Works published in the Serie Universitaria cover the following specialities: Architecture and Town Planning; Fine Arts; Biology; Agricultural Sciences; Social Sciences; Journalism; Law; Economics; Phylosophy; Phisics; Geology; History; Engineering; Literature and Philology; Mathematics; Medicine, Pharmacy and Veterinary; Music; Chemistry; Theology.

Each group of specialities has its own colour on every cover.

The works summarized in this publication were presented by their authors at a Workshop held on 23th to 25th October 1989 at the Parador Nacional "Luis Vives", Valencia (Spain).

There is a limited edition of 450 copies of this volume, available free of charge.

Fundación Juan March

247 Workshop on Pathogenesis - Related Proteins in Plants

SERIE UNIVERSITARIA

Fundación Juan March

Workshop on Pathogenesis-Related Proteins in Plants

Organized by

V. Conejero and L. C. Van Loon

L. C. Van Loon
R. Fraser
J. F. Antoniw
M. Legrand
Y. Ohashi
F. Meins
T. Boller
V. Conejero
C. A. Ryan
D. F. Klessig
J. F. Bol
A. Leyva
F. García-Olmedo

Fundación Juan March

Serie Universitaria

247

Workshop on Pathogenesis-Related Proteins in Plants

Organized by

V. Conejero and L. C. Van Loon

L. C. Van Loon
R. Fraser
J. F. Antoniw
M. Legrand
Y. Ohashi
F. Meins
T. Boller
V. Conejero
C. A. Ryan
D. F. Klessig
J. F. Bol
A. Leyva
F. García-Olmedo

Fundación Juan March
Castelló, 77. Teléf. 435 42 40
28006 Madrid

The works summarized in this publication were presented by their authors at a Workshop held on 23th to 25th October 1989 at the Parador Nacional "Luis Vives", Valencia (Spain).

Depósito legal: M-6.873/1990
ISBN: 84-7075-402-5
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid

I N D E X

PAGE

GENERAL PROGRAMME OF THE WORKSHOP

October 23

Morning: FIRST SESSION

L.C. VAN LOON. An introduction to pathogenesis-related proteins.
R.S.S. FRASER. Plant-pathogen interaction.
J.F. ANTONIW. Pathogenesis-related proteins and induced resistance.

ORAL PRESENTATIONS

W.S. PIERPOINT
E. DUMAS
M.H.A.J. JOOSTEN

Afternoon: SECOND SESSION

M. LEGRAND. Characterization of PR-proteins.
Y. OHASHI. Stress -and salicylic acid- inducible expression of PR1 protein gene in transgenic plants.
F. MEINS. Hormonal regulation and possible functions of the basic isoforms of B-1,3-glucanase and chitinase.

ORAL PRESENTATIONS

G. BURKARD
P. AHL

October 24

Morning: THIRD SESSION

T. BOLLER. Subcellular localization and antifungal function of chitinase and B-1,3-glucanase, two pathogenesis-related enzymes.
V. CONEJERO. Viroid-induced tomato pathogenesis-related (PR) proteins as components of a general mechanism of response to afflicting agents.
C.A. RYAN. Regulation of wound-inducible proteinase inhibitor genes in plants potato and tomato.

ORAL PRESENTATIONS

Y. MEYER
J.J. SANCHEZ-SERRANO
T. PALVA

Afternoon: FOURTH SESSION

ORAL PRESENTATIONS

U. RASMUSSEN
J.A. PINTOR-TORO

October 25

Morning: FIFTH SESSION

D.F. KLESSIG. The PR1 proteins-their synthesis, location, and possible function.
J.F. BOL. Expression of PR genes.

ORAL PRESENTATIONS

T. BRYNGELSSON
J. RYALS

Afternoon: SIXTH SESSION

A. LEYVA. Molecular analysis of phenylalanine ammonia-lyase genes.
F. GARCIA-OLMEDO. Thionins: a family of plant antimicrobial proteins.

ORAL PRESENTATIONS

I.E. SOMSSICH
E. KOMBRINK

POSTER SESSIONS

P. CARBONERO
C. CASTRESANA
F. CÔTE
G. FACCIOLI
A.F. TIBURCIO
M.T. GONZALEZ-JAEN
M.H. HARPSTER
J. KALLERHOFF
H.J.M. LINTHORST

M. OHSHIMA
U.M. PFITZNER
R. RIVERA-BUSTAMANTE
D. ROBY
B. SAN SEGUNDO
I. TOBIAS
S. TUZUN
G. VISEDO

INTRODUCTION

V. CONEJERO

Dep. Biotecnología. E.T.S.I. Agrónomos
Universidad Politécnica. Valencia. (Spain)

L.C. VAN LOON

Dep. Plant Physiology
Agricultural University. Wageningen. (The Netherlands)

INTRODUCTION

Plants are endowed with the capability to produce adaptative responses to maintain their homeostasis. Pathogenesis-related (PR) proteins are induced in plants in response to infection with viruses, viroids, fungi and bacteria and may accumulate up to 10% of the total soluble protein in the leaves. These proteins are apparently involved in the defense of the plant against the attack to pathogens. Their induction is regulated at both the transcriptional and the translational level. All plant species so far investigated can express PR proteins with similar properties. Progress is being made in the molecular biology of PR proteins and the identification of their genes, with the ultimate aim to transform plants in order to increase their resistance to pathogens. Furthermore, the induction of PR proteins is an example of gene expression regulated in response to specific stresses. Artificial induction by hormones, phenol compounds and metal ions indicates that different kinds of compounds can act as inducers. Thus, it is of interest to elucidate the organization and inducibility of PR genes, by studying promoter regions.

Five years ago, workers actively engaged in the study of PR proteins met in Wageningen and compared preparations from different plant species (Neth.J.Pl.Path.89 (1983) 239-325). Now the application of the techniques of molecular biology is yielding information on the expression of PR proteins and the function of these proteins is gradually being elucidated. It was again important to exchange ideas, results and methods to coordinate and synergyze further research. This has been the aim of the "2nd Workshop on Pathogenesis-Related Protein in Plants" held in Valencia last October (22-26) under the generous auspices of the Fundation Juan March.

The program of the Workshop consisted of some review lectures (14) oral presentations (14), posters (21) and extended time of formal and informal discussions.

FIRST SESSION

L.C. VAN LOON
R.S.S. FRASER
J.F. ANTONIW

AN INTRODUCTION TO PATHOGENESIS-RELATED PROTEINS

L.C. VAN LOON
Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

The sedentary plant is bound to adjust its metabolism to the constantly changing external environment. It is subject to varying light conditions, temperature and humidity, and may encounter stress conditions such as drought, flooding, high temperature, chilling, chemical pollution, predation and infection by pathogens. Such conditions invariably induce the plant to synthesize specific proteins that apparently function to reduce the deleterious effects of the imposed stress.

Pathogenesis-related proteins (PRs) are plant-species specific proteins produced in response to infection by viruses, viroids, fungi or bacteria. PRs were identified as early as 1970 as a number of additional protein bands after separation on polyacrylamide gels of extracts from leaves of tobacco reacting hypersensitively to tobacco mosaic virus. Similar proteins have since been detected in several species from the families Solanaceae, Leguminosae, Amaranthaceae, Cucurbitaceae, Rutaceae and Umbelliferae. Moreover, serologically related proteins have been found in some of the Gramineae. Characterization of the major PRs from tobacco has shown them to possess several common properties that set them apart from the cytoplasmic proteins present in leaf cells: they are selectively extractable at low pH, resistant to various endogenous and exogenous proteases, and localized predominantly outside the cell in the intercellular space of the leaf. The cloning of cDNAs of a number of these proteins has allowed their amino acid sequences to be deduced and homologies between proteins in different plant species to be established. Tobacco PRs can now be grouped into at least five families of closely related proteins. Four of these have been well characterized biochemically. Each comprises one or more acidic proteins that are induced to high levels in leaves by necrotizing pathogens, as well as basic counterparts that seem to be expressed at lower levels in a developmentally-controlled manner in leaves and roots. The PR-2 family has been shown to possess β-1,3-glucanase activity; the members of the PR-3 group are chitinases. PR 5 is related to the sweet protein thaumatin and shows homology to a bifunctional proteinase/amylase inhibitor from maize seeds, as well as to osmotin, thought to function in the adaptation of tobacco to osmotic stress. The PR-1 group comprises the most highly induced proteins in tobacco and has been studied most, but its function remains elusive.

However, the members share sequence homology and cross-react serologically with the major allergen of the hornet wasp, dol m V.

The occurrence of PRs not only locally in infected tissues but also systemically, often throughout the entire plant, is associated with the phenomenon of acquired resistance, in which a primary infection affords some degree of protection against subsequent challenge inoculation with various types of pathogens. The glucanases and chitinases may be effective against fungi, but none of the PRs has so far proved to restrict virus or viroid multiplication or spread in intact plants. By differential hybridization additional classes of cDNA clones corresponding to virus-inducible mRNAs have been isolated. Several proteins already present at low levels in plant tissues may share one or more properties with PRs and be stimulated to various extents by pathogen infection, e.g. enzymes of phenylpropanoid metabolism and phytoalexin biosynthesis, hydroxyproline-rich glycoproteins, and oxidative and hydrolytic enzymes such as peroxidase and protease, as well as proteinase inhibitors. These proteins have likewise been implicated in the defense reactions of plants to pathogens. Hence, the classical PRs seem to be the most prominent manifestation of an integrated pattern of responses to infection.

The nonspecific character of these defenses suggests that the factors involved are all concomitantly induced, probably through the same signals. In tobacco PRs are induced not only after infection with different pathogens, but also as a result of various abiotic stresses, as well as application of selected chemicals. Most of these conditions induce the production of high levels of the plant hormone ethylene. Pricking leaves on healthy plants with needles moistened with toxic concentrations of ethephon, induces PRs similarly to virus infection. However, the natural precursor of ethylene, 1-aminocyclopropane-1-carboxylic acid, which does not induce visible symptoms, was significantly less active in inducing the PR-1 group and PRs 2a and 2b, whereas the remaining PRs were induced as effectively as after virus infection. On the other hand, salicylic acid (SA) induced predominantly PRs 1a, 1b, 1c, 2a and 2b. Since treatment of virus-infected tissue with α-aminooxypropionic acid, a competitive inhibitor of phenylalanine ammonia-lyase, reduced the induction of these same PRs, high amounts of these PRs may be induced as a result of the synthesis of a product structurally resembling SA, produced as a consequence of the stimulation of aromatic biosynthesis associated with the development of tissue necrosis. Thus, in tobacco the signal for the induction of a full set of PRs appears to be dual: on the one hand, ethylene, and, on the other hand, a presumably aromatic compound have independent, though complementary and partly overlapping actions.

19

PLANT-PATHOGEN INTERACTION.

R. S. S. Fraser. AFRC Institute of Horticultural Research. Worthing Road. Littlehampton. West Sussex, BN17 6LP, U. K.

In this paper, I have been asked to review plant-pathogen interaction in the broadest sense, to serve as a background for the more detailed contributions dealing with specific aspects of pathogenesis-related proteins. I will concentrate mainly on plant-virus interactions, but refer to some aspects of the interactions between plants and their microbial pathogens.

Many types of interactions occur between plants and pathogens: some determine whether the pathogenesis is successfully established, some are involved in the means by which the pathogen exploits the host to ensure its own multiplication; some are involved in the production of the symptoms of disease on the host, and some involve the interplay of host resistance mechanisms with pathogen countermeasures to overcome resistance. Many of these types of interactions involve an initial recognition event between host- and pathogen-specified molecules, the transduction of signals emanating from this recognition, and the induction of a response.

Several examples of interaction will be discussed in detail. These will focus on mechanisme determining host range; the genetics of resistance and virulence in plan-virus and plant microbial systems, and the links with the types of resistence mechanisms involved; effects of viruses on host growth and symptom formation, and some possible mechanims of induced resistance. It is clear that pathogenesis-related proteins are involved in several of these areas of interaction, although often as a rather "downstream" activity. However, in comparison with the lack of information on the nature of the initial recognition events and on the intervening signal transduction mechanisms, the induction and function of pathogenesis-related proteins are comparatively well understood. The pathogenesis-related proteins provide a useful model system for studies of host-pathogen interaction, as well as being of potential importance in practical disease control.

Pathogenesis-related proteins and induced resistance.

J.F. ANTONIW & R.F. WHITE
Plant Pathology Department,
Rothamsted Experimental Station,
Harpenden, Herts., AL5 2JQ, U.K.

Antiserum prepared to the PR1a protein purified from TMV-infected Xanthi-nc tobacco was used to develop a sensitive, specific enzyme-linked immunosorbent assay (ELISA) for PR1 proteins. This assay was used to investigate the relationship between PR1 proteins and induced resistance. Healthy Xanthi-nc leaves contained very low levels of PR1a (about 0.5 ng/g fresh weight leaf) and this dramatically increased to about 20 µg/g fresh weight leaf by six days after infection with TMV, which is localized in this cultivar. The systemic infection of another tobacco cultivar, Xanthi, with TMV caused no significant increase in PR1 concentration over the same time.

ELISA was also used to measure the distributions of TMV and PR1a protein in and around single, local necrotic lesions from 3 to 14 days after inoculation. The highest levels of TMV were in the centre of the lesion. In contrast there was very little PR1a protein in the centre of the lesion, the largest amounts were in a ring of tissue immediately surrounding the lesion. This is the sort of distribution that might be expected for a protein associated with preventing virus spread.

Investigations of the induction of both PR1 proteins and resistance by aspirin and polyacrylic acid in both localized and systemic TMV infections of different tobacco cultivars also supports the association of PR proteins with both a reduction in virus multiplication and virus spread. Further evidence for and against the association of PR proteins with induced resistance will be discussed.

ORAL PRESENTATIONS

W.S. PIERPOINT
E. DUMAS
M.H.A.J. JOOSTEN

Some salicylate-induced, PR-proteins of potato leaves

W S Pierpoint, P J Jackson and R Evans

(Departments of Biochemistry at
Rothamsted Experimental Station and Leeds University)

Spraying the leaves of young (17 day) potato plants with salicylate induces the appearance of some eight novel proteins that can be recovered from the intercellular fluid and recognised in PAGE gels: It also induces the appearance of material that reacts with an antiserum to the thaumatin-like protein of tobacco leaves. These proteins can be detected in relatively small amounts in the unsprayed leaves of older (35 day) plants, but in yet older (39 day) plants are present in amounts similar to those in sprayed leaves. Three of the proteins have been purified to near homogeneity. One, with Mr near 21,000, reacts with the antiserum to the TL-protein of tobacco leaves. The sequence of 58 amino acids at its N terminus suggests that it is a single peptide with a high (86%) degree of similarity to the two isoforms of the tobacco protein. Another protein appears to have a blocked N-terminus that prevents sequencing; but fortuitous proteolysis reveals an internal sequence of 29 amino acids that has a 68% similarity to a sequence in the basic leaf chitinases of potato, tobacco and kidney beans. This protein, like the acidic chitisases of tobacco is slightly, but distinctly retarded during percolation through a column of colloidal chitin. The third protein also has a blocked N terminus and has yielded no amino acid sequence. However, unlike the other two, it reacts strongly with an antiserum to a basic 1:3-β-glucanase of potato leaves that was kindly given to us by Dr Kombrink. At least four other proteins in our intercellular fluid extracts, two of which have been partially purified, also react positively with this antiserum.

