was disassembled beyond the point at which CP-MR was effective. Wu et al (1990) determined that TMV-CP in transgenic protoplasts prevented TMV from being uncoated and/or associated with 80S ribosomes during the formation of stripasomes, the earliest event yet detected in the process of virus infection. Taken together these results suggest that an early event in the infection process is blocked in CP-MR. Although some of the steps in uncoating and stripisome formation can be demonstrated *in vitro* the precise mechanisms by which endogenous CP blocks these steps *in vivo* remain to be determined. In an ongoing study mutants of the TMV CP with altered assembly characteristics were developed and expressed both during virus infection (by replacing the w.t. coat protein cistron with one encoding the mutant in an 'infectious clone' of TMV) and in transgenic plants that were used in assays for CP-MR. The results of these studies and the implications regarding the mechanisms of CP-MR will be presented.

In complementary studies we expressed the CP under control of the promoters derived from *PAL* and *rol C* genes (from *A. rhizogenes*): the former leads to expression in the upper epidermis and primary xylem and the latter to expression in phloem. Expression from the PAL promoter provided resistance to infection, but to a lesser degree than did the 35S promoter from CaMV. Neither the *PAL* nor the *rol C* promoter provided resistance to systemic infection. Expression of the CP gene primarily in leaf mesophyll cells (Clark et al., 1990) or in phloem or xylem cells provided little or no resistance. The results of these studies support the conclusions of Register and Beachy (1988, 1989) that CP-MR against TMV is largely the result of interference by the endogenous CP with an early event in the infection process, an event which must take place in the cell that is inoculated. In the case of mechanical infections with TMV epidermal cells are the first to be infected: it is therefore logical that accumulation of CP in these cells would provide resistance.

While the expression of CP genes provides resistance to an early event in infection, other genes derived from virus sequence have been shown by other research groups to interfere with subsequent steps in the replication cycle. Of specific interest to us is the attempt to reduce the local and/or long distance spread of the infection. For these studies we are investigating the role of the TMV Movement protein (MP) and embarked on a strategy to develop transgenic plants that interfere with the MP.

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The use of modified viral genes and host defense genes to obtain virus resistant plants

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We have investigated the possibilities to exploit the plant defense mechanisms known as "systemic acquired resistance" and "cross-protection" to engineer virus resistant plants. Some recent data on the first mechanism will be presented but the emphasis will be on a study of the second mechanism by the transformation of plants with (modified) viral genes.

Systemic acquired resistance is the phenomenon that plants reacting hypersensitively to pathogenic attack develop a broad resistance to a wide variety of pathogens in the pathogen free parts of the plant. As a model system we used the hypersensitive response of Samsun NN tobacco to infection with tobacco mosaic virus (TMV). The virus remains localized in necrotic lesions on the inoculated leaf and the virus-free parts of the plant become resistant to a subsequent challenge with viruses, fungi and bacteria. The induction of this resistance is paralleled by the *de novo* synthesis of many pathogenesis-related (PR) host proteins. The proteins from PR groups 1-5 can each be subdivided into extracellular acidic proteins and vacuolar basic proteins. In addition to TMV infection, the genes encoding the basic isoforms are strongly induced by ethylene and wounding and they are constitutively expressed in the roots of healthy plants. The genes encoding the acidic isoforms are not expressed under these conditions. After TMV infection, genes encoding the acidic PR proteins are systemically induced but the genes encoding the basic isoforms are not, indicating that the latter play no role in the systemic acquired resistance. Results obtained by other groups indicate that proteins from groups 1, 2 (ß-1,3-glucanases), 3 (chitinases) and 5 (thaumatin-like proteins/osmotin) are involved in the TMV-induced antifungal response of tobacco. In agreement with this, we did not find any resistance to virus infection of plants transformed with genes from these PR groups. Recently, we have cloned several other TMV-inducible tobacco genes including genes encoding class I and class II proteinase inhibitors. Properties of these genes and their mode of induction will be presented.

Cross-protection is the phenomenon that plants systemically infected by a mild strain of a given virus become resistant to infection with a severe strain of the same virus. A few years ago we showed that plants transformed with the coat protein genes of alfalfa mosaic virus (AlMV), tobacco streak virus (TSV) and tobacco rattle virus (TRV) were resistant to infection with particles of the respective viruses. Subsequently, we have used AlMV as a model to study the phenomenon of genetically engineered protection in more detail. The genome of AlMV is tripartite: RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively, and RNA 3 encodes the movement protein P3 and the viral coat protein (CP). In contrast to our AlMV CP plants, plants transformed with AlMV CP by Monsanto were resistant to both AlMV particles and RNAs. In addition, plants were transformed by Monsanto with a mutant AlMV CP gene encoding a protein of which the first amino acid was changed from Ser to Gly; these plants were not resistant to wild type (wt) virus. In collaboration with Monsanto we engineered the same mutation in an infectious clone of AlMV RNA 3 and the susceptibility of plants transformed with wt and mutant CP to infection with particles and RNAs of the mutant virus were investigated.

Infection of non-transgenic tobacco with AlMV RNAs 1, 2 and 3 requires the presence in the inoculum of a few molecules of CP per RNA molecule. Plants transformed

with full-length AlMV P1 and P2 genes (P12 plants) were found to support replication of RNA 3 when they were inoculated with an RNA 3 T7-transcript without coat protein in the inoculum. This shows that expression of functional AlMV replicase genes in tobacco does not result in virus resistance. Ten independent lines of transformants were tested that express the 3'-terminal region of the P2 gene that encodes the C-terminal part of the 90K P2 protein that is homologous to the TMV 54K sequence. This TMV sequence has been shown to confer resistance to TMV. Although a number of transformants showed expression of the truncated P2 gene at the RNA level, none of the δP2 plants showed a resistance to AlMV infection. cDNAs of AlMV RNAs 1, 2 and 3 could be transcribed into infectious RNAs when fused to the T7 RNA polymerase promoter or were infectious as such when fused to the CaMV 35S promoter. The role of the coat protein in the initiation of AlMV infection was studied by inoculation of non-transgenic, P1, P2 and P12 plants with various mixtures of AlMV T7-cDNAs, 35S-cDNAs and CP. The results indicate that the need for coat protein in the inoculum depends on the timing of the production of AlMV RNAs and gene products after inoculation.

Although our AlMV CP plants are resistant to infection with AlMV particles, they are susceptible to infection with AlMV RNAs 1, 2 and 3. This indicates that CP produced by the plants mainly blocks uncoating of the virus particles but is able to substitute for CP in a RNA inoculum. Recently, we transformed these CP plants with the AlMV P3 gene. When these so called P34 plants were inoculated with 35S-cDNA 1 and 35S-cDNA 2, they supported replication of AlMV RNAs 1 and 2 at a level that was about 10% from the level obtained after inoculation of P34 plants with a mixture of 35cDNA 1, 35S-cDNA 2 and T7-RNA 3. Apparently, expression of a combination of functional P3 and CP genes does not result in a resistance to inoculation with AlMV cDNA/RNA.

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A satellite RNA of a plant virus has linear, multimeric forms and circles as likely intermediates in replication

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Satellite RNAs increase only in cells in which a supporting plant virus is replicating, and they become encapsidated in the supporting virus coat protein. A critical characteristic of a satellite RNA is that it lacks any extensive nucleotide sequence similarity with the RNA of the supporting virus; in contrast, defective-interfering RNAs, which are similar to satellite RNAs biologically, are deletion and rearrangement mutants of the genomic RNA, showing extensive similarity to the genomic RNA. Because association with a satellite RNA often reduces the accumulation of the supporting virus, the satellite RNA behaves as a molecular parasite of the virus. Of potential or actual agronomic importance, depending on the system, is the ability of a satellite RNA to alter the symptoms that the supporting virus alone induces, enhancing or ameliorating the severity of virus-induced disease. The 359 nucleotide residue (nt) satellite RNA of tobacco ringspot virus (sTobRV RNA) is the subject of this abstract.

No evidence indicates that sTobRV RNA is a messenger RNA (Buzayan et al., 1986a). Nevertheless, inoculated sTobRV RNA (Schneider, 1977) or its plasmid cDNA clones (Gerlach et al., 1986), or transgenically-expressed sTobRV RNA (Gerlach et al., 1987), reduce drastically the titer of the supporting virus, tobacco ringspot virus (TobRV) and effectively protect some host plants from the most severe symptoms of TobRV infection. Probable replication intermediates of sTobRV RNA (circles and repetitive sequence, multimeric forms), and chemical reactions presumably involved in replication (self-cleavage; spontaneous ligation to form circles), have been identified (Prody et al., 1986; Buzayan et al., 1986b).

Results presented here and previously are consistent with replication of sTobRV RNA by means of rolling circle transcription. In the postulated replication scheme, the first step is the circularization of the most abundant of the encapsidated sTobRV RNAs, the linear, "monomeric", 359 nt molecule arbitrarily designated as having the positive polarity and

abbreviated L-(+)M. The resulting circular RNA C-(+)M serves as the template for the synthesis of linear, multimeric sTobRV RNA of the (-) polarity, which is processed to L-(-)M. Circularization to C-(-)M, transcription, and processing of the multimeric (+) polarity transcript complete the replication cycle (Bruening, 1990; Bruening et al., 1991).

Certain details of the cleavage and ligation reactions have been elucidated, and mutants show a correlation between the circularization of L-(-)M *in vitro* and the ability of the corresponding L-(+)M to initiate replication (Feldstein et al., 1989, 1990; van Tol et al., 1990, 1991). Transient expression of L-(+)M and mutants thereof was achieved by means of a plant DNA virus-derived vector that allows even non-replicating mutants of sTobRV RNA to be generated *in vivo*. Both complete and certain deleted forms of L-(+)M, transiently expressed in protoplasts and in plants, circularized even in the absence of TobRV, suggesting that the first step in the replication of sTobRV RNA occurs without any contribution from the supporting virus.

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TRANSGENIC TOBACCO EXPRESSING RIBOZYME OR ANTISENSE SEQUENCES HAVE INCREASED RESISTANCE TO VIRUS INFECTION.

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Ribozymes are RNA molecules with catalytic activity. The "hammerhead" form of ribozyme is capable of being targeted against particular RNA molecules and catalysing their cleavage in a site specific manner.

We have tested the ability of a ribozyme targeting the genomic RNA of tobacco mosaic virus to confer resistance in transgenic tobacco. The ribozyme, which cleaves TMV RNA efficiently *in vitro*, contains three catalytic domains embedded within a 1-kilobase molecule that is complementary to the 5' end of TMV. Transgenic tobacco plants expressing the ribozyme under the control of the CaMV 35S promoter were generated. The corresponding antisense sequence and the transformation vector without insert were used to produce control transgenic tobaccos. When progeny of regenerants expressing the transgenes were challenged with TMV, 7 of 43 ribozyme lines and 6 of 51 antisense lines showed delayed onset of symptoms and reduced numbers of affected plants. In contrast, none of the lines transformed with vector alone were resistant to infection. Lines showing a resistant phenotype were not necessarily those expressing the transgenes at higher levels. Segregation analysis on lines having single transgenic loci showed that the resistance phenotype co-segregated with the transgene in every case.

Resistance was maintained in following generations, and was greater for homozygous plants than for heterozygous progeny. Resistance occurred over a wide range of TMV inoculum concentrations, and for inoculation with TMV RNA. Of note, the six resistant antisense lines all showed delayed infection with the related virus tomato mosaic virus (ToMV; 80% sequence homology). However, only two of the six ribozyme lines showed significant resistance to ToMV.

PATHOGEN DERIVED PROTECTION AGAINST HUNGARIAN GRAPEVINE CHROME MOSAIC NEPOVIRUS (GCMV)

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We have constructed an hybrid gene driving the synthesis, in transgenic plants, of the coat protein (CP) of Hungarian grapevine chrome mosaic nepovirus (GCMV). As the GCMV CP coding sequence is located internaly on GCMV RNA2, it was first isolated and modified by the addition of an ATG codon and of a 5' non-coding sequence (Ω leader of TMV). This construction was cloned in an expression cassette composed of CaMV 35S promoter and the terminator from *Agrobacterium* nopaline synthase gene. The transgene was introduced in the genome of tobacco (*Nicotiana tabacum* var. xanthi), transgenic plants regenerated and R1 and R2 progenies carrying the transgene in an homozygous form were finally obtained.

The correct expression of the integrated transgene was verified by northern and by western blotting. The protein expressed has the expected size and accumulates at relatively high levels (approximately 2-6 μg per gram of leaf). This corresponds to levels 3-5 fold lower than those observed during GCMV systemic infection of *Chenopodium quinoa*, the standard multiplication host of this virus.

Analysis of the CP expressing plants (CP^+) demonstates that these plants are resistant to GCMV infection. The resistance seems to have two components which are observed at different inoculum pressures. At low inoculum pressure, fewer CP^+ plants become infected as compared to non-expressor transgenic controls (CP^0). At a higher inoculum pressure, this first component of resistance tends to diseappear but an important inhibition in the accumulation of viral RNAs is still observed.

Surprisingly, the reduction in the accumulation of viral RNAs is also observed when the the transgenic CP^+ plants are inoculated with purified viral RNAs instead of viral particules.

Results derived from inoculation experiments using a nepovirus closely related to GCMV (tomato black ring virus, TBRV) and with a GCMV-TBRV pseudorecombinant will also be discussed, as well as data on the resistance of protoplasts derived from CP^+ plants.

Session IV

ON THE MECHANISM OF ACTION OF A PLANT VIRUS RECESSIVE RESISTANCE GENE.

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Since the proposal of the gene-for-gene model to explain plant resistance to pathogens (1), many examples have been shown to fit into it. In most cases, according to the canonical description of the model, the plant genes that are matched by their corresponding avirulence genes in the pathogen govern a dominant trait: resistance.

The gene-for-gene model can be applied to resistance to plant viruses. Regardless of the difficulty of applying the dominance/recessivity concept to avirulence genes of plant viruses, many virus resistances in plants are dominant monogenic (2). Several of these have been studied in molecular terms and some insight about their mechanisms of action has been obtained (e.g.: 3-8). However, there are significant exceptions to the rule of dominance. One of them is resistance to potyviruses. These viruses conform the largest group of plant viruses, having more than one third of the total of plant viruses described (9). About 40% of the monogenic resistances to potyviruses are recessive (10).

We have been interested in the mechanism of action of the gene y^a that confers resistance to the type member of the potyvirus group, potato virus Y, in pepper (11, 12). This gene has been shown to be recessive by several authors (13, 14). Our results indicate that potato virus Y has an impaired cell-to-cell movement in pepper plants carrying this gene. These results will be presented and discussed, together with a progress report on the screening for new sources of resistance to viruses in pepper.

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INDUCTION OF RESISTANCE IN PLANTS BY TRANSFORMATION WITH VIRAL REPLICASE GENE SEQUENCES

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My laboratory has developed a novel approach to virus resistance, in which we transform plants with sequences derived from the viral replicase to induce resistance. We have shown this to be an effective strategy with both tobacco mosaic virus (TMV) and cucumber mosaic virus (CMV). However, as discussed below, for each virus we have employed a different portion of the replicase sequence. Work from our laboratory and others on such "replicase-mediated resistance" is reviewed in detail in Carr and Zaitlin (1993).

Our initial work on replicase-mediated resistance to TMV employed the so-called 54-kDa protein of TMV, a putative component of the viral replicase (Golemboski *et al.*, 1990). When this work was initiated, the presence of the 54-kDa protein in TMV-infected plants was (and still is) contentious; although there was reasonable evidence to suggest that the protein existed in infected tissue, its detection has eluded both us and one other laboratory. Our interest in it derived from our earlier finding that there was a subgenomic RNA which could be used to translate the protein in vitro, and that this subgenomic RNA was present on polyribosomes of infected plants. However, the 54-kDa protein has so far remained undetectable in infected plants, despite the use of several methods, such as labeling of plant tissues and protoplasts, as well as immunological methods employing several antisera. Nevertheless, studies were initiated to see if a function could be found for the protein. These studies included transformation of plants with the DNA sequence coding for the 54-kDa protein. To our surprise, these transgenic plants were resistant to the tobacco mosaic disease. Their resistance was absolute, in that even very high doses of virus or viral RNA inocula could not bring about the disease. Subsequently however, we found that the cells in the inoculated leaves of these plants actually supported a low level of virus replication (Carr and Zaitlin, 1991). The replication was so suppressed that the virus did not get into the vascular system and thus no systemic infection developed. The resistance was specific to the TMV strain used for the transformation (U1) and to a closely-related mutant, but resistance was not seen to other tobamoviruses and viruses from several other virus groups.

A critical question in understanding this phenomenon is to determine whether this resistance reflected the activity of the 54-kDa protein or its RNA in the transgenic plant. Because we were unable to detect the 54-kDa protein directly in plants, we used an indirect genetic approach to deal with the problem (Carr *et al.*, 1992). By making single nucleotide changes in the 54-kDa sequence, we created two mutants of the 54-kDa protein; one was able to produce a protein which lacked 14 amino acids on the amino end, and a second frame-shift construct engendered one which was only 20% full length. These constructs

were inserted individually into expression plasmids. When they were electroporated into tobacco protoplasts, together with TMV RNA, only the original 54-kDa gene and the mutant which lacked the 14 amino acids were able to suppress replication. The frame-shift mutant, although it had only a single nucleotide change, could not express a functional 54-kDa protein, and was not able to engender resistance. These data speak strongly for the role of the 54-kDa protein in the TMV resistance phenomenon. Very similar results have been recently obtained with pea early browning tobravirus, which encodes a similar 54-kDa replicase protein sequence (MacFarlane and Davies, 1992). In these studies, resistance was induced in *Nicotiana benthamiana*; data suggested that the functional 54-kDa protein was required for resistance induction.

For the induction of resistance to CMV, we took a different approach to that used to obtain replicase-mediated resistance for TMV. In this case, we cloned a truncated version of the RNA2 of CMV strain Fny (Anderson *et al.*, 1992), as opposed to the complete open reading frame used in the TMV study. The sequences deleted from this RNA included a motif characteristic of RNA-dependent RNA polymerases, the gly-asp-asp, or "GDD" box. As with TMV, the resultant transgenic plants were highly resistant to CMV, whether they were inoculated with high concentrations of virus particles or viral RNA. Furthermore, resistance was shown to be active in protoplasts, and like TMV, there was a markedly suppressed replication in the inoculated leaf, precluding systemic disease (Carr and Zaitlin, unpublished). Resistance was shown to the Fny strain used for the transformation and to several other strains in subgroup I of CMV, but not to strains of subgroup II, nor to other cucumoviruses (Zaitlin, Anderson, and Palukaitis, unpublished).

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Title: The development of replicase-mediated resistance as a strategy for producing virus-resistant legumes.

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It has recently been shown that plants transformed with a portion of the replicase gene (54K gene) from tobacco mosaic virus (TMV) are resistant to subsequent infection by the virus. Since all RNA viruses contain replicase genes, this method, termed replicase-mediated resistance, could potentially be applied as a general method for the production of virus-resistant plants. We are therefore investigating whether replicase-mediated resistance can be used to protect legumes against virus infection. Two legume-infecting viruses have been chosen for this study. One, pea early browning virus (PEBV) is a member of the alpha-like supergroup of positive strand RNA plant viruses and has a genome organisation similar to that of TMV. The other virus, cowpea mosaic virus (CPMV), is a member of the picorna-like supergroup and has a very different genome organisation.

Nicotiana benthamiana plants have been transformed with the portion of the PEBV genome which is the direct equivalent of the TMV 54K gene. The transformed plants were resistant to infection with purified PEBV at inoculum doses of up to 1 mg/ml. The plants were also resistant to infection with broad bean yellow band tobravirus but not to other members of the tobravirus group. Details of this work are given in the poster by MacFarlane, S.A., Lomonossoff, G.P. & Davies, J.W. We now intend to transfer the PEBV gene which confers resistance into peas and soybeans.

Although it is known that the CPMV replicase is encoded by the 3' terminal half of the the larger genomic RNA (RNA 1), the precise region equivalent in function to the TMV 54K gene is uncertain. To map the regions of RNA 1 which may be capable of conferring resistance when introduced into plant cells, a 3' co-terminal nested set of RNA 1 deletions have been constructed. These will be used in transient expression studies in cowpea protoplasts to determine which regions of RNA 1 are capable of interfering with CPMV replication. The regions identified will be introduced into transgenic plants and the ability of the inserted sequences to confer resistance against CPMV determined.

REACTION TO CYMBIDIUM RINGSPOT TOMBUSVIRUS INFECTION IN TRANSGENIC NICOTIANA BENTHAMIANA PLANTS EXPRESSING SEQUENCES OF THE VIRAL GENOME OR SATELLITE RNA

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Cymbidium ringspot tombusvirus has a single positive-sense genomic RNA of 4733 nucleotides. The viral genome encodes at least six polypeptides. The 33- and 92-kDa proteins are components of the viral replicase; the 41-kDa protein constitutes the viral capsid; the 22-kDa polypeptide is essential for cell-to-cell movement; the 19-kDa protein and the predicted product of ORF 6 are not essential to virus replication and movement and no specific function has been attributed so far. Defective interfering (DI) RNAs are generated *de novo* upon inoculation with CyRSV genomic RNA. Moreover, genomic RNA supports the replication of a satellite RNA (satRNA) of 621 nt. *Nicotiana benthamiana* plants transformed with satRNA sequences were not protected from apical necrosis and death induced by CyRSV, and the formation and/or replication of DI RNA were inhibited. To test possible mechanisms of resistance against viral infections, *N. benthamiana* plants were transformed with all CyRSV genes sequences.