Biochemical and immunological* studies of the production of pathogenesis-related (PR-b) proteins in tobacco roots infected by *Chalara elegans*.

Abdessamad Tahiri-Alaoui, Eliane Dumas, & Silvio Gianinazzi.

Laboratoire de Phytoparasitologie, Station de Génétique et d'Amélioration des plantes, INRA, BV 1540, 21034 Dijon cedex, France

Summary

All previous studies on PR-b protein induction in tobacco have been carried out on leaves or callus tissue. We report here the production of PR-b proteins also in roots of tobacco plants (*Nicotiana tabacum* cv. Xanthi nc.) infected with the pathogenic fungus *Chalara elegans*. PAGE analysis followed by silver nitrate detection revealed the same PR-b proteins in *C. elegans*-infected tobacco roots as those previouly found in TMV-infected leaves, although the production of these proteins was lower in roots than in leaves. We have confirmed by immunoblotting that the three main groups of PR-b proteins (PR-b_1 group, ß-1,3 glucanases and chitinases) are present in infected roots. Indeed, antiserum against PR-b_1 reacted with PR-b_1, b_2, and b_3 and gave the same pattern of reaction as for leaves. Antiserum against PR-b_5 revealed the presence of PR-b_4, b_5 and, very weakly, b_6. Antiserum against PR-b_7 reacted with both PR-b_7 and b_8.

Immunolabelling at the subcellular level, using a polyclonal antibody against PR-b_1, was essentially found on the walls of hyphae in *C. elegans*-infected cells. The intercellular spaces were only occasionally weakly labelled. No labelling was observed using the anti PR-b_1 serum preadsorbed with purified PR-b_1, either in the intercellular spaces or on hyphal walls. However, when a monoclonal antibody against PR-b_1 was used, very light labelling was observed on hyphal walls of the fungus whilst the host cytoplasm and plasmalemmma appeared labelled in infected cells as well as in distant uninfected ones; labelling was also sometimes found in the intercellular spaces.

No labelling was observed in the intercellular spaces of uninfected roots, either with the PR-b_1 polyclonal antibody or with the corresponding preadsorbed serum. However, with the PR-b_1 monoclonal antibody, labelling was found on the host plasmalemma but this was less intense than in infected roots. The significance of these results in relation to host defence against root pathogens will be discussed.

*Antibodies were kindly provided by Drs.J. Antoniw, R. F. White, J. Carr, B. Fritig and, L.C. Van Loon.

Apoplastic pathogenesis-related proteins from the interaction Cladosporium fulvum (syn. Fulvia fulva) - tomato

Matthieu H.A.J. Joosten and Pierre J.G.M. de Wit, Agricultural University, Department of Phytopathology, P.O. Box 8025, 6700 EE Wageningen, The Netherlands

Cladosporium fulvum is a biotrophic fungal parasite which colonizes the intercellular spaces between the mesophyll cells of tomato leaves and is confined to the apoplast during the main part of the disease cycle. The apoplastic fluid of infected leaves contains proteins which are constitutively produced by the plant, plant proteins specifically induced by the fungus and fungal proteins constitutively produced or specifically induced by the plant.

Several of the induced plant proteins belong to the class of pathogenesis-related (PR) proteins. Two predominant PR-proteins were purified from apoplastic fluid by ion-exchange chromatography, followed by chromatofocusing. One protein (Mr 35 kD) showed 1,3-β-glucanase activity, while the other one (M_r 26 kD) showed chitinase activity. Using antisera raised against these purified enzymes one additional glucanase (M_r 33 kD) and three additional chitinases (M_r 27, 30 and 32 kD) could be detected in apoplastic fluids or homogenates of tomato leaves inoculated with C. fulvum. Upon inoculation with C. fulvum, chitinase and 1,3-β-glucanase activity in apoplastic fluids increased more rapidly in incompatible interactions than in compatible ones.

Antibodies raised against the purified 1,3-β-glucanase and chitinase from tomato were used to study the subcellular localization of these hydrolytic enzymes in tomato leaf tissue inoculated with virulent or avirulent races of C. fulvum. Seven days after inoculation, in the incompatible Cf4/race 5 interaction, the intercellular hyphae that were detected, had lost there contents and the walls showed a high specific labelling. Furthermore, labelling could be detected the cytoplasm, intercellular spaces and electron-dense masses of cells close to the invading hyphae. In the compatible Cf5/race 5 interaction intercellular hyphae were numerous at 7 days after inoculation but no specific labelling could be detected.

Besides the induction of 1,3-β-glucanases and chitinases in tomato leaves by C. fulvum, there is a marked accumulation of several proteins of ca. 14 kD. Apart from a 14 kD protein only present in apoplastic fluid isolated from compatible C. fulvum - tomato interactions, and presumably of fungal origin, three different PR-proteins of about the same molecular weight, to which no biological function has been ascribed, could be identified. These proteins were purified by gel filtration, followed by FPLC using a cation-exchange column at pH 6.0. One of these proteins represents the well characterized PR-protein P14. All three proteins had isoelectric points of about 10.7 and showed immunological relationships. They were identified as protein bands 2, 4 and 6 on low pH polyacrylamide gels.

SECOND SESSION

M. LEGRAND
Y. OHASHI
F. MEINS

CHARACTERIZATION OF PR-PROTEINS

M. Legrand, S. Kauffmann, P. Geoffroy, A. Stintzi and B. Fritig

Institut de Biologie Moléculaire des Plantes, 67000 Strasbourg, France

Since 1970 when Van Loon (Wageningen) and Gianinazzi (Dijon) detected 4 Pathogenesis-Related proteins in tobacco leaves reacting hypersensitively to TMV, many PR-proteins have been described in many plant species.

From TMV-infected Samsun NN leaves we have isolated up to now 23 defense-related proteins with PR-type properties. These include the ten acidic PR-proteins already detected by L.C. Van Loon in the late 1970's and named PR-1a, 1b, 1c, 2, N, O, P, Q, R and S in order of decreasing mobility on native gels. Specific sera have been raised against these latter proteins which have been shown to belong to four distinct serological groups :

- the PR I group with PR-proteins 1a, 1b, 1c whose biological function is still unknown.
- PR-proteins 2, N and O are in fact 1,3-ß-glucanases that are serologically related together and to another acidic 1,3-ß-glucanase (named Q' since it migrates as Q on basic native gels) and to a basic 1,3-ß-glucanase. These five 1,3-ß-glucanases are induced by TMV infection, PR-O and the basic isoform being the most active enzymes with laminarin as substrate.
- PR-proteins P and Q are acidic chitinases serologically related to two other chitinases with higher isoelectric points. The basic hydrolases are known to be hormone-regulated in tobacco cell cultures.
- PR-proteins R and S share sequence homologies with thaumatine and a bifunctional trypsin/α-amylase inhibitor of maize. We were unable to demonstrate any inhibitory activity of the purified proteins towards various serine proteinases. PR-proteins R and S are serologically related to a basic counterpart which accumulates also during the hypersensitive reaction. This basic protein has been purified to homogeneity and amino acid analysis and N-terminal sequencing have indicated that it is identical to a protein named osmotin, which accumulates in salt-adapted tobacco cells.

An activity of proteinase inhibition is, indeed, induced in TMV-infected leaves in a manner similar to PR-proteins but does not correspond to any of the 10 major PR-proteins or to any of four others that migrate in native gels like PR-proteins R and S but have significantly lower MW (13-14.5 kD). The inhibitory activity is born by a small polypeptide (about 6 kD) which has been isolated. N-terminal sequence determination and amino acid analysis have demonstrated that this inhibitor belongs to the inhibitor I family. Unlike other inhibitors of the same family, the tobacco inhibitor is a poor inhibitor of trypsin and chymotrypsin but a very potent inhibitor of serine proteinases of microbial origin. Thus the accumulation of inhibitor I in diseased plants could represent a defense reaction complementary to the lytic action of hydrolases.

In conclusion, among the 23 defense proteins characterized from TMV-infected tobacco leaves, 10 are glycanhydrolases (4 chitinases and 6 1,3-ß-glucanases) with an endo-type catalytic activity. These hydrolases may play a central role in the release of oligosaccharides from the walls of some plant pathogens or the plant cells themselves, some of these oligosaccharides being known as elicitors of defense reactions. Inhibitors of microbial proteases are likely to be involved in defense by counteracting an important pathogenicity determinant. Concerning the other PR- and defense-related proteins, their function and biological significance has still to be established.

STRESS- AND SALICYLIC ACID- INDUCIBLE EXPRESSION OF PR1 PROTEIN GENE IN TRANSGENIC PLANTS

Yuko Ohashi, Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan.

The hypersensitive reaction caused by TMV infection includes the induction of PR1 proteins, which abundantly accumulate in the intercellular spaces of tobacco leaves at the late stage of induction. These proteins are also induced by treatment with chemicals, such as salicylic acid, and by stresses of cutting and mechanical injury under the control of transcriptional level. The function of PR1 proteins has not been clear, however, its involvement in the hypersensitive response is obvious. Therefore PR1 protein genes are good candidates for studying the induction mechanism of this class of stress inducible protein genes.

The primary structures of the genes for PR1a, 1b, and 1c proteins of Samsun NN tobacco were analyzed. Close resemblances were found in the coding and 5' upstream regions of them. The 5' flanking fragments (0.2Kb) just upstream from the coding region of PR1b and 1c genes share more than 90% homology compared with that of PR1a protein gene. In far upstream from this region, however, some insertions and deletions were found. These structural data suggest that this highly-conserved region may be important for the inducible expression of PR1 protein genes, since these three genes are expressed by stresses and some

chemicals in a similar manner.

A series of 5' deletion fragments of PR1a gene was fused to the coding region of the reporter gene encoding β-glucuronidase(GUS), and introduced into tobacco protoplasts by electroporation, or into tobacco, lettuce and potato plants by the Agrobacterium mediated gene transfer. The expression of the introduced genes were observed by quantitative and histochemical analysis of GUS activity. Some stresses and salicylic acid highly induced the GUS activity in the protoplasts and in the transformants which had been introduced by PR(2.4 Kb of 5' flanking)-GUS chimeric gene. Local lesion formation by pathogen attack induced elevated GUS activity around the local lesions as a ring. Salicylic acid induced high GUS activity in all tissues of leaf blade. On the petiole or stem, the induced activity was detected in vascular bundle (especially in sieve area), pith parenchyma and cortical parenchyma. These expression was differently regulated with developmental stage of the plants. For the induced transient expression in protoplasts, about 0.2 Kb of 5' flanking fragment just upstream from the coding region was necessary, and 0.3 Kb for the stable expression in the transgenic plants. These results indicate that cis-acting element(s) responding to the infection of TMV or the treatment of chemicals is present in just upstream region (0 - 0.3Kb) of the 5' flanking region of PR1a gene.

F.Meins and J.M. Neuhaus, Friedrich Miescher-Institut, Basel, Switzerland. "Hormonal regulation and possible functions of the basic isoforms of ß-1,3-glucanase and chitinase".

The basic isoforms of tobacco ß-1,3-glucanase and chitinase exhibit complex regulation. They accumulate to high concentrations (ca.4% of the soluble protein fraction) in lower leaves and roots of the plant. With the onset of flowering the concentration of both glucanohydrolases rapidly drops. Biochemical and immunohistological studies show that the basic isoforms are localized in epidermal cells of the leaf. Both enzymes require ethylene for their biosynthesis and are strongly down-regulated when cultured cells are treated with mixtures of the growth hormones auxin and cytokinin. Northern blot analysis using cDNA probes show that chitinase and ß -1,3-glucanase are regulated developmentally and by hormones at least in part at the mRNA level.

Southern blot analysis and the comparison of sequences of genomic and cDNA clones indicate that basic isoforms of tobacco ß-1,3-glucanase and chitinase are encoded by small gene families, each with ca. 3-5 members. The families appear to have evolved from genes in ancestral parents of tobacco related to the present day species N.sylvestris and N.tomentosiformis . Based on a comparison of amino acid sequences, there are at least three major classes of chitinases in seed plants: class I, basic chitinase with and N-terminal, cysteine-rich domain and a highly conserved catalytic domain ; class II, chitinases with a catalytic domain similar to that of class I enzymes, but lacking a cysteine-rich domain; and class III, chitinases with conserved sequences different from those of class I and class II enzymes. All three classes of chitinase are present in tobacco.

The physiological significance of hormonal regulation of ß-1,3-glucanase and chitinase is not known. Our approach to this problem has been to generate mutants by sense and antisense transformation that over- and under-produce the enzymes. The "tomentosiformis"-type of tobacco chitinase gene 48 was introduced into N.sylvestris cells by Ti-plasmid-mediated leaf disk transformation. The construction in a CaMV 35S plant-expression vector contained the prechitinase coding sequence, 35 bp of the 5'-flanking sequence including the start of transcription and 150 bp of the 3'-flanking sequence including the putative polyadenylation signals. Kanamycin resistance (KmR)was the selectable marker. One transformation event is described here. The evidence for transformation was the co-segregation of the KmR phenotype with the presence of gene 48 coding sequences, mRNA, and chitinase antigen in self classes. Young leaves of homozygous transformants contained up to 100-fold higher concentrations of chitinase antigen and enzyme activity than untransformed or vector transformed plants. Nevertheless, the overall distribution of chitinase in

tissues and organs of central and transformed plants was similar. The chitinase encoded by the foreign gene was also induced by ethylene treatment of the plants. These results suggest that post-translational levels of regulation are important in expression of chitinase genes. Transformed plants with very high levels of chitinase were fertile and normal in appearance. Therefore, precise regulation of chitinase is not essential for the housekeeping functions of N.sylvestris plants.

ORAL PRESENTATIONS

G. BURKARD
P. AHL

Maize Pathogenesis-related proteins : Characterization and cellular distribution

W. NASSER, M. de TAPIA, G. GENOT, L. DIDIERJEAN, P. FRENDO & G. BURKARD

Institut de Biologie Moléculaire des Plantes CNRS ; 12 rue du Gl Zimmer 67000 STRASBOURG

Ten PRm proteins (m for maize) with molecular weights ranging between 14,200 and 34,500 were identified in leaves from mercuric chloride-treated or BMV-infected maize.

In view of the results obtained in the study of their serological properties and their biological functions, maize PR proteins can be divided into at least three families. First the family of the chitinases including five members namely PRm 3-4-5-7 and Ba2 which can again be divided into two groups. The first group includes PR 5-7 and Ba2 chitinases with molecular weights of 29, 34,5 and 30 kDa respectively. They are serologically related to each other but have no serological relationships with PRm3 and PRm4. The second group contains PRm3 and PRm4 which have the same molecular weight (25 kDa), are serologically related to each other and show a lysozyme activity beside the endo-chitinase activity. A high homology can be found between the amino-terminal sequence of maize PRm3 protein and the lysozyme/chitinase from *P. quinquifolia*.