N. benthamiana plants transformed with CyRSV coat protein sequences inoculated with virus at 5.0 and 0.5 μg/ml showed a delay in symptom appearance and apical necrosis compared to control plants, while transformed plants inoculated with virus at 0.05 μg/ml did not show apical necrosis. The coat protein expressed in transgenic plants was also able to encapsidate a CyRSV mutant defective for encapsidation.

Plants transformed with the 33- and 92-kDa proteins sequences, with the readthrough portion of the viral replicase alone and with 22- and 19-kDa proteins sequences showed a variable degree of resistance among different lines, depending on the inoculum concentration. Finally, plants transformed with DI RNA sequences and inoculated with CyRSV genomic RNA showed symptoms typical of viral inocula containing DI RNA and recovered (this part of the work was done by Dr. J. Burgyan's group in Gödöllő, Hungary).

The transposase of maize transposable element Ac forms large rod-like aggregates in nuclei of maize endosperm and transfected petunia protoplasts.

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The maize transposon Activator (Ac) is an autonomous element that encodes all non-host functions necessary to transpose itself and to mobilize the non-autonomous Dissociation (Ds)-elements *in trans*. The element is transcribed into a 3.5 kb mRNA which encodes an 807 amino acid open reading frame (ORFa). In nuclear extracts of maize endosperm a protein with an apparent molecular weight of 112 kDa was detected with five antisera directed against at least three non-overlapping regions of the ORFa-protein. This protein is only found in strains which contain biologically active Ac-elements and the amount is positively correlated with Ac-dose. Overexpression of ORFa in insect cells results in the synthesis of a protein that is indistinguishable immunologically and in size from the maize protein and which is localized within the nuclei of the insect cells suggesting that it is a nuclear protein. The expression of the cDNA segment encoding ORFa in transgenic tobacco plants is sufficient to trigger excision of an Ac-dependent, non-autonomous Ds-element, which makes the protein a likely candidate for the transposase (TPase). The protein binds to subterminally located multimeric AAACGG sequence motifs of the Ac-element. The resulting Protein/DNA-complexes are stabilized by protein-DNA- as well as by protein-protein-interactions. According to a model the interaction between the DNA-bound oligomers might mediate the formation of the active transposition complex in which both ends of the element to be excised are brought into close proximity for excision.

A transient excision assay has been developed, which allows to test the properties of the wildtype TPase and mutant derivatives. Here, a plasmid which encodes TPase and a reporter plasmid which carries a Ds-element inserted in the GUS-gene are cotransfected into petunia protoplasts. In this assay the replacement of the weak Ac-promoter by stronger plant promoters does not lead to the expected increase in excision frequencies. Furthermore, a truncated derivative of the TPase which lacks the 102 N-terminal amino acids triggers higher excision rates than the full-length protein.

The results of experiments will be described in which the protein encoded by transposable element Ac (TPase) of maize has been visualized by immunofluorescence. The protein is found in the nuclei of either the maize endosperm and transfected petunia protoplasts where it appears to be present as large rod-shaped complexes of defined size.

In maize, the number and strength of the signals is positively correlated with Ac-dose and varies between strains which carry Ac at different genomic positions. The correlation between these differences and the occurrence of specific variegation phenotypes on kernels which carry either different Ac-insertions or different Ac-doses suggests that the presence and the number of the complexes is somehow involved in transposition control.

In petunia protoplasts the number of the TPase-complexes which accumulate in the nuclei is directly related to the strength of the promoter which is fused to the Ac-coding region. If protoplasts are compared which do express either the wildtype or the truncated protein no obvious difference in the immunohistochemical signal is seen if both proteins are expressed under the control of the weak Ac-promoter. However, if the weak Ac-promoter is replaced by stronger promoters the two proteins differ in intracellular distribution and in appearance. In contrast to the wildtype TPase, only a fraction of the truncated protein expressed in the protoplasts is found in the nuclei while the majority is localized in the cytoplasm. The structure of the nuclear complexes can not be distinguished from those of the wildtype protein. The cytoplasmic fraction, however, appears to be different in that it consists of few large aggregates that are irregular in shape.

As suggested by the cytoplasmic retention of the truncated protein the N-terminus of the transposase either inhibits premature aggregation in the cytoplasm or contains sequences necessary for efficient nuclear import. The sequence, in fact, contains clusters of basic amino acids which are reminiscent to known plant nuclear localizing sequences.

The reduction in the number of nuclear complexes observed in case of the truncated TPase is correlated with an increase in TPase-activity. In addition, the increase in the amount of complexes as caused by replacement of the weak Ac-promoter by stronger plant promoters is not accompanied by an increase in excision frequency. These and other observations suggest that the TPase bound in the complexes probably is not the active protein. Does the formation of the complexes represent a mechanism which controls the protein's activity by sequestration?

Session V

Insect control with transgenic plants expressing Bacillus thuringiensis insecticidal crystal proteins

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Bacillus thuringiensis sprays have been used for more than three decades as an alternative to chemical insecticides. More recently, transgenic plants expressing B. thuringiensis insecticidal crystal proteins (ICPs) have been produced. Field trials have indicated that effective insect control can be obtained with this technology. Adaptation of the native gene sequence may be necessary for efficient expression in some crops. Transgenic plants expressing ICPs constitutively are considered to increase the risk of development of insect resistance. The incorporation of resistance management tactics is therefore an important element in this technology.

PLANT GENES FOR PROTECTING TRANSGENIC CROPS FROM CHEWING AND SAP-SUCKING INSECT PESTS.

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A reduction in those crop losses which are attributable to insect pests would make a significant contribution to redressing the imbalance between world agricultural production and demand. There is, however, growing concern that the conventional means employed to protect crops from pests are becoming unsustainable; not least because of their potentially disastrous long-term ecological consequences. There is thus an urgent need to develop substitution technologies which would allow a much more limited use of synthetic pesticides yet provide protection for crops within a sustainable agricultural framework. It is in this area of crop protection that the recent advances in biotechnology, especially in genetic engineering, have been looked to as offering the possibility of revolutionary new solutions. Progress directed at using this new technology for the very real, practical ends of crop protection has been rapid. Advances in molecular biology and plant tissue culture mean that it is now possible to produce transgenic plants for a wide range of crop species. However, the source of suitable insect resistance genes remains a key question.

Genes encoding insect control proteins [ICPs] produced by pests and pathogens of insects have received by far the greatest amount of research effort, especially the crystal toxin genes produced by the insect pathogenic bacterium, *Bacillus thuringiensis* [Bt]. The effectiveness of transgenic plants expressing Bt toxin in resisting insect herbivoury, in those situations where it does work, is very impressive. Monsanto Company have now demonstrated that such protection can be translated into commercially acceptable levels of field protection in such important crops as cotton, corn and potato. However, despite the impressive progress made with Bt-expressing crops, some important problems remain to be addressed. There is concern that this further use of Bt toxin may lead to dependence on a single factor resistance, exacerbating the risk of the pests developing resistance to it. Recent reports of broad-spectrum resistance Bt toxins are cause for particular concern. Another feature of Bt toxins is their inherent specificity; given the current pattern of chemical insecticide usage, it is clear that any replacement technology should be applicable to a broad range of insect pests.

The technical strategy we have adopted in the Durham University - AGC Ltd. Insect Resistance Programme is to mimic the natural, multi-mechanistic protection systems which have developed in plants themselves over the course of their long co-evolution with insects: the plants' own solutions to the plants' problem. Plants have evolved a number of mechanisms to protect themselves against insect attack and the combination of these is generally sufficient for durable resistance to most insects. Various factors in the artificial systems of agriculture have led to a breakdown of this balance. We have now isolated more than twelve plant genes encoding different ICPs. Amongst these are representatives from more than six different classes of proteins, each with a quite different mechanism of insecticidal activity. These genes, and selected combinations of them, are being tested in transgenic plants against a variety of insect pests.

The first example of this approach involved the cowpea trypsin inhibitor [CpTI] - the gene for which has been introduced into tobacco plants by *Agrobacterium*-mediated gene transfer [Hilder et al., 1987]. CpTI has been shown

in transgenic plants or in artificial diet bioassays to adversely affect the development and survival of a variety of different pests belonging to the orders Lepidoptera, Coleoptera and Orthoptera.

Since insect control proteins will be delivered to the insects as part of their food in transgenic crops, we have identified a number of candidate genes whose products adversely affect the insects' digestive system in different ways. The classes of compound include:

Serine protease inhibitors : - such as CpTI. Many insects, particularly the Lepidopterans, depend on serine proteases as their primary protein digestive enzymes.
Thiol protease inhibitors : - targetted at those insects, such as the major Coleopteran pests, which have acidic guts and rely on thiol rather than serine digestive proteases.
Alpha-amylase inhibitors : - disrupting carbohydrate metabolism.
Lectins : - sugar binding proteins which appear to interact with receptors in the gut wall of susceptible insects.
Ribosome Inactivating Proteins : - which have been shown to be particularly toxic to Coleopterans

Specific candidate genes for each of these classes have been identified and cloned, as cDNAs or by PCR amplification, for introduction into transgenic plants, where they are being tested singly and in various combinations. Absence of mammalian toxicity was a key consideration in selecting candidate genes. Differential toxicity resulting from the different organisation of the insect and mammalian guts can be exploited to select insecticidal proteins which are not harmful to the intended end-users of the crops. The selection of potentially useful insecticidal lectins provides an example of this strategy. Different lectins show a wide range of toxicity when fed to insects or to mammals. However, whilst both wheatgerm agglutinnin [WGA] and the lectin from snowdrop [GNA] are toxic to beetles, the former is also notoriously anti-nutritional to man, whereas GNA is essentially harmless. Thus, the GNA gene has been selected for transfer into transgenic plants.

The use of these genes in combinations within transgenic plants to give multi-gene, multi-mechanistic resistance is expected to increase both the effectiveness of insect control and its durability, reducing the chances of resistant populations of insects developing. An initial demonstration of the principle of combining these genes has been made by cross breeding transgenic tobacco plants which have been individually transformed with the CpTI gene and the lectin gene from the garden pea. In this case the genes had independent, additive effects in controlling *Heliothis* larvae. Protease inhibitors should be particularly valuable components in such combinations by protecting the other ICPs from premature hydrolysis in the insect gut.

Plant derived insect control proteins tend to be of relatively low absolute toxicity compared to chemical pesticides or *Bt.* toxin. The question of whether expression of a foreign, plant-derived protein in transgenic plants at the levels required for effective insect control incurs a yield penalty has been addressed using our population of CpTI-transformed tobacco plants under carefully controlled conditions in the growth room. Small, but sometimes significant differences were observed between transgenic and control plants, but appear to result from the transformation process, not from expression of the foreign protein.

The pest insects which have been considered so far feed by chewing

plant tissues. Those insects belonging to the order Homoptera - the sap-suckers - include many of the most serious agricultural pests. Crop damage is caused not only directly by the feeding of aphids, planthoppers etc, but some of the most serious losses are indirect, due to their role as vectors of plant viruses. Reduction of Homopteran insect populations would be particularly useful in limiting the spread of viral diseases in crops.

Because of their specialised feeding behaviour and physiology the currently available transgenic plant technologies have so far not been successful in controlling them. Two major problems have had to be solved before this technology could be extended to sap-suckers:

i) the identification of effective gene products - *Bt* toxins with activity against Homopterans have not been described, and most characterised natural plant resistance mechanisms to them appear to be based on small, non-protein secondary metabolites

ii) the development of an effective means of delivery - expression is required in the phloem sap if the protein is to be ingested by the insects.

Using an artificial diet bioassay, whereby the insects feed on artificial sap through a parafilm membrane, we have identified a number of plant proteins which are toxic or anti-metabolic to Homopterans. Amongst these proteins, it has been shown that the snowdrop lectin is effective against rice brown planthoppers [*Nilaparvata lugens*] and rice green leafhoppers [*Nephotettix nigropictus*] - both major sap sucking pests of rice - and against peach potato aphids [*Myzus persicae*].

The promoter associated with the rice sucrose synthase 1 [RSs1] gene has been shown to direct phloem specific expression of *GUS* in transgenic plants. This promoter, and the RSs1 gene intron 1 have been linked to the gene encoding GNA and this construct is being introduced into transgenic rice and tobacco for testing *in planta* against the brown planthopper and peach potato aphids.

Thus, plant biotechnology is well on the way to offering a realistic alternative to total dependence on chemical insecticides [or surrender to insect pests] in a wide variety of crop production systems. The technology is being transferred to 'orphan', third-world crops as well as the major crops of the developed world. Much remains to be done to determine the optimum strategies for using transgenic crops as part of an integrated pest management system for sustainable agriculture, but the rate of progress has been impressive.

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CEREAL INHIBITORS OF INSECT HYDROLASES (α-AMYLASES AND TRYPSIN): GENETIC CONTROL, TRANSGENIC EXPRESSION AND INSECT TESTS

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A substantial fraction of the albumins and globulins from cereal endosperm is represented by a single protein family that includes trypsin inhibitors and monomeric, dimeric and tetrameric inhibitors, of heterologous α-amylases. This group of homologous inhibitors which may play a protective role against insect predators, is encoded by a multi-gene family which is dispersed over five out of the seven homoeology groups of chromosomes in wheat and barley. Only recent advances in our knowledge of barley trypsin inhibitor CMe and of monomeric inhibitor of α-amylase, WMAI-1, will be discussed.

Trypsin inhibitor CMe. cDNA and genomic clones corresponding to trypsin inhibitor CMe have been characterized. Chromosome 3H carries the gene(s) for CMe. A gene duplication in a region of <4 kbp is present in cv. Villa. The 1st gene corresponds to the cDNA and has no introns; the second copy is a pseudogene. In certain high-lysine mutants of barley, such as Riso 1508, this protein is present at less than 2-3% of the wild-type amount in the mature kernel with respect to the wild type barley (cv. Bomi) from which it was derived and the corresponding mRNA levels in developing endosperms are also repressed (<1%). Neither a change in copy number nor a major rearrangement of the structural gene account for the markedly decreased expression. The mutation at the lys 3a locus in Riso 1508 has been previously mapped in chromosome 5H. A single dose of the wild-type allele at this locus (Lys 3a) restores the expression of gene CMe in chromosome 3H to normal levels, indicating that the expression of gene CMe is regulated in trans by the gene at the lys 3a locus. The molecular characterization of this trans-activator is one of the aims of the present research in our group, as well as the functional study of the promoter for CMe in transient expression experiments with barley protoplasts and in transgenic tobacco plants.

Monomeric α-amylase inhibitor WMAI-1. The cDNA corresponding to WMAI-1 has been expressed in Escherichia coli. The protein has the correct N-terminal sequence and the same electrophoretic mobility and specific activity towards the α-amylase from the insect Tenebrio molitor as the mature WMAI-1 isolated from wheat. Thirteen mutants have been obtained at six different sites. Substitution of the highly conserved N-terminal S by the sequence ARIRAR increased the pre-incubation time required for maximum activity. A similar result was obtained by insertion of GPRLPW after position 4, while insertion of EPRAPW at the same position rendered the inhibitor inactive. The substitution D/EGPRL and insertions DGP or D, at position 58, produced complete inactivation. All other mutations had only minor effects on activity.

Transgenic tobacco plants carrying chimeric genes with the 35S promoter followed by the cDNAs for CMe, and WMAI-1 have been obtained and tested against Agrotis ipsilon and Spodoptera littoralis. Increased mortalities in transgenic plants in the range of 30-40% over controls have been observed with larvae of both species.

TOWARDS THE MAP-BASED CLONING OF THE TOMATO ROOT-KNOT NEMATODE RESISTANCE GENE Mi

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Introduction

Root-knot nematodes (*Meloidogyne* spp) cause severe damage worldwide to a wide variety of crop plants of economical importance, including monocotyledons, dicotyledons and herbaceous and woody plants (Sasser, 1980). As utilization of nematode resistant plant cultivars provides an effective and environmentally safe alternative to chemicals for root-knot nematode control, much effort has been put into identifying host resistance against these pathogens in the germplasm of established cultivars or in related wild species. For some plant species useful sources of resistance have thus been identified and successfully incorporated into cultivars through breeding programs. However, for some crops that are severely damaged by these pests, no suitable source of resistance is available.

In principal, recombinant DNA technology has the potential to allow the transfer of appropriate resistance genes into susceptible crops across genetic incompatibility barriers. To date, this powerful approach is not feasible, as no nematode resistance genes have been cloned. Apart from the potential impact on agriculture, cloning of resistance genes would be a major step towards understanding the molecular events involved in the plant's defense response. Tomato offers an accessible model system from which to clone a nematode resistance gene.

Root-knot nematode resistance in tomato

Root-knot nematode resistance in tomato was first observed in 1941 by Bailey in the wild species *L.peruvianum*. Using embryo rescue, Smith (1944) succeeded in introducing this trait into *L.esculentum*. The resistance was subsequently shown to be encoded by a dominant allele at a single locus, referred to as *Mi*, an acronym taken from the first letters of

the root-knot nematode species *Meloidogyne incognita* (Gilbert and McGuire, 1955). The *Mi* locus was mapped to chromosome 6 (Gilbert, 1958) and was found to confer resistance to the three most damaging root-knot nematode species, *M.incognita, M.arenaria* and *M.javanica*. Nematode resistance in all modern tomato lines derives from the single hybrid plant obtained by Smith (1944).

Root-knot nematode juveniles are attracted to both resistant and susceptible plant roots. Following host penetration, generally near the root tip, nematodes migrate intercellularly to the region of cell differentiation (Eisenback and Triantaphyllou, 1991). From this point on, the course of events depends on the compatibility of the interaction between the nematode and the plant genotype. In a susceptible host, plant cells adjacent to the head of the nematode enlarge, in response to signals from the nematode, to form "giant cells", which are large, multinucleate, metabolically active cells that serve as a source of nutrients for the developing, endoparasitic form of the nematode. Concurrent hyperplasia and hypertrophy in the surrounding tissues leads to the formation of the galls or root-knots, characteristic of *Meloidogyne* spp. infection. In a nematode resistant genotype, however, a localized necrosis of plant cells around the anterior end of the nematode occurs within one day of infection and no giant cells or galls are formed. How the *Mi* gene triggers this sequence of events is unknown. Conceivably, a clone of *Mi* would provide a powerful tool for investigating the mechanism of this plant/pathogen interaction.

Map-based cloning of the root-knot nematode resistance gene *Mi*

The past decade has been a major advance in the development of gene isolation strategies. Nowadays, essentially every segment of a eukaryote genome is accessible for detailed identification and manipulation, irrespective of its coding potential. When the protein product of a gene of interest is known, cloning of the gene is relatively simple using techniques which are in every molecular biologist's tool box. Thus, once the protein has been purified antibodies directed against it can be used to screen a cDNA expression library. Alternatively, oligonucleotides synthesized on the basis of corresponding amino acids from parts of the protein may be applied to screen a cDNA library or to prime a polymerase chain reaction (PCR). The latter approach, in particular, has shown to be extremely powerful in isolating genes encoding low abundant proteins (Aarts *et al.*, 1991).

Because the product of *Mi* is unknown, strategies for gaining access to the gene are limited. Basically, two approaches are available, transposon tagging and map-based cloning. Map-based cloning, which is also referred to as positional cloning or reverse genetics, involves the identification of DNA markers surrounding the target gene, the physical

mapping and cloning of all sequences between the two closest flanking markers, and, finally, the complementation of a susceptible host through transformation so as to identify the cloned chromosomal fragment carrying the resistance gene (Wicking and Williamson, 1991). Thusfar, most examples of genes approached by a map-based cloning strategy are to be found among human heriditary disease genes, but more recently also plant genes start entering the scene (Giraudat *et al.*, 1992).

Although simple in its concept, gene isolation by a map-based cloning approach is laborious requiring many steps to be taken before the gene of interest is finally at hand. Irrespective of the nature of the target gene, map-based cloning boils down to the following steps:

(i) determine chromosomal location (genetic map position) of gene of interest by linkage analysis
(ii) identify tightly linked molecular markers
(iii) determine genetic order of molecular markers around target gene
(iv) construct long range physical map showing the physical order of and distances between molecular markers
(v) clone all sequences between two closest flanking markers
(vi) identify the cloned chromosomal fragment carrying the gene of interest by complementation of a genotype with the corresponding mutant phenotype.

As to the root-knot nematode resistance gene *Mi* in tomato, step (i) had already been accomplished more than 30 years ago as described above. To start a collection of molecular markers surrounding the *Mi* gene (step (ii)) we have isolated the acid phosphatase gene Aps-1, that is tightly linked to *Mi* and amenable to cloning using a product-based cloning approach (Aarts *et al.*, 1991; Williamson and Colwell, 1991). Furthermore, by screening a set of well-defined genetic stocks of tomato which differ only for a small chromosomal region carrying the resistance gene, we have been able to identify a variety of additional linked RFLP and RAPD markers (Klein Lankhorst *et al.*, 1991a,b; Ho *et al.*, 1992). These markers have been ordered into a high resolution molecular linkage map of the *Mi* region (step iii) that provides the landmarks for a chromosomal walk in the *Mi* region (Ho *et al.*, 1992). Among the four cDNA markers that are tightly linked to *Mi*, three are dominant (*L.peruvianum* specific). One cDNA marker (LC379) corresponds to a gene family comprising 20-30 members, one of which is present in the smallest introgressed region (500-1000 kb) associated with *Mi*. Whether the lack of hybridization to *L.esculentum* DNA is due to rapid evolutionary divergence or to the presence of *L.peruvianum* specific genes is not clear, but it raises the possibility that *Mi* (and perhaps other plant resistance genes) may not

have a homologue in susceptible genotype DNA. As a first step in dissecting the molecular organisation of the introgressed region around the *Mi* gene, we have constructed two long-range physical maps of the closest flanking markers (GP79 and Aps-1) which together span over 1200 kilobases. A chromosomal walk in the *Mi* region is now in progress. To this end, a Yeast Artificial Chromosome (YAC) library of tomato carrying chromosomal segments of 100-500 kilobases is being screened with markers from the *Mi* region. Eventually, this should result in the cloning of all DNA sequences contained between the two closest flanking markers and the molecular identification of the *Mi* gene.