The second family of maize PR proteins contains the 1,3 ß-glucanases PRm6a - 6b and Ba1. They are serologically related to each other and cross-react with antisera raised against 1,3-ß-glucanases from other plant species (tobacco, bean).

Protein PRm2 belongs to the group of tobacco PR1 proteins as shown by its serological relationships with these proteins and also the similarities in the aminoacid composition of PRm2 and tobacco PR1b.

The cellular distribution of the maize hydrolases has been investigated ; extraction of ICF and preparation of protoplasts were used to localize, by immunological reactions, the different hydrolases.

Results with ICF extracts indicate that the acidic chitinases (PRm3, PRm4, PRm5 and PRm7 proteins), the acidic 1,3-ß-glucanases (PRm6a and PRm6b proteins) and also PRm2 protein are located in the extracellular spaces.

The maize basic hydrolases have not been detected in the ICF. A convincing explanation could be that PRm Ba1 and PRm Ba2 are both located inside the cell. Further support for this conclusion comes from experiments with protoplasts suggesting strongly that in maize the basic chitinase and the basic 1,3-ß-glucanase have an intracellular localization.

CHITINASE AND B-1,3-GLUCANASE ARE INDUCED IN TOBACCO BY INFECTION WITH *PSEUDOMONAS TABACI* AND *PHYTOPHTHORA PARASITICA* VAR. *NICOTIANAE*.

P. Ahl Goy (Ciba-Geigy AG, CH-4002 Basle, Switzerland) and F. Meins jr (Friedrich Mischer Institut, P.O. Box 2543, CH-4002, Basel, Switzerland.

The basic isoforms of endo-1,3-B-glucosidase (B-1,3-glucanase, EC 3.2.1.39) and of endochitinase (chitinase, EC 3.2.1.14) are PR-proteins induced in tobacco in response to viral infection. We have measured by rocket immunoassay the content of B-1,3-glucanase and of chitinase in *Nicotiana tabacum* cv. Havana 425 plants following infection with *Pseudomonas tabaci* (wildfire disease) or *Phytophthora parasitica* var. *nicotianae* (black shank disease). Both enzymes were induced, relative to non-infected controls, in leaves infected with *P. tabaci* and in stems and leaves infected with *P. parasitica*. Increases in enzyme content were confined to the infected parts of the plants indicating that the induction is local rather than systemic.

To investigate if regulation occured at the transcriptional or at the translational level, the B-1,3-glucanase- and chitinase-mRNA content of the tissues was measured by RNA blot hybridization using the inserts of cDNA clones for the tobacco mRNAs as probes. *P. tabaci* and *P. parasitica* infection increased the mRNA content relative to controls indicating that both pathogens act at least in part at the level of mRNA accumulation. As judged by immunological tests and RNA hybridization, the B-1,3-glucanase and chitinase induced by *P. tabaci* and *P. parasitica* var. nicotianae are the same basic isoforms known to be induced by tobacco mosaic virus infection. Therefore the induction appears to be a nonspecific response of the hosts to pathogens.

THIRD SESSION

T. BOLLER
V. CONEJERO
C.A. RYAN

SUBCELLULAR LOCALIZATION AND ANTIFUNGAL FUNCTION OF CHITINASE AND ß-1,3-GLUCANASE, TWO PATHOGENESIS-RELATED ENZYMES

Thomas Boller

Botanisches Institut, Hebelstrasse 1, CH-4056 Basel (Switzerland)

A survey of the available literature provides interesting information about the localization and function of chitinase and ß-1,3-glucanase, two pathogenesis-related enzyme in plants. Ethylene co-ordinately induces chitinase and ß-1,3-glucanase in bean leaves. The two ethylene-induced enzymes have isoelectric points in the basic range. Cell fractionation studies and immunocytochemical evidence indicate that the two enzymes are primarily localized in the central vacuole of ethylene-treated bean cells. Homologous chitinases and ß-1,3-glucanases have been observed among the "basic pathogenesis-related proteins" in infected plants, e.g. in tobacco, pea or bean. Several of the acidic pathogenesis-regulated proteins of tobacco and of other plants have been identified as chitinases and ß-1,3-glucanases. Cell fractionation studies indicate that these activities are located in the extracellular space.

The amino-acid sequence derived from the basic and acidic forms in tobacco show a considerable degree of homology. In the case of chitinase, both the basic and the acidic isoforms possess a sequence of about 20 amino acids which is absent in the mature proteins and is thought to represent the "signal sequence" responsible for entry into the endoplasmic reticulum. Next, the basic isoforms contain a sequence of 40 amino acids which is related to hevein, a small protein present in the vacuoles of the latex of rubber trees, and to wheat germ agglutinin, a chitin-binding lectin with a vacuolar localization. In particular, the positions of the eight cysteines is maintained. This domain constitutes the N-terminus of the mature proteins and is lacking in the acidic isoforms. The amino acid sequence following the hevein domain in the basic chitinases bear a clear homology to the acidic isoforms, except for the 6 C-terminal amino acids which are present in the basic isoforms but absent from the acidic isoforms. It is interesting to speculate that the differences in amino acid sequences are related to the differential localization of the basic and the acidic isoforms of chitinase.

In cucumber, chitinase is induced both locally and systemically in response to necrotizing pathogens. This chitinase has an acidic isoelectric point and is extracellular both in healthy and systemically-induced leaves. Surprisingly, cucumber chitinase has a completely different primary sequence from the one of bean leaves.

It has long been postulated that chitinase and ß-1,3-glucanase have an antifungal function, based on the observation that the two enzymes degrade isolated fungal cell walls. More recently, such an antifungal activity has been demonstrated directly. For example, bean chitinase inhibits growth of the saprophytic fungus, Trichoderma viride, at concentrations as small as 100 nM. Interestingly, bacterial chitinases appear to lack a similar antifungal activity.

Subsequent studies with a number of different fungi have shown that the pure chitinase alone has an antifungal activity only in exceptional cases. Likewise, pure ß-1,3-glucanase alone inhibited growth of only one of seventeen test fungi examined. In contrast, combinations of chitinase and ß-1,3-glucanase have been found to inhibit growth of all fungi tested with chitin-glucan cell walls. Microscopical observations indicate that the inhibitory activity of enzyme combinations is related to lysis of the hyphal tips.

The differential localization of chitinase and ß-1,3-glucanase is interesting with regard to their possible antifungal function. Since pathogenic fungi invade the plant from outside, it may be speculated that extracellular chitinases and ß-1,3-glucanases form a first line of defense. They may also be involved in the release of fungal cell wall fragments (elicitors) that act as chemical signals to induce defense reactions. The vacuolar chitinases and ß-1,3-glucanases may form a second line of defense. The vacuolar enzymes get into contact with an invading fungus only if there is a way for the enzymes to leave the cell. This is the case when the whole cell dies in the hypersensitive response. It remains to be seen whether the established antifungal activity of combinations of chitinase and ß-1,3-glucanase observed in vitro operates in a similar way in plant-pathogen interactions in vivo.

VIROID-INDUCED TOMATO PATHOGENESIS-RELATED (PR) PROTEINS AS COMPONENTS OF A GENERAL MECHANISM OF RESPONSE TO AFFLICTING AGENTS

V. Conejero , J. M. Bellés, F. García-Breijo, R. Garro, J. Hernández-Yago, I. Rodrigo and P. Vera.

Departamento de Biotecnología, E.T.S.I. Agrónomos, Universidad Politécnica de Valencia, Spain.

The finding that the polypeptides associated with viroid infection are not specified by the viroid but result from a disease-induced alteration of host metabolism prompted the search for native proteins possibly implicated in the pathological response, later on termed pathogenesis-related (PR) proteins (1). Since the non-denaturing electrophoretic system normally used to detect tobacco PRs excluded basic proteins, a system with the capability of separating cationic proteins was tested. This approach led us to the detection of 10 PR proteins (C1 to C10) in tomato plants infected with CEV (2). These tomato PR proteins shared with those of tobacco the characteristics of being preferentially extracted at low pH and being relatively resistant to proteinase digestion. Tomato PR proteins were found to accumulate also as a consequence of silver and ethephon treatments in association with viroid-like pathogenic reactions (3). These results suggested that PRs and developmental aberrations are reflection at different levels of a general response mediated by ethylene.

The discovery of these proteins posed a number of challenging questions, among which the most important are their biochemical function and biological role, not only in viroid pathogenesis but in a more general system for transducing and responding to pathogenic or stressing signals. First findings in this respect have been made recently: characterization of P69 as an alkaline cysteine proteinase and P32 and P34 (respectively C7 and C6) as chitinases. Also, a β-1,3 glucanase has been assigned to a CEV-induced PR protein in tomato.

Critical to the unraveling of the biological role of these proteins is their *in vivo* localization. Immuno-gold-EM technique applied to study P1(p14) and P69 led to the discovery of two main locations for these proteins: the vacuole, in association with inclusion bodies, a newly described location, and the intercellular spaces of CEV-infected tomato leaves.

An interesting feature of PR proteins is their reputed resistance to degradation by endogenous proteases. Consistently, hey have long half-lives. Nevertheless, they need to be turned-over. The fact that the intercellular space seems to be the compartment where they finally accumulate suggests that their degradation must be carried out there. To this respect, it has been found that some of the tomato PR

proteins are selectively degraded by an extracellular 37-kDa constitutive aspartyl proteinase that could be implicated in a regulatory mechanism for the biological action of PR proteins. The relevance of this enzyme in the PR metabolism is reinforced by the fact that we have found an analogous enzyme in intercellular washing fluids of tobacco plants.

Although the biological role of most of these proteins is as yet unknown the following must be stressed: i) chitinases and β-1,3 glucanases could be implicated in the defense against pathogens containing chitin or β-1,3 glucans as a component of their structure (bacteria and fungi); ii) the role of P69 proteinase either in symptom production or as a defense tool remains to be elucidated. iii) Although P1(p14), the most abundant tomato PR protein, was the first PR proteins whose entire sequence was determined (4), no biochemical function could be assigned to this protein. Nevertheless, P1(p14) has been found in leaves from healthy (non-infected) plants and always associated with cell material under disorganization. This suggested the idea that P1(p14) is involved in cell degeneration, either naturally activated as a normal event of the biological cycle of the plant (i. e. lysigenous development of intercellular spaces by cell ageing) or exogenously provoked by afflicting agents. The possible involvement of P1(p14) with the resistance induced in the systemic reaction of the host or with some other biological role is not discarded. The biological significance of P1(p14) is also sustained by the fact that this protein is synthesized as a pre-protein, then targetted to the vacuole and to the apoplast in association with P69. The vacuolar and apoplastic localization of chitinases have also been reported (5); iv) the reported evidence to date indicates that ethylene is an intermediary step (second messenger) in the coordinate activation of PR synthesis as part of the response to viroid infection.

All this suggests that the viroid-induced PR proteins, although with diferent biochemical activities, might have the common biological role of being part of an adaptative response against potential agressions coming from the environment.

One could hypothesize that these coordinately interconnected defense tools have been surely builded up in two steps: i) specific and individual development of each tool by coevolution with a given pathogen (in our case those containing chitin or β-1,3 glucan as part of their structure) or with certain agressive environmental conditions; ii) interlinking of the individual specific responses. Thus, what it appears to be a non specific response results from evolutionary integration of specific individual components. With this strategy, the battery of defensive tools would be progressively enriched with new experiences. A prediction of this model would be the possible existence or future development of proteinases and nucleases against viruses and viroids.

The lack of specificity of this response could have biological advantages for the plant. This type of mechanism would broaden the scope of potential pathogens against which the plant would have acquired a certain degree of immunity after having been non specifically triggered.

REFERENCES

1.- Camacho-Henriquez, A. and Sänger H. L. (1982) Arch. Virol. 74, 181-186.
2.- Granell, A., Bellés, J. M. and Conejero, V. (1987) Physiol. Mol. Plant Pathol. 31, 83-90.
3.- Conejero, V. and Granell, A. (1986) Physiol. Mol. Plant Pathol. 29, 317-323.
4.- Lucas, J., Camacho-Henriquez, A., Lottspeich, F., Henschen, A. and Sänger, H. L. (1985) EMBO J. 4, 2745-2749.
5.- Mauch, F. and Staehelin, L. A. (1989) The Plant Cell 1, 447-457.

Regulation of Wound-Inducible Proteinase Inhibitor Genes in Plants Potato and Tomato

Clarence A. Ryan, Gynheung An, Michael Costa, Curtis Palm, Ted Farmer and Tom Moloshok. Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340.

Deletion analysis from the 3' to the 5' end of the promoter region of the wound inducible potato proteinase Inhibitor IIK gene has identified a 30 base sequence at -136 to -165 that is necessary for expression. Within this 30 base sequence, DNA-band shift assays using nuclear extracts from tomato leaves have identified a wound-inducible protein that binds to a 10 base region of the promoter. This sequence is adjacent to an 8 base consensus sequence at -147 to -155 that is present in eight elicitor-inducible genes from various other plants. The evidence suggests that a complex set of cis- and trans-acting elements within the -136 to -165 region of the potato IIK gene are involved with the final signalling steps that regulate the wound inducibility of this gene.

In vitro phosphorylation of at least two potato or tomato plasma membrane proteins of Mr ~34 kD and 100 kD is enhanced in the presence of a polygalacturonic acid of DP ~20 from tomato leaves. Poly- and oligouronides can activate genes coding for several plant defense proteins, including biosynthetic enzymes leading to phytoalexins and lignins, as well as proteinase inhibitor proteins. The membrane protein pp34 is phosphorylated at a threonine residue(s) in response to the addition of the uronide fragments. Uronide oligomers of DP 2-10 were assayed for their abilities to induce *in vitro* phosphorylation of pp34. These oligomers, that can activate proteinase inhibitor synthesis in leaves, were totally inactive in enhancing *in vivo* phosphorylation. Larger oligomers are currently being isolated and assayed. (Supported in part by grants from the National Science Foundation and EniChem Americas, Inc.)