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INTRODUCTION TO MEGABASE MAPPING AND CLONING STRATEGIES

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The past decade has seen a major advance in the development of gene mapping and gene isolation strategies. Nowadays, essentially every segment of a eukaryote genome is accessible for detailed characterization and manipulation, irrespective of its coding potential. Techniques for analysing complex eukaryote genomes have become so powerful that, in principle, entire chromosomes or even genomes are amenable to physical mapping and sequence analysis. For yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, mouse, human and *Arabidopsis*, genome programs have been launched to construct physical maps of ordered, contiguous clones of chromosomal DNA covering the entire genome. These maps will serve in cloning any gene of interest through map-based cloning strategies.

A breakthrough in the separation technology of DNA molecules was the development in 1984 by Schwartz and Cantor of pulsed-field gel electrophoresis (PFGE). By using two perpendicularly oriented electric fields in an alternating mode, rather than a constantly applied single field as in conventional electrophoresis, a dramatic increase in separation range was obtained. Individual yeast chromosomes, ranging in size from 230 kb to 2000 kb were clearly resolved and upon blotting to a carrier amenable to hybridization analysis. Since then, there has been a plethora of techniques and types of apparatus which are variations on this theme and allow for the resolution of DNA molecules as large as 10,000 kb (10 Mb). Thus, the gap in resolution between standard techniques in molecular biology and the 100-5000 kb size range that is covered by genetic recombinant analysis has been closed.

In 1987, another major advance in the analysis of complex genomes was made by Burke et al., describing an elegant vector system capable of cloning several hundred kilobase fragments of linear DNA as yeast artificial chromosomes (YACs). With their ability to accommodate 5-20 fold greater amounts of DNA than cosmids, YACs thus brings DNA cloning technology to resolution of genetic/cytogenetic maps.

The past five years has seen an explosion in the application of YAC-technology towards the study of complex eukaryote genomes. YAC-libraries have been made of a variety of organisms and used to isolate (large) genes, gene clusters or other regions of biological interest present in a single YAC or in a relatively small number of overlapping YAC clones. In some systems, YAC-technology is already taken one step further in its application to assemble contigs of overlapping YACs covering an entire chromosome or genome.

This lecture aims to review the basic aspects of PFGE- and YAC-technology that are relevant to long-range genome analysis.

Molecular Plant Breeding: Plant Protection and Subsequent Goals.

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There can be little doubt that plant protection will be the first and for some time to come, the major field of application of genetic engineering in plants. Practical results will determine whether or not this method becomes established as a powerful new tool in breeding schemes.
Whereas much progress has been obtained with regard to breeding of herbicide-, insect- and virus resistant crops, there still is room for significant improvements particularly with regard to fungal, bacterial and nematode attack. It will be of interest to study how introduced resistance genes can be coordinated with resistance mechanisms already operative in the plants. New approaches will be needed. We shall discuss a new method of gene-tagging that produces dominant "gain of function" mutations and will illustrate its usefulness in studying various mechanisms operative in plants resulting in tolerance/resistance to biotic and abiotic stresses.
The experience gained in producing plants with improved plant protection properties will be invaluable in the next phases of molecular plant breeding leading to plants with improved quality (e.g. ripening control) or for the production of tailor-made lipids, carbohydrates, fine chemicals, pharmaceuticals etc., etc. as well as plants with improved resistance to abiotic stresses (drought, temperature, heavy metals etc., etc.).

POSTERS

DEVELOPMENT OF A PEPPER TRANSFORMATION SYSTEM

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The most widely used technique for plant cell transformation is based on the natural gene transfer ability of *Agrobacterium tumefaciens*. In this work, we have tried to develop an *A. tumefaciens* -mediated transformation system for pepper, *Capsicum annuum* cv. Piquillo, based on a previous protocol for pepper in vitro regeneration system (Arroyo and Revilla, 1991).

In an analysis of all the putative steps related with the transformation process in pepper, we have observed that the developmental stage of the plant material and the host range of the Agrobacterium strain depends largely on the species used, which hampers the formulation of a general procedure. However, some general guidelines for the development of a transformation protocol can be depicted. Overall, we have noted that adittion of silver nitrate during the transformation and regeneration process reduces the stress in the plants, and that the use of feeder layers in the preculture improves the induction of the vir genes. Also, the time lengh of coculture is important in regulating the growth phase of the bacteria at the time of infection. In our case, 4-6 days in the optimum for the highest percentage of transformed shoots. These and other experimental details will be discussed, together with other approaches taken.

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PROGRESS ON USE OF DEFECTIVE INTERFERING RNAs TO ELICIT RESISTANCE AGAINST TOMATO BUSHY STUNT VIRUS IN TRANSGENIC PLANTS

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Tomato bushy stunt virus (TBSV) is a tombusvirus which is transmitted through soil and often induces lethal necrosis. In California it has been described as the causal agent of the "tomato decline" disease in the Imperial Valley. The virus has a single strand positive sense RNA of 4.8 Kb with four ORFs (the replicase, the capsid protein, p19 and p20 which maybe involved in movement). Expression of the capsid protein and nested p19 and p20 genes is mediated through subgenomic RNAs. Under certain circumstances TBSV produces defective interfering RNAs (DIs), whose sequences are derived from the virus. These DI RNAs are encapsidated by the viral coat protein and replicate much more rapidly than the viral genomic RNA . Thus, they presumably inhibit virus replication because they compete with the viral RNA for the replicase.

In order to use the DIs in transgenic plants as a method to engineer resistance to TBSV infection, the ends of the transgenic DIs have to represent precisely the termini of native DIs to be recognized by the replicase. For this purpose ribozymes specific for each end were designed that would specifically cleave DI RNAs from longer transcripts produced in transgenic plants. A polymerase chain reaction procedure was used to clone the ribozymes expressing the DI, into a Bluescript plasmid (BRz-DI). The RNA obtained by the *in vitro* transcription of BRz-DI is able to self-process in vitro to yield the native sized DI RNA plus the two ribozymes. This processed DI RNA is replicated in protoplasts when cotransfected with the virus, and it is able to attenuate TBSV symptoms in *N. benthamiana* as effectively as the native DI. Moreover the processed DI RNA inhibits the replication of the virus in *N. benthamiana* and in *N. clevelandii*. Experiments are underway to subclone the Rz-DI into an *Agrobacterium* vector to obtain transgenic plants resistant to TBSV.

Gene Expression in Barley in Response to Mildew Infection

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Many plants show race-specific resistance (RSR) to disease. If the pathogen has an avirulence gene which corresponds to a host resistance gene, there is an incompatible interaction, which limits pathogen development. While in a compatible interaction there is extensive disease. Our aim is to understand the mechanism of RSR of barley to powder mildew (causal agent *Erysiphe graminis* f.sp. *hordei*). We are especially interested in the Mla locus, which has over twenty different RSR alleles.

In this study we have looked for differernces in the response of barley to infection by virulent and avirulent mildew isolates. Several genes with defence-associated functions are induced in both compatible and incompatible infections. These include a chitinase, peroxidase, phenylalanine ammonia-lyase (PAL) and a gene homologous to the pathogenesis-related R protein (PR-R) of tobacco. We have related the expression of these genes to the development of mildew infection structure.

The expression of these genes is similar in compatible and incompatible interactions in several ways. Differences in the levels of chitinase, peroxidase and the PR-R protein only appear 30 hours after infection (h.a.i), when mildew starts to form haustoria. At this time mRNA levels fall in virulent-infected plants, but not in avirulent infections. The growth of virulent and avirulent mildew is similar up to 30 h.a.i, but thereafter avirulent isolates grow more slowly. This suggests that the differences in gene expression is related in some way to RSR. There is a similar fall in PAL expression in compatible interactions at 30 h.a.i, but recently we have found that PAL mRNA levels are higher in incompatible than compatible interactions at 5 h.a.i, the time at which primary germ tubes (PGT) develop. This suggests that the plant is capable of recognising the difference between virulent and avirulent mildew isolates even at this early stage.

ANALYSIS OF THE EFFECTS OF PROTEASE INHIBITORS ON *HELIOTHIS VIRESCENS* USING *IN VITRO* AND *IN VIVO* ASSAYS.

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Transgenic tobacco plants expressing a cowpea trypsin inhibitor (CpTI) have been shown to have enhanced resistance against the tobacco budworm *Heliothis virescens* (Hilder *et al.*, 1987, *Nature* 330:160-163). The cowpea trypsin inhibitors comprise a small family of four major iso-inhibitors belonging to the Bowman-Birk type protease inhibitor group (i.e. double-headed inhibitors). Three are trypsin-trypsin inhibitors and the fourth is a trypsin-chymotrypsin inhibitor (CpTCI). The gene originally introduced into tobacco had only trypsin inhibitor specificity. However, this may not be the most effective choice since characterisation of the gut enzymes of *H. virescens* has demonstrated two major enzyme activities:- trypsin-like and chymotrypsin-like, and a lower level of an elastase-like activity. Therefore, an inhibitor with more than one type of inhibitory activity may be more effective against this insect pest. Inhibition assays have been performed on *H. virescens* gut extracts using total cowpea trypsin inhibitors (CpTI+CpTCI), soybean Bowman-Birk trypsin-chymotrypsin inhibitor (SBBI) and soybean Kunitz trypsin inhibitor (SBTI) to determine which is the most effective. Furthermore, to ascertain whether the results from *in vitro* assays could predict the effectiveness of inhibitors *in vivo*, the inhibitors have been tested in artificial feeding trials and the genes for CpTCI and SBTI have been cloned and introduced into tobacco plants via *Agrobacterium*-mediated transformation. The results from artificial diet studies and preliminary results from bioassays on transgenic plants will be described.

Although protease inhibitors have been shown to successfully enhance resistance to insects it is generally accepted that the introduction of combinations of genes, which target different areas of the insect gut biochemistry, should afford a wider range of protection. To examine this strategy, various genes have been combined and introduced into tobacco and are currently being tested in bioassays.

Genetic analysis of different plum pox potyvirus isolates

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Plum pox virus (PPV) produces the perhaps most important disease of fruit trees of *Prunus* genus. This virus is a member of the potyvirus group, one of the most important group of plant viruses both because of the large number of members which belongs to it and the severe diseases which produce some of these members. Their genome consist of a unique molecule of single-stranded, positive-sense RNA of about 10Kb in length plus a 3' poly(A) tail,. having a unique ORF which is translated into a large polyprotein of about 340 K which is protealytically processed to give the functional products (1).

In order to unravel mechanisms of plant-virus interaction during the potyvirus infection, whose knowledge would allow to approach different strategies to obtain resistant plants against pathogens, we are studying, at molecular level, mechanisms by which viruses induce disease symptoms in plants. So, we began the analysis of three PPV isolates: PPV-Rankovic, whose genome has been completely sequenced (2) and cloned as a full length cDNA clone from which infectious transcripts can be synthesized (3), and PPV-o6 and PPV-PS that in their origin differed in the host range (PPV-o6 infected plum and apricot while PPV-PS infected plum, peach and apricot). All these isolates show different symptomatology when they are propagated in *Nicotiana clevelandii*. The sequence analysis of the 3' terminal region strongly suggests that PPV-PS constitutes a novel PPV strain different from PPV-El Amar and from that formed by PPV-Rankovic, PPV-D and PPV-NAT, and that the 3' terminal region of PPV-o6 derived from a homologous recombination event between a virus similar to PPV-PS and another of the PPV-consensus type, this is the first evidence of homologous RNA recombination within the potyvirus group (4). In order to localize determinants of pathogenicity in the plum pox virus genome, we are constructing several chimeric viruses by in vitro recombination using a full length cDNA clone of PPV-Rankovic, from which infectious trascripts can be synthesized, and cDNA of PPV-PS. Infectious in vitro transcripts were obtained from the chimeric cDNA and although none of the chimeric constructions caused the typical necrotic lesions of PPV-PS, they differed in the type of symptoms and in the time of their appearance.

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Fungal infection induces the expression of a proteinase inhibitor in germinating maize embryos.

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Proteinase inhibitors occur widely among plants, particularly in the families Leguminosae, Gramineae, and Solanaceae (1). Since they have been found to inhibit animal and microbial proteolytic enzymes they are considered to be protective against insect pests, or fungal pathogens (1,2). The best characterized proteinase inhibitor genes are those coding for the two nonhomologous proteinase inhibitors (I and II) from potato and tomato plants. In potato plants, these inhibitors accumulate to very high levels in tubers. In addition, they accumulate in leaves by chewing insects or severe mechanical damage (3,4). Here we report the isolation of a cDNA clon for a gene encoding a proteinase inhibitor, a chymotrypsin/subtilisin inhibitor from maize (Zea mays, pure inbred line W64A). The expression of this gene is induced after fungal infection of germinating embryos.

A cDNA library was prepared from two-day germinated maize embryos infected with Fusarium moniliforme. The cDNA library was subjected to diffential screening with 32P-cDNA probes prepared from poly $(A)^+$ RNA obtained either from sterile or from infected germinating embryos (the later one, was previously substrated with a biotin-labeled cDNA prepared from sterile germinating embryos). One clone was found to contain an open reading frame encoding a protein inhibitor homologous to the barley chymotrypsin/subtilisin inhibitors (61% and 56% homology to the barley CI-2 and CI-1 inhibitors, respectively) (5,6). These inhibitors belong to the potato inhibitor I family.

RNA blot analysis of sterile and F.moniliforme-infected germinating embryos (from day 0 to day 3) indicates that fungal infection induces the expresion of the maize proteinase inhibitor gene. Moreover, infection with other fungi (Penicillium spp., Trichoderma ssp.) also results in an increase in the level of the maize proteinase inhibitor mRNA. Although a body of evidence has accumulated which supports the concept that proteinase inhibitors are involved in plant defense against attacking pests, there are very few examples in which a relationship between the production of a proteinase inhibitor and infection by a fungus has been demonstrated. In our plant-pathogen system, the induction of the maize proteinase inhibitor in response to fungal infection could represent a defense mechanism complementary to the induction of other known defense-related genes. Related to this, we have found that infection with F.moniliforme induces the expression of several Pathogenesis-Related proteins in germinating maize seeds (7,8).

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A putative nutrient stress-induced cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*

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Summary

A cDNA clone encoding a putative cell wall protein (Qid3) was isolated from a library prepared from chitin-induced mRNA in cultures of the mycoparasitic fungus *Trichoderma harzianum*. The predicted 14 kDa protein shows a potential signal peptide, several hydrophobic domains and certain motifs structurally similar to extensin-like and glycine-rich plant cell wall proteins. *qid3* gene is induced by chitin and nutrient-stress and, when introduced in yeast, the cell division is arrested in cytokinesis and cell separation.

THE POSSIBLE USE OF CYSTEINE PROTEASE INHIBITORS IN GENETIC ENGINEERING FOR RESISTANCE TO *DIABROTICA* SPECIES.

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Crude enzyme preparations from larval guts of *Diabrotica undecimpunctata howardii*, the Southern Corn Rootworm, have been shown to contain high levels of protease activity which is almost completely inhibited by the specific inhibitors of cysteine proteases, E64 and cystatin. Using gelatin copolymerised into polyacrylamide gel as substrate, a number of protease isoenzymes have been identified, all of which appeared to be inhibited by E64 and stimulated by cysteine. As cysteine proteases are not employed in mammalian digestive systems, these enzymes would be a highly suitable target for genetic engineering aiming to confer to plants resistance against *Diabrotica*.

Transgenic plants expressing serine protease inhibitors have already been produced and shown to exhibit resistance to insects with serine proteases as their major digestive proteases. Thus the idea of successfully conferring resistance to insects whose main gut proteases are of the cysteine-type, via the expression of cysteine protease inhibitors, seems quite plausible.

Rice seeds are known to contain proteinaceous inhibitors of the cysteine proteases. One, oryzacystatin-I, which is highly effective against papain activity and has significant sequence homology to animal cystatins, was partially purified from rice seed and the effects of this preparation on larval enzymes *in vitro* and on larval development *in vivo* was investigated. With *in vitro* assays using a synthetic substrate, larval gut cysteine proteases were almost completelyinhibited (94%), while the same amount of the oryzacystatin preparation only inhibited an equivalent level of papain activity by approximately 60%. This indicates that oryzacystatin is more effective against the larval gut cysteine proteases than against papain. For *in vivo* assays the oryzacystatin preparation was incorporated into an artificial diet at1% dry weight. Larval development on this diet was monitored according to weight gain and survival. After 9 days on the diet both of these parameters had decreased by approximately 20% when compared to controls.

As the oryzacystatin-I content of rice seeds is rather low it is extremely difficult to accumulate enough protein for use in bioassay. To provide enough purified oryzacystatin for extensive bioassays, a full length oryzacystatin encoding sequence has been introduced into *Escherichia coli* and a functional inhibitor expressed.

GENETIC ENGINEERING OF BACTERIAL DISEASE RESISTANCE IN PLANTS

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We are studying the possibilities to genetically engineer bacterial disease resistance in plants using two antibacterial proteins, the barley endosperm hordothionins and the giant silkmoth cecropinB. Research is focussed on expression, proper processing and folding, and correct targeting.

The hordothionins are small ($M_r \approx 5000$) proteins that are processed from a larger precursor protein consisting of a signalpeptide, the mature hordothionin and an acidic polypeptide. Constructs have been made that code for one or more of these distinct domains and have been expressed in tobacco. Expression levels up to 0.7% of total soluble protein in young tobacco leaves could be obtained using a full-length gene. Without the acidic polypeptide coding region, expression was at least tenfold lower. The recombinant-hordothionins were properly processed from the precursor proteins, but could not be identified in the intercellular spaces.

A full-length gene was also expressed in tomato and transgenic plants with high expression levels were made homozygous for the transgene. Spraying with a bacterial suspension of *Xanthomonas campestris* pv. *vesicatoria* indicated some resistance in these plants, but bacterial growth was not inhibited *in vivo*, as estimated by infiltration of leaves, reisolation and plating. These plants were also not resistant to infection with *Clavibacter michiganensis* subsp. *michiganensis*.

CecropinB, another small protein ($M_r \approx 3800$), is also processed in the insect from a precursor protein. Gene constructs were made that code for the mature cecropinB, a plant signalpeptide and the cecropinB, and the full-length (cDNA-derived) gene. These were expressed in tobacco. At the RNA level, cecropinB-specific transcript levels were increased in plants containing the construct with the plant signalpeptide, which was made chemically with a codon usage adapted to *Solanaceae*. The cecropinB protein could not be detected. The offspring of transgenic tobacco plants with high cecropinB-mRNA levels were evaluated for resistance to *Pseudomonas solanacearum* and some plants survived inoculation with as much as 5 ml of a 107 CFU/ml bacterial suspension per plant. None of the control plants survived this treatment. Surviving plants are being multiplied for renewed screening.

Future research is focussed on targeting the hordothionins to the intercellular spaces, increasing the levels of cecropinB expression and stabilizing the protein by making a fusion protein with the hordothionins.

THE HAMMERHEAD RIBOZYMES OF PEACH LATENT MOSAIC VIROID AND OF A VIROIDLIKE RNA FROM CARNATION: VARIATIONS ON A THEME.

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Cis-acting ribozymes conforming to the hammerhead structure model have been recently identified in peach latent mosaic viroid (PLMVd) (1) and in carnation stunt associated viroidlike RNA (CarSAV) (2). These two small RNAs can adopt hammerhead structures in the strands of both polarities, devoting approximately one-third of their genome to the formation of the self-cleaving domains, which in CarSAV are partially overlapping. Plus and minus partial- and full-length RNA transcripts of PLMVd containing the hammerhead structures self-cleaved during transcription and after purification as predicted by these structures; these data are consistent with the high stability of the PLMVd hammerhead structures and indicate that the corresponding reactions most probably occur through an intramolecular mechanism. A similar situation was observed with minus full- and partial-length transcripts of CarSAV including the hammerhead structure, whose autolytic processing should also proceed intramolecularly. In the case of plus CarSAV transcripts only a dimeric RNA, but not a monomeric one, self-cleaved efficiently during transcription and after purification, suggesting the implication of a double-hammerhead structure theoretically more stable than the corresponding single cleavage domain. However, monomeric plus CarSAV RNA self-cleaved after purification by the predicted site in a concentration-independent reaction of slow rate.

The variations observed in PLMVd, CarSAV and other hammerhead structures probably reflect different adaptations to regulate the action of these cis-acting ribozymes. They should be active at some stages of the rolling circle-type replication proposed for these infectious RNAs (mediating the autolytic processing of their multimeric transcripts) and inactive when the synthesis of the final monomeric circular RNA has been completed (preventing its self-cleavage).

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Genetic variation of viral, and satellite, RNAs.