ORAL PRESENTATIONS

Y. MEYER
J.J. SANCHEZ-SERRANO
E.T. PALVA

DEDIFFERENCIATION OF TOBACCO MESOPHYLL PROTOPLASTS IS A WOUND RESPONSE: THE POSSIBLE IDENTITY OF "IN VITRO CALLUS AND "WOUND CALLUS"

Y. MEYER, Y. CHARTIER, J. GROSSET and I. MARTY. Université de Perpignan Laboratoire de Physiologie Végétale - Avenue de Villeneuve - 66025 - PERPIGNAN-Cédex (FRANCE)

We have shown previously that tobacco mesophyll protoplasts in culture synthesize proteins which are undetectable in the leaf tissue from which protoplasts are isolated. The synthesis of six of these, which we have named a, a', a_1 (MW 31,000 to 33,000), b, b' (MW 26,000 and c (MW 14,000), is greatly reduced (up to ten fold) by the presence of auxin in the culture medium, while the synthesis of other proteins is not modified by the hormonal treatment (MEYER et al. 1984a,1984b). We have further shown that these proteins represent the majority of the polypeptides secreted by protoplasts into their vacuole and that they continue to be synthesized in *in vitro* cultivated tobacco cells (MEYER et al. 1987). The production of antibodies against proteins a', b' and c has allowed us to show a close immunological relationship between proteins a_1 and a' and between proteins b' and b, respectively, while proteins a and c appeared to be completely unrelated to these two groups and to each other (MEYER et al. 1988). More recently we have described the results of immmunodetection experiments using antibodies directed against "Pathogenesis-Related" (PR) proteins extracted and purified from tobacco mosaiv virus (TMV)-infected Samsun NN tobacco leaves, as well as against a basic 26 kd protein induced in tobacco cells by culture in the presence of NaCl. The results enable us to identify 5 of the 6 major protoplast proteins whose synthesis is diminished by auxin, as two chitinases (polypeptides a' and a_1) a 1,3-β-glucanase (polypeptide a) and two osmotins (polypeptides b' and b) (GROSSET et al., 1989).

We are now analysing factors which induce PR protein synthesis during the isolation of Tobacco mesophyll protoplasts. It appears that wounding alone, is sufficient to trigger these synthesis. In addition wounding induces changes in the mRNA population (as shown by in vitro synthesis followed by two-dimensional electrophoresis) which are very similar to those induced by protoplast isolation. This suggests, that PR protein synthesis and possibly dedifferenciation of mesophyll cells (including cell division and callus formation) is a wound response. In addition, sustained synthesis of PR proteins in callus suggests that *in vitro* callus and wound callus represent the same state of differenciation.

MEYER Y., L. ASPART, Y. CHARTIER, 1984a. Plant Physiol. 75: 1028-1034.
MEYER Y., L. ASPART, Y. CHARTIER, 1984b. Plant Physiol. 75: 1035-1040.
MEYER Y., Y. CHARTIER, G; ALIBERT, 1987. Plant Physiol. 83: 713-718.
MEYER Y., J. GROSSET, Y. CHARTIER, J.C. CLEYET-MAREL. 1988. Electrophoresis, 9: 704-712.
J. GROSSET, MEYER Y., Y. CHARTIER, S. KAUFFMANN, M. LEGRAND, B. FRITIG. (submitted).

The regulation of the potato proteinase inhibitor II gene expression

Jose J. Sánchez-Serrano, Simone Amati, Michael Keil, Hugo Peña-Cortés, Salomé Prat and Lothar Willmitzer.
Institut für Genbiologische Forschung GmbH. Ihnestrasse 63, 1000 Berlin 33, F.R.G.

The proteinase inhibitor II gene family is constitutively expressed in tubers and in young floral buds of potato plants. Mechanical wounding triggers the accumulation of its mRNA in the aerial parts of the plant. This activation of the gene expression is not restricted to the tissues in the immediate vicinity of the wound site but rather leads as well to its accumulation in the non-wounded, systemically induced, aerial organs.

The phytohormone abscisic acid (ABA) mediates the wound induction of the proteinase inhibitor II gene. Proteinase inhibitor II mRNA accumulation is detected after spraying potato leaves with ABA and a systemical induction of non-sprayed leaves is also observed. Consistant with its involvement in the wound response, the endogenous ABA levels increase 2 to 3 fold upon wounding, both in the directly injured as well as in the systemically induced leaves. To provide further support for the involvement of ABA in the wound induction we have taken advantage of the ABA-deficient potato (droopy) and tomato (sit) mutants which show reduced levels of the hormone upon water stress. The proteinase inhibitor II gene is not activated by wounding in these mutant plants. However, ABA treatment leads to its accumulation thoughout the aerial organs in both droopy and sit mutants.

A reporter gene (GUS) was cloned into a proteinase inhibitor II cassette, consisting of a 1.3 kb promoter fragment and a 0.27 kb fragment containing the polyadenylation signals, and subsequently transferred to both tobacco and potato by Agrobacterium-mediated techniques. This cassette is able to confer wound-inducible and tuber constitutive GUS activity to the transgenic plants, thus following the expression pattern of the proteinase inhibitor II gene family. Histochemical analyses of the transgenic plants show the proteinase inhibitor II promoter driven GUS activity being strongest in the mesophyll cells close to the vascular tissue.

Deletion analyses of the proteinase inhibitor II promoter were carried out using transgenic tobacco plants. A 800 bp promoter drives wound-inducible activity of the fused CAT gene, which is several fold weaker than the activity shown in plants transformed with the 1.3 kb promoter. The CAT activity from a 550 bp promoter is below the detection limits, thus indicating the presence of elements in the 5´ upstream region required for gene expression . When the CaMV 35S enhancer is fused to these proteinase inhibitor II truncated promoters, wound-inducible CAT activity is recovered with the 550 bp and in some cases with a 440 bp promoter. Plants transformed with a 210 bp promoter construction display only constitutive CAT activity. Moreover, a promoter fragment from -1300 to -195 is able to confer in both orientations wound-inducible activity to an otherwise inactive 35S truncated promoter-CAT gene construction, thus displaying features of an enhancer element. These results suggest that sequences downstream of -195 are not required for the wound induction of the proteinase inhibitor gene.

To gain an insight into the mechanisms of the proteinase inhibitor II wound response we have searched for protein factors acting on the cis-regulatory sequences responsible for the wound induction. For this purpose we have divided the proteinase inhibitor II promoter fragment in subclones suitable for analysis of DNA-protein interactions by the gel retardation assay. Protein-DNA complexes are formed with some of the fragments used. The binding activities remain constant upon wound induction and are also present in nuclear extracts from potato tubers. The interaction of a nuclear protein to an upstream element has been further characterized by DNAse I footprinting and DNA methylation interference experiments. The sequence requirements of the interaction have been determined from the binding properties of a series of mutated oligonucleotides. The protein binds to the sequence GAGGGTattttCGTAA where mutations at the nucleotides indicated with lower case letters do not disturb the binding specificity.Deletion analysis of the promoter points to a constitutive enhancer function for this upstream element.

INVOLVEMENT OF PATHOGENESIS-RELATED (PR) PROTEINS IN ERWINIA CAROTOVORA - ARABIDOPSIS THALIANA INTERACTION

E.Tapio Palva, Tuula K.Palva, Kjell-Ove Holmström, Minna Pirhonen, Pekka Heino, Hannu Saarilahti, Jianping Xin and Maj-Britt Karlsson

Department of Molecular Genetics, Uppsala Genetic Center, Swedish University of Agricultural Sciences, Box 7003, S-75007 Uppsala, Sweden

Arapidopsis thaliana is susceptible to infection by soft-rot Erwinias (e.g. by Ecc and Eca strains). As both the pathogen and the host plant are amenable to genetic and molecular studies, Arapidopsis thaliana - Erwinia carotorova provides an excellent model for basic studies of plant-pathogen interaction.

Infection of A. thaliana by Erwinia leads to rapid maceration of infected plantlets. The most important factor that determines virulence in Erwinia is the massive production of exoenzymes: e.g. pectic enzymes, cellulases and proteases. Mutants of Erwinia impaired in production or secretion of these enzymes exhibit lowered virulence or are fully avirulent.

Treatment of A. thaliana plantlets with Erwinia exoenzymes leads to rapid induction of several classes of PR-proteins in A. thaliana including proteins related to PR1 and β-glucanases of tobacco, as shown by immunological techniques and Northern analysis. By employing clones encoding individual exoenzymes of Erwinia we could asses the role of each exoenzyme as an inducer of the plant response. Results of this analysis suggest that pectic enzymes, both pectate lyases and polygalacturonases, elicit the synthesis of PR-proteins. Apparently pectic fragments released from the plant cell wall by these enzymes function as endogenous elicitors of the plant response.

Infection of A. thaliana by mutants of Erwinia carotovora that are avirulent or show clearly reduced virulence results in rapid induction of PR-proteins provided that these Erwinia mutants still produce and secrete pectic enzymes. However, PR-proteins are not induced (or are induced only transiently) during infection by wild-type Erwinia carotovora, suggesting that the bacterium can suppress the plant response.

On the other hand induction of PR-proteins, e.g. by treatment of plants by pectic enzymes, prior to Erwinia carotovora infection renders A. thaliana plantlets more resistant to subsequent infection.

FOURTH SESSION

ORAL PRESENTATIONS

U. RASMUSSEN
J.A. PINTOR-TORO

CHITINASE IN BRASSICA NAPUS

Ulla Rasmussen
Agricultural Research Department, Risø National Laboratory,
DK-4000 Roskilde, Denmark

Chitinase activity is strongly induced in Brassica napus after infection with Phoma lingam or Cylindrosporium concentricum. Seven days after infection of one cotyledon with Phoma lingam the chitinase activity is increased 14 times. No induction in chitinase activity was found in the other cotyledon or in the primary leaves that subsequently developed in the same plant.

Seven basic chitinase isozymes have been partially purified from infected leaves. The MW of the isozymes were estimated to be in the range of 29-38.

Polyclonal antibodies to chitinase from sugar beat cross-react specifically with a 30 kD chitinase polypeptide in B. napus. By immuno-blot assay it was confirmed that this polypeptide is strongly induced after pathogen infection. Results from a Canadian group have shown that a 38.5 kD chitinase is induced by ethylene treatment. The two polypeptides are serologically different. Immuno-blot assay using specific antiserum to the 38.5 kD polypeptide demonstrated slight induction after pathogen infection.

Using immuno-histological techniques we are currently studying the localization of chitinase during the plant defence reaction.

MOLECULAR ANALYSIS OF SALT STRESS.

J.A. Pintor-Toro, J.A. Godoy, S.Torres-Schumann and O. del Pozo. Instituto de Recursos Naturales y Agrobiologia. CSIC. Apdo.1052. Sevilla. 41080.

Salt stress induces important changes on gene expression. Salt effects on protein synthesis in tomato germinating seeds were investigated by 2-D PAGE of proteins labeled in vivo with (35-S)Methionine. Seeds germinating in NaCl were analized at three germination stages (4 mm long radicles, 15 mm long radicles and expanding cotyledons) and compared to seeds germinating in water. At the first germination stage several basic proteins of M.W. 13 Kd, 16 Kd, 17 Kd and 18 Kd were detected only in salt germinating seeds. Other basic proteins of M.W. 12 Kd, 50 Kd and 54 Kd were salt-induced in the second and third stages of germination. One 15 Kd acid protein was observed in every stage assayed and showed several phosphorylated forms. Pulse-chase experiments revealed that the phosphorylated molecules of this polypeptide were stable only while the stress is present. All of these proteins, except 17 Kd, were induced also by abscisic acid (ABA) in the same germination stages.

A cDNA library was constructed from poly(A+)RNA of NaCl- and ABA-treated tomato germinules. Through differential screening several cDNAs clones were obtained which hybridized preferentially to RNA from treated germinules. One of these clones , TAS15, has been characterized: it is induced in a) germinules treated with ABA, NaCl or mannitol b) roots, stems and leaves of ABA- or NaCl-treated tomato plants grown in an aerated hydroponic system. TAS 15 codes for the 15 Kd phosphorylated polypeptide. The protein sequence, deduced from nucleotide sequence, is glycin-rich (22.4%), contents phosphorilable aminoacids (Thr, Tyr, Ser) and a cluster of 5 Ser residues. TAS15 shows similarities with genes of barley, cotton and maize induced in late steps of embryogenesis (Lea genes).

FIFTH SESSION

D.F. KLESSIG
J.F. BOL

THE PR1 PROTEINS - THEIR SYNTHESIS, LOCATION, AND POSSIBLE FUNCTION

D. F. Klessig[1], J. R. Cutt[1], D. C. Dixon[1], J. P. Carr[1], J. Malamy[1], M. Metzler[1], M. H. Harpster[2], P. Dunsmuir[2], R. N. Beachy[3], and I. Raskin[4]

[1]The Waksman Institute at Rutgers, the State University of New Jersey, P. O. Box 759, Piscataway, NJ 08855; [2]DNA Plant Technology Corporation, 6701 San Pablo Avenue, Oakland, CA 94608; [3]Department of Biology, Washington University, St. Louis, MO 63130 and [4]E. I. du Pont de Nemours and Company, Inc., Wilmington, DE 19898

As a model to study how plants react to pathogenic attack the response of tobacco (*Nicotiana tabacum*) to tobacco mosaic virus (TMV) is being investigated. In response to a variety of pathogenic and chemical agents tobacco plants produce large amounts of pathogenesis-related (PR) proteins, whose synthesis has been associated with resistance to TMV. Our work has focused on the synthesis, location and function of the PR1 family of these proteins. Functional mRNAs encoding the PR1 proteins are present only when the synthesis of these proteins has been induced, suggesting that their synthesis is controlled in part at the level of mRNA accumulation. This has been confirmed by direct RNA analysis using primer extension and northern blot analyses.

The PR1 proteins are synthesized and processed like polypeptides destined for the endoplasmic reticulum. This pathway of synthesis is consistent with our biochemical and immunomicroscopy studies which indicate that some of these proteins are secreted and accumulate in the intercellular spaces and xylem elements of infected leaves. In addition, PR1 (or PR1-related) proteins accumulate within the vacuoles of crystal idioblast cells of TMV-infected leaves. High levels of PR1 mRNA were detected by *in situ* hybridization suggesting that crystal idioblast cells synthesize and then retain, rather than secrete, large quantities of PR1 proteins. These proteins are found in close association with crystals of calcium oxalate within the vacuoles of the crystal idioblast cells.

The synthesis of PR1 proteins is associated with the hypersensitive response and systemic acquired resistance although their function is unknown. We have used several approaches to determine whether PR1 proteins are involved in resistance to TMV. Roger Beachy and co-workers have shown that transgenic plants, constitutively expressing the coat protein (CP) of TMV, exhibit enhanced resistance to this virus. To determine if this enhanced resistance might be mediated through induction of the PR1 genes, their expression was examined. In transgenic plants derived from NN genotypic tobacco (NN plants are genetically resistant owing to the presence of the dominant resistance allele N), a high proportion (~80%) of those producing CP also expressed the PR1 genes at low levels. However, this correlation between TMV CP and PR1 gene expression was not observed in similarly transformed nn genotypic tobacco plants (untransformed nn plants are susceptible to TMV). Therefore, it is unlikely that PR1 proteins play a critical role in genetically engineered resistance in transgenic plants producing TMV CP.