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Plants resitant to viral diseases have been obtained by introducing into the plant's genome sequences from: **(1)** the virus coat protein gene, **(2)** the virus replicase gene, **(3)** attenuative satellite RNAs associated to the virus. These three types of engineered "resistance genes" result in differently expressed resistances, that are always specific for the virus from which the sequences come, or for closely related viruses. Thus, the efficiency of engineered resistant plants may be affected by the genetic variation of viruses, through mutation, pseudorecombination or true recombination. In addition, natural evolution of attenuative satellite RNAs, or new combinations satellite RNA/helper virus/host plant, may be at the origin of possible hazards associated with strategy (3).

Cucumber mosaic virus (CMV) is a virus for which all three strategies for engineered resistance have been proposed. We have studied the evolution of field populations of CMV and its satellite RNA. Fast evolution of CMV-satRNA in field populations occurs by sequential accumulation of mutations. Genetic change results in the appearance of new phenotypes, that may render inefficient, and unwise, to seek engineered resistance by strategy (3). Mutations in CMV genomic RNAs do not accumulate at high rates, as for CMV-satRNA. On the other hand, variation through reassortment of the genomic RNAs of different strains is frequent, and true recombination may also occur. Reassortment in the field has only been shown between closely related strains, so that it would not affect the efficiency of strategies (1) and (2). Nevertheless, the interchange of genetic material between distantly related strains cannot be ruled out, and a wider field prospection is underway to detect its occurrence and quantify its frequency.

Detection of the protein encoded by transposable element Ac *in situ*

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The protein encoded by transposable element Ac, which is a likely candidate to be the transposase (TPase) of the Ac/Ds transposable element system of maize, has been labeled *in situ* by immunofluorescence using five different antisera recognizing at least three non-overlapping epitopes of the Ac-protein. As suggested by the results of earlier experiments in which the protein has been stained in the nuclei of transformed insect cells the protein is localized in the nuclei of the maize endosperm. Surprisingly, the protein appears not to be evenly distributed in the nuclei. Instead, large rod-like structures are observed, which are approximately 2 μm in length. These structures are seen only in endosperms containing active Ac-elements. The amount of the complexes is positively correlated with Ac-dose and differs between maize lines which carry Ac in different genomic positions.

Plant defence reactions expression and induction in sunflower cell suspensions

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Plant cell suspension cultures have proven to be a very useful experimental system to study plant defence reactions at both biochemistry and molecular biology level and to obtain new crop varieties with enhanced resistance to microbial pathogens and insects through genetic engineering and somaclonal variation techniques. By understanding which genes are activated in response to microbial or insect attack and which molecular processes promote plants'natural defences we can stablish strategies of gene expression alteration or manipulation for increased resistance against specific pathogens.

In our laboratory we are currently studying plant defence mechanisms using the sunflower (*Helianthus annuus* L.)-*Plasmopara halstedii* (causing agent of the downy mildew) interaction as model experimental system. At whole plant level we have observed that the resistance character is due more than to the ability of defence mechanisms expression (which is also observed in susceptible cultivars) to its induction at early stages of the infection process and at the proper place and amount to be efficient for fungal growth inhibition (it seems to happen in the resistant cultivars). It would be very important to stablish which one are the molecular bases of the early induction in an attempt to stablish strategies of plant resistance improvement.

The characterization of the plant defence mechanisms expression and induction is now being carried out in our laboratory using sunflower cell suspensions. In the present communication we describe preliminary results on the level of expression of defence reactions at different stages of the cell growth an its induction by a elicitor preparation coming from yeast extract. The reactions we are focused include: production of the sunflower cumarinic phytoalexins scopoletin and ayapin, induction of enzymes implicated in the biosynthesis of specific phenolic compounds (phenylpropanoids and lignins) and cell-wall hydrolitic enzymes (β-1,3-glucanase and chitinase). Not differences in the level of expression of the above biochemical parameters have been found at different times of the sunflower cell growth curve, by the contrary, yeast extract originated a marked increase in phytoalexin, phenolic and hydrolitic enzyme activity levels, although the pattern of induction was different for each one.

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ENGINEERED RESISTANCE AGAINST POTYVIRUSES USING TRUNCATED *TOBACCO ETCH POTYVIRUS* COAT PROTEIN MOIETIES

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Coat protein mediated resistance (CPMR) has proved to be successful against several families of plant viruses (Beachy *et al.* 1990). In the case of the Potyviridae family, so-called heterologous resistance is observed when we inoculate a transgenic line expressing the coat protein of a potyvirus different to the challenging virus (Stark and Beachy, 1989). The possibility that a domain of the potyviral coat protein, most likely a highly conserved domain, could account for the broad range of CPMR lead our group to design a set of truncated TEV-CP moieties corresponding to a N-terminal deletion of 28, 63 or 112 amino acids. The amino acid sequence of each moiety increases the percentage of homology with a phylogenically conserved sequence among potyviruses. For control purposes, tobacco Xanthi plants were engineered to express an RNA slightly longer to that of the coat protein coding sequence (it includes, for cloning convenience reasons, part of the NIb gene sequence) in sense and antisense orientation.

R2 populations were challenged by mechanical inoculation with several concentrations of TEV. Resistance is scored according to several criteria, among which the delay in the appartion of symptoms, the maximum percentage of infected plants and symptom severity. Out of nine transgenic lines expressing the longest TEV-CP moiety, one line was scored resistant. Out of nine transgenic lines expressing the medium-size TEV-CP moiety, two lines were scored resistant. Out of ten lines expressing the shortest TEV-CP moiety, two lines were scored resistant. A clear attenuation of symptoms was observed at all levels of inoculum. The largest delay in the apparition of symptoms is observed with the medium size TEV-CP expressing plant. This is the first report of CPMR obtained by expressing a truncated potyviral coat protein.

The same experiments were carried out with two different concentrations of PVY. A clear attenuation of symptoms was observed with the transgenic lines expressing the two shortest moieties of the TEV-CP at all levels of inoculum, but no significant delay in the apparition of symptoms was observed.

No resistance was observed against TEV or PVY when inoculated onto transgenic lines expressing the TEV-CP RNA antisense construct. The symptomatology is however different from that of normal virus infection. A short delay in the apparition of symptoms was observed with transgenic lines expressing the TEV-CP RNA sense construct at low levels of homologous inoculum. The same lines are not resistant against an heterologous inoculum.

Possible mechanisms of genetically engineered resistance against potyviruses will be discussed.

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STUDIES ON KIWIFRUIT TRANSFORMATION USING *AGROBACTERIUM* -MEDIATED AND DIRECT DNA TRANSFER TECHNIQUES

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The superior qualities shown by the female Hayward cultivar of kiwifruit (*Actinidia deliciosa* var. *deliciosa*) led to the worldwide selection of this cultivar for kiwifruit production. Most of kiwifruit orchards are still young and not very much affected by pests or diseases. However there are several reports of infections and infestations with acari, insects, nematodes, fungi and bacteria. Considering that the world production of kiwifruit is primarily based on one clone of genetically uniform plants the production becomes very sensitive to pathogens that may spread contaminating different agricultural areas. Some biological approaches have been suggested to control some kiwifruit plagues and infections. However in most cases the choice is still the wide application of poisoning chemicals. The progress observed in the last years at the level of the molecular biology, together with the development of efficient methods for introduction of foreign DNA in the plant cell may offer real alternatives for the control of kiwifruit pathogens. The efficient use of genetic transformation for crop improvement is primarily based on the development of good biological systems to allow the application of transformation protocols. In this perspective we developed methods for kiwifruit (cv. Hayward) propagation using apical and axillary buds, different explant types (root, stem and leaf) and protoplast cultures.

Kiwifruit transformation was tested using agroinfection and direct DNA transfer. For agroinfection we used a virulent agropine strain of *Agrobacterium rhizogenes* (AGL1) carrying a plasmid with GUS and NPT II chimeric genes and a disarmed octopine strain of *A. tumefaciens* (LBA 4404), carrying a plasmid with a NPT II chimeric gene and a GUS chimeric gene containing a plant intron. Leaves of micropropagated plantlets were infected with *A. rhizogenes*. Five to six roots developed per leaf, on hormone-free medium, when kanamycin (30 $\mu g.ml^{-1}$) was added 3 weeks after infection. These roots were induced to develop shoots in a medium containing 15 $\mu g.ml^{-1}$ of kanamycin. Under these conditions some green shoots were recovered. For infection with *A. tumefaciens* the explants were pre-cultured for 1 or 2 days. From these explants green shoot buds could be regenerated when kanamycin (25-75 $\mu g.ml^{-1}$) was added 15-45 days after infection. Some green shoots recovered from these buds, showed different levels of resistance to 100 $\mu g.ml^{-1}$ of kanamycin. However no sustained multiplication was observed under this kanamycin concentration. Protoplast transfection was accomplished using PEG 4000 (20%) or electroporation (4 pulses with a duration of 40 μs and a field strength of 200 $V.cm^{-1}$). For stable transformation we used plasmids carrying the NPT II chimeric gene. Some regenerated colonies showed NPT II activity before kanamycin selection was applied. A mean value of 31 green shoots was regenerated per 10^6 electroporated protoplasts after 8 months of selection on media supplemented with kanamycin (15-25 $\mu g.ml^{-1}$). This value increased to 66 when 5 months of selection were followed by 3 months on kanamycin-free medium. For 10^6 PEG-treated protoplasts a selection period of 8 months yielded 31 green shoots, while 5 months of selection followed by 3 months without selection yielded 86 green shoots. In control plates an average of 1360 shoots was obtained per 10^6 protoplasts. The green shoots regenerated after the different transformation procedures have not yet been submitted to molecular analysis to verify the integration of the DNA fragment.

GENETIC TRANSFORMATION OF *Pinus nigra* AND *Pinus radiata*: AN ALTERNATIVE METHOD TO OBTAIN FOREST PLANTS PROTECTED FROM INSECT ATTACK.

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In southern Europe *Pinus nigra*, *P. pinaster*, *P. sylvestris* and *P. radiata* are the most valuable pines in reafforestation programmes. *P.nigra* is affected by acid rain and insect attack, being necessary its continuous reafforestation. Radiata pine is not native, but it is a rapid growing species; its diffusion is limited due to insect attack, which causes defoliation, death of individuals and changes in the quality and chemical composition of the wood, being the larva of the genus *Thaumetopoea* the most aggressive in mediterranean forests.The obtention of transgenic pine plants expressing the δ-endotoxin of *B. thuringiensis* and the mass clonal propagation of these plants will allow to carry out reafforestation programmes with resistant trees. This imply the reduction of costs and ecological problems, by eliminating pesticide treatments in the field.