To more directly test PR1 involvement in TMV resistance, transgenic plants were constructed which constitutively expressed the PR1b member of this small gene family. This protein was secreted from cells of these transgenic plants

and accumulated in the intercellular space at levels equivalent to those found in nontransgenic plants after infection or chemical induction. Transgenic plants derived from the nn genotypic parents failed to exhibit delayed onset or reduction in the severity of systemic symptoms after TMV infection. In transgenic plants derived from NN genotypic parents the time of appearance, the size and general morphology, and the number of viral lesions produced were similar to the parental control plants after TMV infection. These data indicate that the PR1b protein of tobacco is not sufficient for TMV resistance, and imply that the PR1 proteins may not function as antiviral factors. Since a variety of PR proteins synthesized during the hypersensitive reaction to TMV infection have activities consistent with roles in protection against fungi, bacteria and insects, the PR1 transgenic plants are being challenged with a variety of other pathogens. We have found that the PR1b transgenic plants are as susceptible to tobacco hornworn (*Mandusa sexta*) as the nontransformed control plants.

Originally the PR1 proteins of tobacco were found only in leaf tissue after pathogen or chemical induction. However, we have recently found that they are also developmentally expressed in floral structures of uninfected plants. Large amounts of PR1 proteins are synthesized in the sepals where they accumulate in the intercellular spaces, in the xylem, within the vacuoles of crystal idioblast cells, and in intracellular bodies within parenchyma cells associated with vascular bundles. The PR1 genes are also expressed at low levels in the developing embryo.

Arabidopsis thaliana also contains and expresses two PR1-related genes. Representatives of each subfamily have been cloned. Partial sequence analysis indicates ~50% homology with the tobacco PR1 genes. Preliminary studies suggest that they are also developmentally regulated, though the organ specificity may be somewhat different between tobacco and *Arabidopsis*.

Salicylate has been used extensively as a chemical inducer of PR proteins in tobacco and is known to induce local acquired resistance to TMV. We have found that endogenous salicylate concentrations increase at least 20-fold over basal levels in leaves of the TMV-resistant (NN) plants after infection with TMV. No increase in salicylate or PR1 message was seen after infection of the TMV-sensitive (nn) plants. This rise of endogenous salicylate levels with or preceding the appearance of PR1 mRNA in both infected leaves and uninfected leaves of inoculated NN plants suggest that salicylate may be part of the natural induction pathway for PR gene expression during the hypersensitive response and systemic acquired resistance.

EXPRESSION OF PR GENES

J.F Bol, M.D. van de Rhee, M.T González Jaén and H.J.M Linthorst.
Department of Biochemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 5, 2333 CC Leiden, The Netherlands.

Initially, the pathogenesis-related (PR) proteins induced by hte hypersensitive response of tobacco to infection with tobacco mosaic virus were defined as acidic, protease-resistant proteins that accumulated in the intercellular space of the inoculated and systemically induced leaves (for reviews see Van Loon, 1985; Bol, 1988; Fritig et al., 1989, Bol et al., 1990). More recently, it became clear that basic isoforms of the majority of acidic PR proteins accumulate in the vacuoles of cells in TMV-infected tobacco leaves. These basic isoforms have been identified also in many other plant species in which they accumulate under various stress conditions either in the vacuoles or the apoplast. Based on serological relationships and molecular properties, the tobacco PR proteins have been classified into five groups. The proteins in each group are encoded by multigene families in the genome of Samsun NN tobacco.

Group 1 contains the PR-1 proteins of which the function is not known. The tobacco genome contains about eight genes for acidic PR-1 proteins and a similar number of genes encoding basic PR-1 proteins. After TMV-infection three genes encoding basic PR-1 proteins (PR-1a, -1b and -1c) and at least one of the genes encoding basic PR-1 proteins are expressed.

Group 2 contains acidic and basic isoforms of enzymes with β-1,3-glucanase activity. The acidic PR proteins are represented by PR proteins 2, N, O and Q' whereas a single basic β-1,3-glucanase has been identified. Genomic blots indicate that the acidic and basic isozymes are each encoded by families of four to eight genes, most of which are expressed after TMV-infection.

Group 3 contains two acidic chitinases, the PR proteins P and Q, and two basic chitinases. The tobacco genome contains two to four genes for both the acidic and basic isozymes.

Group 4 contains a class of acidic low molecular weight proteins of unknown function. Two proteins of 14.5 kd (named r1 an s1) and two proteins of 13.0 kd (named r2 and s2) are induced by TMV infection but these proteins have not yet been characterized in detail.

Group 5 consists of two acidic proteins and at least one basic protein with a molecular weight of 24 kd. Different nomenclatures are used for group 4 and 5 proteins by various research groups. In the nomenclature of Fritig et al. (1989)

the acidic proteins are called PR-R and -S. The function of these proteins is not known. They show extensive sequence similarity to the sweet-tasting protein thaumatin and to a maize inhibitor of α-amylase and protease of insects.

We have cloned cDNAs and genes corresponding to the acidic and basic proteins from groups 1, 2, 3 and acidic proteins from group 5. In addition, we have cloned genes enconding several proteins that are strongly induced by TMV-infection but do not correpond to known PR proteins. One of these is a low molecular weight glycine-rich protein (GRP). The cloned genes have been used in plant transformation experiments to study regulatory elements involved in gene expression and to shed light on the putative role of these proteins in plant defence mechanisms.

Regulatory sequences have been analysed by fusing upstream sequences of the PR-1a and GRP genes to GUS and CAT reporter genes, respectively. These constructs have been introduced in the genome of Samsun NN tobacco by an Agrobacterium tumefaciens mediated transformation procedure. In addition to TMV-infection, the PR-1a and GRP genes are also strongly induced by spraying plants with 5 mM salicylate. Upstream sequences of 689 bp of the PR-1a gene or longer sequences were sufficient for induction of the reporter gene in transgenic plants by TMV or salicylate while 633 bp or shorter sequences were inactive. Similarly, an upstream sequence of 645 bp of the GRP gene was sufficent for induction of the reporter gene by TMV-infection or salicylate-treatment, while reduction of this sequence to 400 bp abolished the induction.

The function of PR proteins was studied by expressing the PR-1a, GRP and PR-S genes in transgenic plants both in the sense and anti-sense orientation under the control of the CaMV 35S promoter. Transgenic plants with a high level of expression of PR-1a, GRP or PR-S were as susceptible to infection with TMV or alfalfa mosaic virus as were non-transformed control plants. This indicates that these PR proteins alone are not responsible for the TMV-induced resistance of tobacco to virus-infection. Moreover, the transgenic plants were not protected against insect attack.

In transgenic plants expressing antisense RNA to the PR-S gene the induction by TMV infection of PR proteins R and S was reduced by about 90%. This reduction did not affect the formation of local lesions or the induction of other PR genes, indicating that PR proteins R and S have no role in these processes.

References

Bol JF (1988) Structure and expression of plant genes encoding pathogenesis-related proteins. In: Verma DPS, Goldberg RB (eds) Plant gene research; temporal and spatial regulation of plant genes, pp 201-221. Springer Verlag, Wien/New York.

Bol JF, Linthorst HMJ, Cornelissen BJC (1990) Plant pathogenesis-related proteins. In: Gould H (ed). Annual Review of Phytopathology, Vol 28. Annual Review Inc. Palo Alto, in press.

Fritig B, Rouster J, Kauffmann S, Stinzi A, Geoffroy P, Kopp M, Legrand M. (1989) Virus-induced glycanhydrolases and effects of oligosaccharide signals on plant-virus interactions. In: Lugtenberg BJJ (ed) Molecular signals in microbe-plant symbiotic and pathogenic systems. Springer Verlag, Wien, in press.

Van Loon LC (1985) Pathogenesis-related proteins. Plant Mol Biol 4: 111-116.

ORAL PRESENTATIONS

T. BRYNGELSSON
J. RYALS

Pathogenesis-related proteins in barley

Tomas Bryngelsson and Mats Gustafsson, Dept. of Crop Genetics and Breeding, The Swedish Agricult. Univ., S-268 00 Svalöv, Sweden.

Pathogenesis-related proteins have up till now been detected in some 20 species, mainly dicotyledonous. We have analyzed changes in the soluble protein pattern in the leaves of eight lines of barley, carrying different resistance genes to mildew (*Erysiphe graminis* f. sp. hordei). New host proteins were induced in five barley lines carrying the laevigatum resistance gene (hypersensitive reaction) by an incompatible mildew isolate. They were not detectable in two lines with the Ml-a1 gene (immune reaction) or the susceptible variety Tellus by analysis of soluble proteins with SDS-PAGE and IEF. These proteins have low molecular weights, and either low or high isoelectric points. Thus, they resemble the PR proteins found in dicotyledonous species.

One of the induced proteins, Hv-1, was purified and characterized. *Hordeum vulgare* cv. Alva was challenged with an incompatible race of mildew and the Hv-1 protein was isolated from necrotized tissue by a combination of ammonium sulphate precipitation, anion exchange chromatography, hydrophobic interaction chromatography and chromatofocusing. The protein has a molecular weight of 19 kD and an isoelectric point of 3.4. It is not a glycoprotein as judged from the lack of reaction to Concanavalin A and peanut agglutinin. The Hv-1 protein was partially sequenced and compared to known protein sequences. The sequence of 28 amino acids had a 48 % homology with the N-terminus of osmotin and NP24, proteins which are synthesized in response to salinity stress in tobacco and tomato, respectively. The same level of homology was also present in a maize trypsin/α-amylase inhibitor and in thaumatin, the sweet-tasting protein from *Thaumatococcus daniellii*. Even higher homology (58 %) was discovered with the N-terminus of the PR-5 (syn. PR-R, PR-S) protein of tobacco.

Two other PR proteins have recently been purified (Hv-2 and Hv-3). Both are highly acidic with pI-values below 3.4 and molecular weights of approximately 33,000 and 36,500 kD, respectively.

70

John Ryals
CIBA-GEIGY Biotechnology
P.O. Box 12257
Research Triangle Park, NC
USA

Complementary DNA clones encoding two isoforms of the acidic endochitinase (chitinase, EC 3.2.1.14) from tobacco were isolated. Comparison of amino acid sequences deduced from the cDNA clones and the sequence of peptides derived from purified proteins show that these clones encode the pathogenesis-related proteins, PR-P and PR-Q. The cDNA inserts were not homologous to either the bacterial form of chitinase or the form from cucumber but shared significant homology to the basic form of chitinase from tobacco and bean. The acidic isoforms of tobacco chitinase did not contain the amino-terminal, cysteine-rich, hevein domain found in the basic isoforms indicating that this domain, which binds chitin, is not essential for chitinolytic activity. The accumulation of mRNA for the pathogenesis-related proteins PR-1, PR-P, PR-R and PR-Q in Xanthi, nc tobacco leaves following infection with tobacco mosaic virus (TMV) was measured by primer extension. The results indicate that the induction of these proteins during the local necrotic lesion response to the virus is coordinated at the mRNA level.

Complementary cDNA clones were also isolated encoding a novel type of acidic β-1,3-glucanase. Analysis of these clones is presented along with evidence suggesting that there are at least three structural classes of glucanases in tobacco. Two of the classes are acidic, extracellular PR-proteins. One of which comprises PR-2, PR-N and PR-O and the other which is a novel PR-protein previously undescribed in tobacco.

SIXTH SESSION

A. LEYVA
F. GARCIA-OLMEDO

MOLECULAR ANALYSIS OF PHENYLALANINE AMMONIA-LYASE GENES.

Antonio Leyva, Xiaowu Liang, Ernst Natt, Jose A. Pintor-Toro, Michel Dron and Christopher J. Lamb. Plant Biology Laboratory, Salk Institute for Biological Studies, PO Box 85800, San Diego, California 92138, USA.

Plants exhibit a number of adaptative and protective responses by synthesizing an array of natural products designed to protect the plant against environmental stresses. The phenylpropanoid natural products elaborated from phenylalanine are of particular significance in this regard. Examples include lignin which is a structural polymer of cell walls and a stress-induced barrier to infection, flavonoid pigments and UV protectants as well as isoflavonoid phytoalexins. Some of these natural products are also responsible for the activation of symbiotic and virulence genes in *Rhizobium* and *Agrobacterium* , respectively.

Phenylalanine ammonia-lyase (PAL) catalyzes the first reaction specific to general phenylpropanoid metabolism. PAL activity is highly regulated during development and by an array of environmental stimuli including wounding, infection and light. The control of PAL enzyme activity is a key element in the regulation of phenylpropanoid synthesis and has been shown to reflect the *de novo* synthesis of PAL enzyme subunits. RNA blot hybridization and nuclear run-off experiments have shown that elicitor, wounding, infection and irradiation rapidly stimulate transcription of PAL genes leading to marked acumulation of PAL mRNA and hence increased enzyme synthesis and activity. In addition treatment with aminooxiphenylpropionic, a specific inhibitor of PAL activity, converts a normally resistant interaction of soybean seedlings with an avirulent race of *P. megasperma* into a susceptible interaction. These observations focus attention on the organization and structure of PAL genes in relation to their activation by environmental stimuli as a key element of plant defense responses (Lamb *et al.*, 1989).

PAL genomic sequences have been isolated from genomic libraries of *Phaseolus vulgaris* using elicitor-treated bean PAL cDNA sequences as a probe (Cramer *et al.*, 1989). These studies revealed that PAL is encoded by a family of at least three genes which encode distinct polypeptide isoforms. The transcripts corresponding to each of the PAL genes show a complex pattern of regulation during development. All three genes are expressed to high levels in root tissue. However, only PAL1 and PAL2 are expressed in shoots, while only PAL1 is expressed in leaves. In addition PAL2, but not PAL1 or PAL3, is highly expressed in floral tissue. Likewise there is a differential regulation in response to environmental cues. All three genes are induce by mechanical wounding of hypocotyls, but infection only induces PAL1 and PAL3, whereas light induces PAL1 and PAL2 but not PAL3 (Liang *et al.*, 1989a).

Molecular analysis of the mechanisms underlying the differential expression of PAL genes will reveal how plants incorporate defense responses into developmental programs.

In a first step in this analysis we have shown that a PAL2-GUS gene fusion is regulated in an appropiate manner in transgenic tobacco plants with respect to organ specificity, cell type specificity and environmental regulation by light, wounding etc (Liang *et al.*, 1989b). Histochemical analysis revealed that the PAL2 promoter is highly active in precise zones in the apical tips and in vascular tissue. Environmental signals such as wounding and light were accompanied by changes in the tissue-specific expression of the PAL2-GUS fusion. These results indicate that the pattern of expression of PAL2 is established at the transcriptional level. In suspension cultured bean cells, PAL is activated 2-3 min after elicitor treatment. This rapid activation suggests that there are few steps in the signal transduction pathway between signal perception and transcriptional activation of the gene.

A major challenge will be to establish the mechanism by which these different developmental and environmental stimuli are integrated to achieve a coherent pattern of PAL2 expression. Conceivably integration may occur at the level of the promoter by the operation of a mosaic of independent *cis*-acting regulatory sequences or alternatively integration may occur at earlier points in the signal transduction pathway.

To analyze the PAL2 promoter region, initial studies have focused on the generation of mutants in the PAL2 promoter region and the consequences of these changes on the expression of GUS in transgenic tobacco plants. In parallel, we have deliniated several regions of the PAL2 promoter that interact with nuclear factors *in vitro*, and are currently assaying the functional significance of these regions *in vivo*. Some results derived from these studies will be discused.

REFERENCES

Cramer, C.L., Edwards, K., Dron, M., Liang, X., Dildine, S.L., Bolwell, G.P., Dixon, R.A., Lamb, C.J. & Schuch, W. (1989) *Plant Mol. Biol.* **12**, 367-383.

Lamb, C.J., Lawton, M.A., Dron, M., & Dixon, R.A. (1989) *Cell* **56**, 215-224.

Liang, X., Dron, M., Cramer, C.L., Dixon, R.A. & Lamb, C.J. (1989) *J. Biol. Chem.* **264**, 14486-14492.

Liang, X., Dron, M., Schmid, J., Dixon, R.A. & Lamb C.J. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, in press.

THIONINS: A FAMILY OF PLANT ANTIMICROBIAL PROTEINS. F. García-Olmedo, M.J. Carmona, J.J. Lopez Fando, J.A. Fernandez, C. Maraña and P. Carbonero. Catedra de Bioquímica y Biología Molecular. E.T.S. Ingenieros Agrónomos, U.P. Madrid, 28040-Madrid, Spain.

The thionins are a family of cysteine-rich polypeptides, 45-47 amino acid residues in length, which were first identified in wheat endosperm (Balls et al., 1942) and have been subsequently designated with various names, depending on the species from where they have been isolated (see García-Olmedo et al., 1982, for a review of early work). The purpose of this communication is to assess the potential of thionin genes as possible targets for manipulation in plant breeding and to briefly report on recent progress of the molecular biology of this protein family. In connection with the possible breeding objective, two features merit special consideration: their distribution in the plant kingdom and their toxicity to different cells and organisms.

A crystalline protein, designated purothionin, was characterized by Balls et al. (1942) in wheat endosperm and subsequently similar proteins were found in the endosperms of many species of the Aegilops/Triticum group (Carbonero and García-Olmedo, 1969), barley (Redman and Fisher, 1969), rye (Hernández-Lucas et al., 1978) and oats (Bekes and Lasztity, 1981). The first member of this family isolated from leaves was viscotoxin A3 from the european mistletoe (Samuelsson et al., 1968), which was followed by other viscotoxins (Samuelsson and Petersson, 1970), phoratoxin (Samuelsson, 1974) and ligatoxin and denclatoxin (Samuelsson and Petersson, 1977), within the Loranthaceae, as well as by the thionins from the leaves of Pyrularia pubera, Santalaceae (Vernon et al, 1985) and, more recently, by those from barley leaves (Gausing, 1987; Bohlmann et al., 1987). Also belonging to this protein family is crambin, isolated by Van Ethen et al. (1965) from the cotyledons of Crambe abyssinica, Cruciferae.

Thionins are toxic to bacteria, including phytopathogenic ones (Stuart and Harris, 1942; Fernandez de Caleya et al., 1972), to yeast (Stuart and Harris, 1942; Hernandez-Lucas et al., 1974), to phytopathogenic fungi (Bohlmann et al., 1988), to laboratory animals, intraperitoneally but not orally (Coulson et al., 1942), to insect larvae (Kramer et al., 1979), and to cultured mammalian cells (Nakanishi et al., 1979; Carrasco et al., 1981). They are inhibitors of in vitro protein synthesis (García-Olmedo et al., 1983) and affect membrane permeability and inhibit DNA, RNA and protein synthesis in mammalian cells (Carrasco et al., 1981).

To explore their potential in plant protection, cDNAs and genomic DNA corresponding to thionins from cereal endosperm have been cloned, and used to construct chimaeric genes in combination with various promoters. Transformed tobacco plants have been obtained and significant levels of the corresponding mRNAs have been detected. Constructions based on the genomic DNA, which has

two introns, seem to be correctly processed. Kanamycin-resistant progreny from the transformed plants will be used to tests for disease resistance with appropriate pathogens.

Balls, A.K., Hale, W.S. and Harris, T.H., 1942, Cereal Chem., 19:279.
Békés, F. and Lasztity, R., 1981, Cereal Chem., 58:360.
Bohlmann, H. and Appel, K., 1987, Mol.Gen.Genet., 7:446-454.
Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reinmann-Philipp, U., Schrader, G., Barkholt, V. and Apel, K., 1988, EMBO J., 7:1559.
Carbonero, P. and Garcia-Olmedo, F., 1969, Experientia 25:1110.
Carrasco, L., Vazquez, D., Hernandez-Lucas, C.,Carbonero, P. and Garcia-Olmedo F., 1981, Eur. J. Biochem., 116:185.
Coulson, E.J., Harris, T.H. and Axelrod, B., 1942, Cereal Chem., 19:301.
Daley, S.L. and Theriot, L.J., 1987, J. Agric. Food Chem., 35:660.
Fernández de Caleya, R., Gonzalez-Pascual, B., García-Olmedo F. and Carbonero P., 1972, Appl. Microbiol., 23:998.
Garcia-Olmedo, F., Carbonero, P., Hernández-Lucas, C., Paz-Ares, J., Ponz, F., Vicente, O., and Sierra, J.M., 1983, Biochim. Biophys. Acta , 740:52.
Gausing K., 1987, Planta, 171:241.
Hernández-Lucas, C., Fernández de Caleya, R. and Carbonero, P., 1974, Appl.Microbiol., 28:165.
Hernández-Lucas, C., Carbonero, P. and García-Olmedo, F., 1978, J.Agric.Food Chem., 26:794.
Hernández-Lucas, C., Royo, J., Paz-Ares, J., Ponz, F., García-Olmedo and Carbonero, P., 1986, FEBS LETT., 200:103.
Kramer, K.J., Klassen, L.W.,Jones, B.L., Speirs, R.D. y Kammer, A.E., 1979, Toxicol. Appl. Pharmacol., 48:179.
Nakanishi, T., Yoshizumi, H., Tahara, S., Hakura, A. and Toyoshima, K., 1979, Gann, 70:323.
Samuelsson, G., 1974, Syst. Zool., 22:566.
Samuelsson, G. and Petersson B., 1970, Acta Chem. Scand., 24:2751.
Samuelsson, G. and Petersson, B., 1977, Acta Pharm. Suec., 14:245.
Samuelsson, G., Seger, L. and Olson, T., 1968, Acta Chem. Scand. 22:2624.
Stuart, L.S. and Harris, T.S., 1942, Cereal Chem., 19:288.
Vernon, L.P., Evett, G.E., Zeikus, D. and Gray, W.R., 1985, Arch. Biochem. Biophys., 238:18.
Van Etten, C.H., Nielsen, H.C. and Peters, J.E., 1965, Phytochemistry, 4:467.

ORAL PRESENTATIONS

I.E. SOMSSICH
E. KOMBRINK

Isolation, characterization and functional analysis of the "pathogenesis-related " PR1 and PR2 protein genes of parsley.

Imre E. Somssich, Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, FRG.

Parsley cells treated with a fungal elicitor derived from the soybean pathogen Phytophthora megasperma f.sp.glycinea respond rapidly and synchronously with many of the typical defense reactions seen in host-pathogen and nonhost-pathogen interactions. We have demonstrated that in many cases gene activation leading to de novo mRNA and protein synthesis play a key role in numerous early induced biochemical responses.This is true for the genes encoding two enzymes of the general phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL) and 4-coumarate:CoA ligase (4CL), for an enzyme specific to the furanocoumarin pathway,S-adenosyl-L-methionine bergaptol O-methyltransferase (BMT), for a hydroxyproline-rich glycoprotein (HRGP) an important structural component of the cell wall, and for a number of small acidic proteins termed "pathogenesis-related" PR proteins.
Despite our lack of knowledge concerning their function,there exists a number of reasons why the analysis of the PR protein genes are of interest. Activation of PR gene transcription occurs rapidly and transiently in elicitor-treated cells followed by massive accumulation of the respective mRNAs. As demonstrated by in situ hybridization a similarly strong and rapid PRmRNA accumulation is also observed localized at fungal infection sites in intact parsley tissue.

All three menbers of the PR1 gene family have been cloned and characterized. Comparison of their promoters have revealed both conserved and non-conserved DNA regions. Search for putative cis-regulatory elements the PR1-1 promoter by means of in vivo DNA footprinting has revealed two regions showing changes in the reactivity of G -residues to DMS. Within one region at position -240, 5 guanosin residues change their reactivity in vivo after elicitor treatment of the cells. Experiments to test the functional relevance of these finding are now in progress.
Finally, the single copy PR2 gene has also been cloned and analysed. Some 800 bp of its promoter have been fused to the reporter gene ß-Glucuronidase (GUS) and elicitor induced transient gene expression tested in transformed parsley protoplasts. Deletion contructs in combination with in vivo DNA footprinting experiments are in progress to define cis-elements within the promoter of this gene.

STRUCTURAL AND FUNCTIONAL ANALYSIS OF PR-PROTEINS AND THEIR GENES IN POTATO

Erich Kombrink, Max-Planck-Institut für Züchtungsforschung, Abteilung Biochemie, D-5000 Köln 30, Fed Rep Germany

Among the numerous 'pathogenesis-related' proteins (PR-proteins) of potato which are newly synthesized upon infection of leaves with the late blight fungus *Phytophthora infestans*, we have recently identified the enzymes 1,3-β-glucanase and chitinase. Both enzyme activities increase massively and coordinately not only after infection but also after treatment of leaves with fungal elicitor and accumulate, together with other PR-proteins, to a significant amount in the intercellular leaf space. Induced expression of 1,3-β-glucanase and chitinase is transcriptionally regulated as revealed by mRNA blot analysis and *in vitro* transcription assays using isolated nuclei of elicitor-treated potato leaves.

The participation of 1,3-β-glucanases and chitinases in the expression of resistance in potato remains unclear. When compatible (plant susceptible) and incompatible (plant resistant) interactions of two potato cultivars with appropriate races of *P. infestans* were analyzed, no significant differences in accumulation of the two hydrolytic activities could be detected in whole leaf extracts.

The cytological and cellular distribution of PR-proteins and their mRNAs in infected leafs was studied by immunohistochemistry and *in situ* hybridization. Both techniques revealed that accumulation of 1,3-β-glucanase and expression of the respective genes is not restricted to the vicinity of infection sites or to specific cell types. The uniform labelling of tissue sections suggests that the signal causing accumulation of 1,3-β-glucanase rapidly spreads throughout the tissue, resulting in systemic activation of gene expression upon infection. In contrast to 1,3-β-glucanase, other genes activated upon infection (e.g. PAL and PR-protein 1) are locally expressed around infection sites.

Besides their potential participation in the defense program of plants to pathogen attack, both enzymes are suspected to have other physiological functions in plant metabolism and development. This is concluded from the finding that both enzymes exsist in different classes of isoenzymes which differ not only in size, charge, and catalytical activity, but also in their expression in response to infection and organ specificity as well as cytological and subcellular localization. To elucidate the mechanisms responsible for differential expression of 1,3-β-glucanases and chitinases and controlling their localization and targeting to different cellular compartments and to evaluate their participation in the expression of disease resistance in potato, the enzymes and their genes, are being studied in detail. Antisera produced against the purified enzymes and heterologeous cDNA probes have been used to isolate numerous cDNA and genomic clones form potato, which are now being characterized.

81

POSTER SESSIONS

P. CARBONERO
C. CASTRESANA
F. CÔTE
G. FACCIOLI
A.F. TIBURCIO
M.T. GONZALEZ-JAEN
M.H. HARPSTER
J. KALLERHOFF
H.J.M. LINTHORST
M. OHSHIMA
U.M. PFITZNER
R. RIVERA-BUSTAMANTE
D. ROBY
B. SAN SEGUNDO
I. TOBIAS
S. TUZUN
G. VISEDO

A FAMILY OF α-AMYLASE/TRYPSIN INHIBITORS FROM CEREALS ACTIVE AGAINST INSECT AND OTHER HETEROLOGOUS ENZYMES. P. Rodriguez-Palenzuela, J. Royo, E. González-Hidalgo, L. Gómez, R. Sánchez-Monge, G. Salcedo, F. García-Olmedo and P. Carbonero. Bioquímica y Biología Molecular. Escuela Técnica Superior de Ingenieros Agrónomos. Universidad Politécnica de Madrid y Centro Nacional de Biotecnología. Madrid. (Spain).

A substantial fraction of the albumins and globulins of wheat and barley endosperms is represented by a single protein family, which includes inhibitors of heterologous α-amylases and of trypsin. Wheat and barley α-amylase inhibitors can be classified according to their degree of aggregation into monomeric, dimeric and tetrameric forms. The trypsin inhibitors characterised so far are all monomeric. This group of homologous inhibitors is encoded by a multi-gene family which is dispersed over several chromosomes, as has been determined by electrophoretic analysis of the proteins and by Southern hybridization analysis with cDNA probes, using the appropriate aneuploid lines. The homology among the different components has been established through direct aminoacid sequence of the purified proteins or that deduced from the DNA sequence of cDNA clones |1|.

Among the more recent advances in our knowledge of the different types of inhibitors are the following:

a) Trypsin inhibitors. Genomic and cDNA clones of barley trypsin inhibitor CMe have been isolated and sequenced. The longest ORF of the cDNA codes for a typical signal peptide, a feature common to all cDNAs characterized so far |3,4,5|, followed by a sequence identical to the known aminoacid sequence of the CMe protein. The genomic clone has no introns. The expression of gene CMe and its regulation by a gene located in trans has been demonstrated |2|. Transgenic tobacco plants, carrying a chimeric gene constructed with the cauliflower-mosaic-virus 35S promoter and the structural sequence from the CMe cDNA, have been obtained and insect tests have been initiated.

b) Monomeric α-amylase inhibitors. Similarly, a cDNA clone corresponding to the monomeric inhibitor 0.28 from wheat has been sequenced, transgenic tobacco plants carrying a 35S-0.28 construction have been obtained, and insect tests have been initiated.

|1| García-Olmedo, F., Salcedo, G., Sanchez-Monge, R., Gomez, L., Royo, J. and Carbonero P. (1987) OXFORD SURVEYS OF PLANT MOLECULAR AND CELL BIOLOGY. Vol. 4. Oxford University Press. pp. 275-334.

|2| Rodriguez-Palenzuela, P., Royo, J., Gomez, L., Sanchez-Monge, R., Salcedo, G., Molina-Cano, J.L., García-Olmedo, F. and Carbonero, P. (1989) MOL. GEN. GENET. (in press).

|3| Paz-Ares, J., Ponz, F., Rodriguez-Palenzuela, P., Lázaro, A., Hernández-Lucas, C., García-Olmedo, F., Carbonero, P. THEOR. APPL. GENET. (1986) 71, 842-846.

|4| Lazaro, A., Sanchez-Monge, R., Salcedo, G., Paz-Ares, J., Carbonero, P. and García-Olmedo, F. (1988) EUR. J. BIOCHEM. 172, 129-134.

|5| Lazaro, A., Rodriguez-Palenzuela, P., Maraña, C., Carbonero, P. and García-Olmedo, F. (1988) FEBS Lett. 239, 147-150.