We consider the possibility to use *Agrobacterium tumefaciens*-mediated T-DNA transfer to express chimeric genes in *Pinus nigra* Arn. and *Pinus radiata* D. Don. plants. This technology, along with tissue culture for transgenic plant recovery, clonal selection and propagation will allow to obtain elite trees with resistance to insect attack, which is responsible, to a large extent, for conifer forest decline in southern Europe. Obtention of an efficient and reproducible plant regeneration system for *P. nigra* and *P. radiata* tissue cultures is an essential prerequisite for the obtention of transgenic plants through *Agrobacterium*-mediated transformation protocols. Our objectives can be summarized as follows:

- Establishment of an efficient plant regeneration system with tissue cultures of both pine species.
- Obtention of transgenic plants through *Agrobacterium*-mediated transformation of *Pinus nigra* and *P. radiata* tissue cultures.
- Assessment of other techniques for transformation of pine plants.

The use of traditional and recent methods for plant genetic manipulation will contribute to crop improvement in *Pinus* species.

RESISTANCE TO BROAD BEAN MOTTLE VIRUS USING DEFECTIVE INTERFERING RNAs

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Defective interfering RNAs (DI-RNAs) have been reported associated to some African strains of broad bean mottle virus (BBMV) (Romero *et al.*, 1992). In addition to three genomic and one subgenomic RNA components, BBMV encapsidate DI-RNAs that are formed by in frame deletions in the RNA 2 open reading frame. A comparison of the intensities of full-length RNA-2 bands from DI-containing and DI- deficient virion RNA preparations revealed that the DI-RNAs decreased the level of RNA-2 components in both total and encapsidated. *De novo* generation of DI-RNAs has been observed after subsequent serial passages of BBMV strains through broad bean plants. Some of the *de novo* DI-RNAs were similar to originally observed DIs whereas the other differed considerably in size.

As a novel approach for the control of plant viral infections using BBMV as a model system, the only bromovirus that has DI-RNAs and can infect *Nicotiana* plants, our experiments are designed to confer resistance in transgenic plants to viral infections by expressing DI-RNAs. The DI-RNAs sequences will be introduced into the genome of *Nicotiana clevelandii* and *N. benthamiana*, by an *Agrobacterium*-mediated transformation system and tested for viral resistance.

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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF A SOLUBLE SALCYLIC ACID-BINDING PROTEIN FROM CUCUMBER

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Previously we identified a soluble salicylic acid (SA) binding protein (SABP) in tobacco leaves whose properties suggest that it may play a role in transmitting the SA signal during plant defense responses (Z. Chen and D.F. Klessig, 1991. PNAS, 88: 8179-8183). A screen of different plant species indicates that many contain a similar binding activity. High levels were detected in cucumber. Since several studies (J.P. Métraux et al, 1990. Science, 250: 1004-1006; J.B. Rasmussen et al., 1991. Plant Physiol, 97: 1342-1347) have shown that dramatic increases in endogenous SA levels correlates with the development of systemic adquired resistance (SAR) in cucumber, its SA-binding activity was characterized. The cucumber SA-binding activity exhibits several similarities with the tobacco SABP. It is soluble and precipitates in the 0-45% amonium sulfate cut. The activity is SDS-sensitive and pH dependent with an optimum pH range between pH 5.5 and 6.5, like that of tobacco. Scatchard analysis reveals the same level of SABP in cucumber as in tobacco (5 pmole per mg of total soluble protein) but a lower affinity for SA with Kd of 30 μM compared to the tobacco's 14 μM. The cucumber SABP, like the tobacco SABP, exhibits very stringent binding specificity; only those analogues of SA which are biologically active for induction of disease resistance or defense-related (eg. PR) genes, are bound by SABP. In contrast, its apparent native molecular weight as determined by gel filtration is markedly different. The SABP of cucumber is ~55 kDa compared to the ~180 kDa tobacco SABP. Purification of both proteins is in progress to eventually determine whether or not they have similar functions. Perhaps one acts as a receptor while the other is involved in SA metabolism or in mediating other physiological effects of SA.

ISOFORMS OF THE SO-CALLED NON-SPECIFIC LIPID TRANSFER PROTEINS ARE PRESENT IN SPINACH AND ARABIDOPSIS AERIAL PARTS AND ARE ACTIVE AGAINST FUNGAL AND BACTERIAL PLANT PATHOGENS.

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Non-specific lipid transfer proteins (LTPs) from plants were so designated because it was initially thought that they were involved in cytoplasmic lipid shuttling between organelles, but more recent findings, which show that they are synthesised as precursors with signal peptides and that they are secreted, seem to contradict the above possibility. On the other hand, it has been recently found in this laboratory that LTPs isolated from barley leaves were potent inhibitors of bacterial and fungal plant pathogens. It was therefore of interest to investigate whether this antimicrobial activity was a general property of the protein family or was just restricted to the barley LTPs.

Two homogeneous proteins were isolated from a crude cell wall preparation from mature spinach leaves by extraction of the insoluble pellet from a Tris-HCl (pH 7.5) homogenate with 1.5 M LiCl and fractionation by reverse-phase high-performance liquid chromatography. N-terminal amino acid sequences of the two proteins allowed their identification as LTPs and one of them corresponded to that previously isolated from the soluble fration. The two proteins were active against the pathogens tested at micromolar concentrations. The bacterial pathogens *Clavibacter michiganensis subsp. sepedonicus* and *Pseudomonas solanacearum* and the fungus *Fusarium solani* were the pathogens used in this study.

Two homogeneous LTPs from Arabidopsis were similarly obtained and also found to be active against the same pathogens at micromolar concentrations. One of the proteins seemed to correspond to that encoded by a previously reported cDNA, while the second one was quite divergent.

TOBACCO PLANTS TRANSFORMED WITH THE 54kDa REGION OF THE PMMV REPLICASE GENE ARE RESISTANT TO VIRUS INFECTION.

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Pepper mild mottle virus (PMMV), a member of the tobamovirus group, is one of the most destructive viruses of pepper crops grown under plastic in the Southeast region of Spain (1). Several PMMV isolates have been described infecting pepper cultivars with incorporated resistance genes to Tobacco mosaic virus (TMV) and Tomato mosaic virus (ToMV) and they have been differentiated by breeders as pathotypes P_1, P_{12} and P_{123}, based upon their ability to overcome the resistance conferred by an allelic series of Capsicum spp. genes known as L1, L2 and L3, respectively (2). The complete genome sequence of PMMV-S, a Spanish isolate identified as a P_{12} pathotype, has been recently determined in our laboratory (3). Since there are no comercial pepper cultivars with resistance genes readily available, we are analyzing a genetically engineered resistance based on the replicase-mediated strategy (4).

A cDNA fragment from 3411 to 5016 nt of the PMMV-S sequence was introduced into the disarmed binary vector pGSJ780A and used to transform Nicotiana benthamiana plants. This sequence covers PMMV-S 54 kDa gene, which encodes a putative component of the viral replicase complex.

The presence of the quimeric gene in trasgenic plants was determined by a polimerase chain reaction (PCR) assay of total plant DNA extract using primers flanking the viral sequence insert. Transformed plants were challenged with either PMMV-S, PMMV-I, an Italian isolate identified as a P_{123} pathotype, or TMV. Preliminary results shows an absolute resistance to PMMV-S and PMMV-I infection in some of the trasgenic plants while the others presented only a partial resistance. No resistance was obtained against TMV.

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OUTLINE OF THE ROUNDTABLE DISCUSSION

G. Bruening

CONCEPTS OF NATURAL RESISTANCE AND RESISTANCE PHENOMENA

General classification of resistance phenomena

Non-host resistance (immunity or incompatible reaction exhibited by sets of species, genera, families of plants)

Cultivar resistance (genotypic immunity or genotypically-controlled incompatible reaction exhibited by plant lines)

Induced resistance

Cross protection (prior or simultaneous systemic infection by protecting virus)

Acquired resistance (prior and usually localized infection by protecting pathogen)

Local
Systemic (SAR)

Concurrent protection (protecting and challenging pathogen probably must reach the same cells)

Gene-for-gene hypothesis

Very well established concept; does new information justify revision of the concept at this time?

Application of concept in resistance against viruses

Signaling in pathogen-plant interactions

Within cell

Cell-to-cell

Systemic (long distance)

Common and potentially common features of resistance against disparate pathogens

Common signals (e.g., avirulence gene product)

Common transduction pathways

Common responses (e.g., hypersensitivity)

Common lines of research?

ANALYSES OF NATURAL RESISTANCE AND RESISTANCE PHENOMENA

Documenting and isolating plant genes involved in a susceptibility and resistance phenomenon

What genes are involved?
- Genes necessary and sufficient for resistance
- Back-up genes activated after loss of resistance gene function

Genes of known resistance capability and function
- Hydrolases
- Subcellular targetting of hydrolases
- Requirement of two or more hydrolases to acheive protection

Genes of known resistance or anti-pathogen capability and unknown function

- SAR8.2
- Antibiotic peptides
- Elucidation of function by antisense and ribozymes against resistance genes and putative resistance genes
- New, more efficient methods for identifying genes and pathways in resistance phenomena

Contributions of plant genes to disease susceptibility
- Plant hydrolase action and increased susceptibility

Strategies for isolating resistance genes

- Map-based cloning procedures
- Cloning from functional gene product

Non-resistance functions of resistance genes

Documenting and isolating pathogen genes involved in a susceptibility and resistance phenomenon

Avr genes as pathogenicity genes

Other functions of pathogen genes for avirulence and pathogenicity

ENGINEERED GENES FOR RESISTANCE

Based on application of natural resistance genes

Non-host resistance as source

Genotypic resistance as source

Based on natural anti-microbial agents

Satellite and DI RNAs
Bacillus thuringiensis insecticidal crystal protein
Proteinase and amylase inhibitors against insects
Chitinase as antifungal agent
Insecticidal receptor-binding lectins

Based on application of pathogen genes

Virus coat protein
Virus replicase genes and gene fragments
Virus mutant movement protein
Bacterial ornithyl transcarbamylase

Based on synthetic resistance (non-resistance genes applied to resistance; synthetic nucleotide and amino acid sequences)

Inhibitors of virus enzymes

Hydrolytic enzymes (Barnase) for synthetic HR

Ribosome inhibitors

Expression systems and expression

Wound-inducible promoters and other specialized promoters

Lack of correlation between expression level and efficacy of construction; mechanism?

Optimizing expression
- Transcription and processing
- Translation
- Stability of mRNA and protein

POTENTIAL PROBLEMS IN AND/OR TECHNICAL BARRIERS TO RAPID INTRODUCTION OF ENGINEERED RESISTANCE

Performance of engineered resistance genes under field conditions

Field levels of disease pressure and their relationship to laboratory standards for resistance test protocols, inoculum level, etc.

Resistance gene performance
- For reliable resistance in the short term
- For durable resistance (e.g., use of multiple Bt ICPs or multiple resistance genes with distinct modes of action)

Maintenance of resistance gene function in progeny
- Silencing of resistance gene expression

Consequences of resistance gene expression and overexpression on plant agronomic properties

List of Invited Speakers

Workshop on

ENGINEERING PLANTS AGAINST PESTS AND PATHOGENS

List of Invited Speakers

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