DIFFERENTIAL EXPRESSION OF ß(1,3)-GLUCANASE IN NICOTIANA PLANTS

C. Castresana, F. De Carvalho, G. Gheysen, M. Van den Bulcke, G. Bauw, D. Inzé, J. Vandekerckhove, and M. Van Montagu

Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent (Belgium)

Plants respond to the attack of pathogenic microorganisms by inducing the expression of specific genes whose encoded products are directed to protect the plant against the infection. A group of proteins, designated "pathogenesis-related" or PR proteins, have been shown to be secreted into the extracellular spaces of the plant during the establishment of an incompatible interaction, typically recognized by a hypersensitive response in the plant.

Among these proteins, a family of hydrolytic enzymes identified as ß(1,3)-glucanases and chitinases have been shown to act as inhibitors of growth and/or fungal sporulation *in vitro*. Both groups of enzymes also contain intracellular isoforms whose expression has previously been shown to be hormonally regulated.

To evaluate the role of the different isoforms in the plant defence mechanisms, we have characterized both extracellular and intracellular ß(1,3)-glucanases by subcellular fractionation and partial amino acid sequence determination using the electroblotting and microsequencing techniques. These analyses revealed the presence in tobacco plants of at least two vacuolar and three extracellular isoforms with unique amino acid sequence and, therefore encoded by different genes. Two genomic clones corresponding to a vacuolar (pGN6) and a extracellular (pGN1) ß(1,3)-glucanases, respectively, have been isolated and characterized from *Nicotiana plumbaginifolia* plants. Both genes are expressed in the roots of healthy plants, but in addition the vacuolar isoform is also expressed in leaf tissue at approximately the same level of that in roots.

Chimeric constructs have been prepared by fusing the 5'-non-coding region of pGN1 to the reporter gene *gus*. Analysis of transgenic plants have revealed that although the expression conferred by this promoter is restricted primarily to the root tissue it is also possible to detect *gus* activity in the leaves.

Finally, we have examined the alterations in the level of gene expression conferred by this promoter in response to the infection by different bacteria, pathogenic and nonpathogenic, for this host.

EXPRESSION OF CHITINASE, LYSOZYME, ß-1,3-GLUCANASE IN TOBACCO AND IMMUNOCYTOCHEMICAL LOCALIZATION OF PR-1 PROTEINS

Côte François [1], Trudel Jean [1], Cutt John R [2], Audy Patrice [1], Benhamon Nicole [1], Grenier Jean [1], Letarte Jocelyne [1], El Ouakfaoui Souad [1], Klessig Daniel F [2] and Asselin Alain [1]

[1] Département de Phytologie, Université Laval, Canada
[2] Waksman Institute of Microbiology, Rutgers University, New Jersey, USA.

PR proteins had been lately divided for classification into functional groups. Two of these are ß-1,3-glucanase and chitinase. In several plant-pathogen interactions, they are known to be coregulated and to act synergically. For each of these enzymes, we have developed rapid and sensitive methods of detection in polyacrylamide gels. These methods allowed us to distinguish the different isoforms involved in the interaction.

Recently, several chitinases have been identified as extracellular pathogenesis-related (PR) proteins in potato, cucumber, maize, tomato and tobacco. Up to now, three virus-plant combinations have been studied for stimulation of chitinases: tobacco, mosaic, virus (TMV)-Samsun NN tobacco, tobacco necrosis virus (TNV)-hypersensitive cucumber and brome mosaic virus (BMV)-systemic maize. In the case of TMV-infected hypersensitive tobacco, four PR proteins were identified as chitinases: two acidic proteins (corresponding to PR-P (or PR-b7b) and PR-Q (or PR-b8b) and two basic proteins not related to previously known PR proteins. These four proteins are all endochitinases, are serologically related and account for all chitinase activity in TMV-infected tobacco leaves. Acidic isoforms (PR-P and PR-Q) have relatively low specific activity when compared to basic chitinases and represent about one third of all chitinase activity in leaves exhibiting necrotic local lesions to TMV.

ß-1,3-glucanase (laminarinase) activity was detected after polyacrylamide gel electrophoresis under native conditions by using laminarin or lichenan as substrate. After electrophoresis and incubation of gels, the glucans were stained with aniline blue flurochrome. Under UV illumination, lysis zones appeared as dark bands against a fluorescent background. As low as 0.001 unit of commercial Penicillium lamina-rinase could be observed after incubating the polyacrylamide gel for 45 minutes at pH 5.0. Extracts of commercial Penicillium laminarinase exhibited 4 bands with lytic activity towards laminarin. Analysis of intercellular fluid extracts of tobacco mosaic virus-infected tobacco leaves revealed 4 ß-1,3-glucanases corresponding to 3 acidic pathogenesis-related proteins b4 (2), b5 (N) and b6b (O) and one basic protein.

We have isolated a cDNA clone corresponding to one Nicotiana tabacum cv.Xanthi-nc PR-ß-1,3-glucanase mRNA from a

λGT11 cDNA library. The library was screened using an antiserum recognizing extracellular proteins of TMV-infected tobacco and ^{125}I-labelled PR-2 and -N. One clone was obtained. The insert was subcloned in plasmid vector and both strands were sequenced. Comparison of published tryptic peptide sequences with the deduced amino acid sequence from the DNA sequence data showed that this cDNA clone was from the gene family of extracellular stress-induced β-1,3-glucanases. There was 98% homology between the translated sequence of nucleotides and the extracellular 36 kDa glucanase (PR (36),PR-N or b5).

Unfortunately no function for the PR-1 group has been found. Despite numerous studies about PR proteins on a biochemical basis, there is little information about the precise localization of such proteins within infected plant tissues. Therefore, additional ultrastructural data on PR-1 proteins in Nicotiana tabacum cv. Xanthi-nc reacting hypersensitively to TMV infection were needed. Because of the close serological relationship between tomato PR p14 protein and tobacco PR-1 proteins, a monospecific polyclonal rabbit antiserum raised aganist electrophoretically purified tomato PR p14 was used for investigating the subcellular localization of PR-1 proteins in tobacco necrotic tissue and bordering cells. We present evidence in the last section of this poster, for the specific localization of these proteins in electron-opaque deposits along cell walls and in intercellular spaces.

ANTIVIRAL FACTORS (AVF) : PROTEINS RELETED TO PATHOGENESIS WHICH INDUCE AQUIRED RESISTANCE IN PLANTS

G.FACCIOLI a M.FORNI

Instituto di Patologia Vegetale,
Universitá di Bologna, Italy

Chenopodium amaranticolor plants locally infected by TNV contain, in both inoculated and uninoculated upper leaves, an antivirally-active substance(s), which confers resistance against a TNV or TMV challenge inoculation. Such antiviral factor(AVP), extractable by hydrated calcium phosphate and partially pururifiable by DEAE column chromathography, is probably a phosphorylate glyco-protein , which is active in variours virus-host combinations.Its formation, like that of interferons, is prevented by actinomycin-D (AMD) and vitamin C, and stimulated by polyacrylic acid (PA) and Poly (I:C). Also its mechanisms of action looks to be partially similar to that of interferon: a sinthetase activity capable of polymerizing ATP into antivirally-active oligonucleotides seems to be present in AVP-treated leaves and synthetic oligonucleotides (ppp2'-5'A, 2'-5 A) are active against TNV infection. AVF could be recovered also from intercellular fluid of TNV-infected leaves, and SDS-PAGE experiments of AVF obtained in both ways, revealed the presence of at least, 2 additional bands with respect to control estracts. In non-denaturing PAGE experiments at least 2 antivirally active peaks could be separated. Similar AVFs, the antiviral action of which was tested in the system TNV- C.amaranticolor , were isolated from both inoculated leaves of locally-infected plants (Gomphrena globosa - PVX), and systemically infected species ("White burley" tobacco PVX, Nicotiana glutinosa-PVY , C.quinoa-PMV and "Taylor"bean-BCMV); BCMV-infected bean seeds also contained AVF. Therefore production of non specific antivirally active proteins seems to be a general defense response of plants to viral infections.

MOLECULAR ANALYSIS OF PLANT RESPONSES TO UV-C INDUCED STRESS

A.F. Tiburcio[1], L. Campos[1], E. Mansur[1], X. Figueras[1], C. Caelles[2], I. Claparols[3], Mª T Piñol[1], A. Boronat[2]

Unidad de Fisiologia Vegetal[1] y Unidad de Bioquímica[2]. Facultad de Farmacia. Universidad de Barcelona. 08028 Barcelona. [3] C.I.D-C.S.I.C. J. Girona Salgado,18-20. 08034 Barcelona.

The influence of UV-C (254 nm) irradiation on growth, alkaloids and soluble proteins was previously studied in *Nicotiana rustica* plants (Tiburcio, 1981; Tiburcio et al. 1985). Plants irradiated at 960 Jm^{-2} during 66 days were retarded in growth and delayed in flowering. The irradiation with UV-C induced an accumulation of carotenoids with a simultaneous decrease in chlorophyll (Chl) pigments. Analysis of putrescine-derived alkaloid contents revealed the existence of a 'stimulatory' effect induced by the UV-C treatment. When the soluble proteins of the leaves of irradiated plants were separated by SDS-PAGE, changes in the banding pattern were observed with respect to the control leaves. Densitometric analysis showed that the most significant differences occurred in the 74 and 38 kD bands. There was no measurable 74 kD band in the control, but it was enhanced after UV-C irradiation. The 38 kD band was increased about 6-fold by UV-treatment (Tiburcio et al. 1985). These changes were similar to those reported to other plant stress conditions, such as heat-shock (Barnett et al., 1980), acid and osmotic (Young, 1984) stresses.

In current experiments, we are investigating the effect of UV-C irradiation on *Arabidopsis thaliana*. Plants irradiated at 960 Jm^{-2} during 5 consecutive days showed a decrease (about 2-fold) in fresh weight. Analysis of Chl contents revealed a decrease in Chl b (about 3-fold), without significant changes in Chl a. In contrast, carotenoid levels increased about 2.5-fold. The increase in the carotenoid contents has been correlated to the induction of HMG-CoA reductase gene, thought to play an important regulatory role in plant isoprenoid biosynthesis (Caelles et al., in press). The UV-C treated plants have also shown a dramatic increase in the polyamine contents, especially both the free and the hydroxycinnamic acid conjugated forms of spermidine and putrescine.

References

- Tiburcio, AF (1981) **Resumen de la Tesis Doctoral**. Universitat de Barcelona, pp. 1-28.
- Tiburcio, AF, Piñol, MªT, Serrano, M (1985) **Environ Exp Bot** 25: 203-210.
- Barnett,T, Altschuler, M, McDaniel, C, Mascarenhas, JP (1980) **Devl Genet** 1: 331-340.
- Young, N (1984) **Ph D Thesis**, Yale University, New Haven, CT, U.S.A.
- Caelles, C., Balcells, L., Ferrer, A., Hegardt, F.G., Boronat, A. **Plant Mol Biol (in press)**.

ANALYSIS OF THE PROMOTOR REGION OF THE TOBACCO PR-1 GENE

M.T. Gonzaléz-Jaen * , M.D. van de Rhee and J.F. Bol
Department of Biochemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 5, 2333 CC Leiden, The Netherlands.
* Departamento de Genética, Facultad de Biologia, Universidad Complutense, 28040 Madrid, Spain.

PR-1 proteins represent a subset of the "Pathogenesis-Related" proteins (PR proteins) induced in tobacco (Nicotiana tabacum cv. Samsun NN) following infection with Tobacco Mosaic Virus (TMV) (1,2). These proteins whose function is still unknown have also been found in other dicotyledonous and monocotyledonous species (3) and apparently are serologically related.

The cloning of cDNA allowed to test the occurrence of transcriptional control of the synthesis of PR-1 proteins (4) and their pattern of induction at mRNA level in relation with infection (5). mRNA for PR-1 was detected two days after inoculation with TMV in infected leaves showing a maximum value at day 4th to 5th. Eight days after inoculation mRNA was also detected in virus-free upper leaves reaching a maximum value at day 14 th. On the other hand, salicylate treatment of tobacco leaves induces locally the synthesis of PR-1 proteins (6, 7).

In order to identify putative regulatory elements involved in the expression of PR-1 gene a genomic clone for PR-1 (8) was used to generate upstream fragments of different length (287, 436, 516, 633, 689, 742, 792 and 902 bp) which where fused to GUS reporter gene. Tobacco plants (N. tabacum cv. Samsun NN) were transformed with these constructs by an Agrobacterium tumefaciens mediated procedure.

Significative induccion of GUS activity was obtained in leaves treated with salicylate or TMV infected when upstream fragments of PR-1 gene were 689 bp or longer. Systemic induction of GUS reporter gene was observed in the upper non-infected leaves fourteen days after inoculation of the lower ones in the progeny of plants carrying the 902 bp upstream fragment; shorter constructs were not tested yet. Since upstream sequences of 633 bp or smaller had shown no significative induction of GUS expression either by salicylate treatment or TMV infection a relevant sequence involved in gene expression could be located somewhere between positions -633 and -689 upstream the coding region. These results are compared with those obtained so far for other promotors of PR genes or related (9, 10, 11). These data would agree with the possibility that the putative sequence located between -633 and -689 would be a sequences "enhancer-type" and sequence responsible for induction by salicylate treatment or TMV infection would be located much closer to the coding region. This hypothesis is currently being tested.

References

1. van Loon, L.C. 1982. Regulation of changes in proteins

and enzymes associated with active defense against virus infection. In: Active Defense Mechanisms in Plants E.K.S. Wood, Ed. N. York, Plenum Press, pp. 247-273.
2. Bol, J.F. 1988. Structure and expression of plant genes encoding pathogenesis-related proteins. In: Temporal and Spatial Regulation of Plant Genes. D.P.S. Verma and R.B. Goldberg, Eds. Springer-Verlag, Vienna, pp. 201-221.
3. White et al. 1987. J. Gen. Virol. 68: 2043-2048.
4. Hooft van Huijsduijnen et al. 1985. EMBO J. 4: 2167-2171.
5. Hooft van Huijsduijnen et al. 1986. EMBO J. 5: 2057-2061.
6. Antoniw, J.F.; White, R.F. 1980. Phytopathol. Z. 98: 331-341.
7. Hooft van Huijsduijnen et al. 1986. J. Gen. Virol. 67: 2135-2143.
8. Cornelissen et al. 1987. Nucleic Acids Res. 15: 6799-6811.
9. van Kan et al. 1989. Plant Mol. Biol. 12: 153-155.
10. van Kan et al. 1988. Mol Plant-Microbe Int. 1: 107-112.
11. Lamb et al. 1989. Cell 56: 215-224.

DISEASE RESPONSE OF TRANSGENIC TOBACCO PLANTS WHICH CONSTITUTIVELY EXPRESS PR1b TO TOBACCO MOSAIC VIRUS (TMV).

M. H. Harpster *, J. R. Cutt, D. C. Dixon, J. P. Carr, P. Dunsmuir * and D. F. Klessig. The Waksman Institute at Rutgers, State University of New Jersey. P.O. Box 759, Piscataway, New Jersey 08855. * DNA Plant Technologies Inc., 6701 San Pablo Ave., Oakland, California 94608.

The positive correlation of PR1 (PR1a, -1b and -1c) protein accumulation with viral resistance in a number of tobacco cultivars has prompted speculation that these proteins may constitute an important component of disease resistance. As a test of this hypothesis, we have generated transgenic Nicotiana tabacum plants of cultivars Xanthi NN (resistant to TMV) and Xanthi nn (sensitive to TMV) which constitutively express the PR1b gene. Assuming that PR1b functions as an antiviral factor, transgenic plants of both genotypes should exhibit some degree of enhanced resistance to TMV.

The subsequent inoculation of transgenic plants derived from the cv. Xanthi NN showed a similarity to the temporal appearance, size and number of viral lesions wich develop in untransformed control plants. Likewise, inoculated transgenic plants of the cv. Xanthi nn exhibited no delay in the onset or severity of disease symptomology (vein clearing and chlorosis) relative to control plants. Collectively, these results provide strong evidence that the PR1 proteins do not constitute part of a generalized resistance response to viral pathogens or, alternatively, are alone insufficient for providing resistance.

In our future work we will be testing whether plants which constitutively express a combination of the PR proteins show resistance to TMW. We are also exploring the possibility that the PR1 proteins may have antibacterial and/or antifungal activities.

REGENERATION AND TRANSFORMATION IN MELON (CUCUMIS MELO L.)

Sofia Ben Tahar, Michiel de Both, Laurence Thion and Jean Kallerhoff.
Biosem-Groupe Limagrain
24, Avenue des Landais
63170 AUBIERE- FRANCE

The application of genetic transformation to crop plants requires in the first place a good system for the regeneration of plants through adventitious shoot buds or somatic embryogenesis. An efficient method for adventitious shoot induction on cotyledonary explants of muskmelon (Cucumis melo L.) has recently beeen described (Niedz et al , 1989). Basing ourselves on this system, we studied in more detail some of its aspects.

The time, during which the dissected mature embryos were left to germinate before the cotyledonary explants were taken into culture, was found to be an important factor. Futhermore, the response of different parts of the cotyledons was tested, and optimal BAP and ABA concentrations for shoot induction were identified.

Subsequently, cotyledonary explants were subjected to cocultivation with Agrobacterium tumefaciens carrying the binary vector pBI121.

Shoot buds were induced on kanamycin-containing media, and they were found to express GUS. Fertile, transgenic plants could be regenerated and were brought to the greenhouse.

References:

Niedz R.P., Smith S.S., Dumbar K.B., Murakishi H.H. and Stephens C.T. (1989)- Plant Cell, Tissue and Organ Culture 18 : 313-319.

ISOLATION OF RECOMBINANT DNA CLONES ENCODING HYDROLASES OF TOBACCO

H.J.M. Linthorst, L.C. van Loon[1], J. van Roekel[2], C. van Rossum, B.J.C. Cornelissen[2] and J.F. Bol, Biochemistry Department, Leiden University, Leiden; [1]Department of Plant Physiology, Agricultural University, Wageningen and [2]Mogen International NV, Leiden, The Netherlands.

Infection of Samsun NN tobacco with tobacco mosaic virus results in the induced synthesis of so-called pathogenesis-related (PR-) proteins. cDNA clones from mRNA for PR-proteins P/Q (extracellular chitinase) and intracellular chitinase were isolated using a differential hybridization technique, and sequenced. The basic chitinase cDNA clone shows an open reading frame coding for a protein which is very homologous to a intracellular chitinase from tobacco (Shinshi et al., 1987). In the deduced amino acid sequence of the extracellular chitinase a 23 residues long signal peptide is present followed by a 231 amino acid residues long mature protein. This protein is homologous to intracellular chitinase but lacks the approximately 50 residues long N-terminal region which has been shown to be a chitin-binding region in e.g. weat germ agglutinin, intracellular chitinases, certain lectins and wound-induced proteins. After transformation of tobacco with chimaeric genes containing the 35S CaMV promoter and either extra- or intracellular chitinase coding regions, transgenic plants were shown to accumulate the chitinases without prior induction. PR-P/Q appeared to be localized in the intercellular fluid of the leaves.
With a probe homologous to an intracellular β-1,3-glucanase cDNA from tobacco (Shinshi et al., 1988) clones were isolated from a genomic tobacco library and sequenced. The sequence of the open reading frame of one of the clones, was shown to be identical to one of the cDNA clones of Shinshi et al. (1988). The coding region of the gene is devided over two exons containing the putative signal peptide and the mature pro-protein reading frames, respectively. The other clone contains a pseudogene. Furthermore, cDNA clones from an expression library were isolated with an antiserum against PR-2/N and used as probe in Northern and Southern hybridization.

Shinshi et al., (1987). PNAS 84, 89-93.
Shinshi et al., (1988). PNAS 85, 5541-5545.

COMPARISON BETWEEN ACIDIC PR1 PROTEIN GENES

Masahiro Ohshima, Department of Molecular Biology, National Institute of Agrobiological Resources, Tsukuba,Ibaraki 305, Japan.

We have isolated and characterized the genomic clones of PR1a, 1b and 1c protein genes of Samsun NN tobacco. The sequence studies and southern blot analysis indicated that one copy each of the three PR1 protein genes per haploid genome would be active in the plant. Their structures resemble each other, and the nucleotide sequences of PR1a and 1b are almost identical in the coding and the 5' upstream regions. The sequence of PR1c has high homology with these of PR1a and 1b in the coding and the 5' upstream sequence of the 0.2 Kb fragment which places just upstream of first ATG. However, in far upstream of this conserved region, there are some sequence insertions and deletions in PR1c gene. The three PR1 protein genes express inducibly under a similar situations, e.g. in stress condition or after the treatment with some chemicals. Therefore, this conserved sequence (180 bp) of PR1 genes might be important for the induced expression of the genes.

To confirm the possibility experimentally, the chimeric genes consisted with a deletion series of 5' upstream of PR1a gene and β-glucuronidase (GUS) gene was constructed, and introduced into tobacco mesophyll protoplasts by electroporaton. GUS activity was induced in the protoplasts by salicylic acid-treatment when the chimeric gene containing the conserved 180 bp or the longer fragment was introdued. These results indicate the common region in all active PR1 genes might contain cis-acting element(s) for the regulated expression of PR1 genes.

Functional analysis of the PR-1a promoter by transient and by stable transfections

Andrea Beilmann[1], Artur J. P. Pfitzner[1,2], Howard M. Goodman[2], Reinhold G. Herrmann[1] and Ursula M. Pfitzner[1,2]

[1]Botanisches Institut der Ludwig-Maximilians Universität and Genzentrum München, Menzingerstr. 67, D-8000 München 19, FRG.

[2]Department of Genetics, Harvard Medical School and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA.

The tobacco PR-1 genes are controlled by various external stimuli such as pathogen attack or exposure of the plants to certain chemicals as well as by developmental stimuli. In order to understand the complex regulation of these genes, the 5´ flanking region of the PR-1a gene (1) was joined by a transcriptional fusion to the *E. coli* β-glucuronidase (GUS). Expression of the reporter gene was monitored in transient expression assays and in stable transformants.

The PR-1a 5´ flanking sequences from -335/-149/-71 to +28 are functional promoter elements in tobacco and carrot protoplasts as determined by transient expression directing correct initiation of the PR-1a/GUS hybrid transcripts from the native transcription start site of the PR-1a gene. The level of gene expression was about two-fold less than that obtained with the cauliflower mosaic virus 35S RNA promoter. Regulation of gene expression by acetylsalicylic acid, however, could not be detected in the transient assays. The same constructs were stably introduced into tobacco via *Agrobacterium* mediated transformation. By Southern analyses, the chimeric genes were found to be correctly integrated in the plant genome. Moreover, based on the Southern blot experiments and on genetic analyses of the progeny plants, many of our transgenic lines had encountered from 2 to several independent integration events. However, neither a constitutive nor an induced GUS activity could be observed in the primary transformants or in the F1 offspring. It therefore appears that the controlled expression of the PR-1a gene may depend on sequences not contained in our constructs and/or on factors which are able to exert their function only when the gene is integrated in the plant chromosomal background.

(1) Pfitzner et al. (1988) Mol. Gen. Genet. 211, 290-295.

PARTIAL CHARACTERIZATION OF A 70 Kd PROTEIN INDUCED IN TOMATO BY TOMATO PLANTA MACHO VIROID.

Segura-Nieto, M. , Rivera-Bustamante, R. , Ruiz-Medrano, R. , Herrera-Estrella, L. and Galindo-Alonso, J.

Viroids are the smallest pathogenic agents reported so far. Those pathogens, as well as other plant pathogens and stress conditions, induced several host proteins upon infection of susceptible plants (PR proteins). It has been reported earlier that tomato planta macho viroid (TPMV) induces a 70 kd protein (p70) in tomato. This protein shows some differences with the PRP already reported in tomato (e.g. its extractability at neutral pH, the failure to be induced by TMV and CMV).

p70 protein was purified from TPMV-infected tomato crude extracts by ammonium sulfate precipitation (50-60 %), gel filtration and RP-HPLC. The peak containing the protein was identified by an immunodiffusion test and SDS-PAGE. The aminoacid sequence of the amino terminal was determined by sequencing in a 470 ABI gas phase sequenator.

Sequence:

NH $_2$ -VYAPPDFLGLAQNMGVFKAGN
D

Aminoacid composition (%)

B (D+N)	= 8,60	MES	= 1,75	Z (E+Q)	= 6,19
CA	= 1,07	A	= 8,38	P	= 6,26
S	= 6,90	G	= 8,78	H	= 1,59
R	= 3,23	T	= 4,74	K	= 9,93
F	= 5,72	L	= 8,67	I	= 6,28
V	= 7,76	Y	= 4,08		

An oligonucleotide based on the end terminal was synthesized and it has been used to screen a cDNA lybrary from TPMV-infected tomato tissue to look for the gene of the 70Kd protein.

FUNCTIONAL ANALYSIS OF DNA SEQUENCES REGULATING THE EXPRESSION OF THE PATHOGENESIS-RELATED GENE, CHITINASE, IN TRANSGENIC PLANTS AND IN ELECTROPORATED PROTOPLASTS.

D. ROBY[1], K. BROGLIE[2], J.J. GAYNOR[3] and R. BROGLIE[2]

1 Centre de Physiologie Végétale, URA CNRS 241
Université Paul Sabatier, 118 route de Narbonne
31062 Toulouse, France.
2 E.I. DuPont de Nemours & Co, Agricultural Products Dept
Wilmington DE 19880, USA.
3 Rutgers University, Dept of Biology
Newark, NJ 07102, USA.

Chitinase is a lytic enzyme found in most higher plants and has been recently identified as several of the "Pathogenesis-Related" proteins ; this enzyme catalyzes the hydrolysis of chitin, a β-1,4-linked homopolymer of N-acetyl-D-glucosamine. The production of chitinase by higher plants is thought to be part of a natural defense mechanism against chitinous pathogens. In bean, this enzyme is encoded by a small multigene family and its synthesis is strongly regulated at the level of gene transcription by a number of inducers including oligosaccharide elicitors and the phytohormone, ethylene. To define the DNA sequence elements that are involved in mediating this response and to understand better the role of ethylene in controlling the expression of specific defense-related proteins during pathogen attack, we have introduced a 4.7 kb fragment of bean genomic DNA containing the CH5B gene into tobacco using Ti-plasmid vectors. Northern blot analysis indicates that the expression of the foreign gene in tobacco is dependent upon treatment with exogenous ethylene or elicitor. Expression of a chimeric gene consisting in 1.7 kb of 5' flanking DNA derived from the CH5B gene fused to the coding sequence of β-glucuronidase, in electroporated bean protoplasts, indicate that this region is sufficient for ethylene and elicitor-regulated expression. Deletion analysis of the promoter region has allowed us to localize the ethylene/elicitor responsive element to a region situated between -382 and -59 upstream of the transcription start. DNA sequence comparison of the 5' flanking regions of two ethylene-regulated chitinase genes reveal two short DNA sequence motifs which are exactly conserved between the two bean genes.
The expression of the chimeric gene was also analyzed in transgenic tobacco plants in response to infection by a fungal pathogen, Botrytis cinerea. The data show that the chimeric gene expression is highly induced around the fungal lesions (0 to 5mm), and to a less extent, at distance from the inoculation site.

IDENTIFICATION OF A cDNA CLONE ENCODING A BASIC FORM OF A PATHOGENESIS-RELATED PROTEIN FROM MAIZE SEEDS.

J. M. Casacuberta, P. Puigdomènech and B. San Segundo.

Department of Molecular Genetics, CID, CSIC. Jordi Girona, 18. 08034 Barcelona, Spain.

A considerable amount of data on PR-proteins in dicotyledoneous plants has been published lately. PR-proteins were first described as an enigmatic group of proteins induced in large amounts in tobacco leaves which respond hypersensitively to infection by viruses or other pathogens, or by treatment with specific chemical agents (revised in references 1-5). Several of these PR-proteins have been reported to display β-1,3 gluconase and chitinase activity. Their mRNAs and genes have also been characterized (2,6). In contrast, limited information about PR-proteins in monocotyledoneous plants is available. Eight PR-proteins (PRm proteins) have been found to accumulate in maize leaves after mercuric chloride treatment or brome mosaic virus (BMV) infection (7). Four of these PRm proteins were found to be chitinases. The presence of proteins serologically related to the tobacco PR-1 type PR-proteins in BMV infected maize have also been reported (8).

Here we describe a cloned cDNA representing a maize protein homologous to PR-proteins from tomato (p14) (9) and tobacco (PR-1 group and cluster G cDNA). A cDNA library, enriched in aleurone-specific sequences was prepared from poly(A)$^+$ containing RNA from the two-day dark germinated maize seeds (Zea mays, pure inbred line W64A). By differential screening (RNA from 2-day germinated seeds or ungerminated seeds was used to prepare ^{32}P-cDNA probes), one clone was found to contain an open reading frame encoding for a protein with 53% homology to protein p14 from tomato, and 55% and 63% homology to PR-1a protein and to the cluster G cDNA from tobacco, respectively. The deduced amino acid sequence starts at amino acid 2 from their homologous proteins (11% Arg+Lys, 7% Asp+Glu). We have named this protein ms-PR (maize seed-pathogenesis related protein). The mRNA size for this ms-PR protein is about 800 bp and the genomic blot analysis suggests that the maize genome contains a single ms-PR gene. Northern blot hybridization experiments indicate also a pattern of seed-specific expression for ms-PR mRNA during germination. Furthermore, when the germination process is carried out under sterile conditions its expression is drastically reduced. The importance of the protein and cloned cDNA presented here lies more in the tissue specificity and the extend of its expression. Its abundant expression in the aleurone layer lead us to propose a role for the PR-proteins as part of a defense mechanism against pathogens during seed germination.

REFERENCES

1.- van Loon, L.C. (1985) Plant Mol. Biol. 4 , 111-116.
2.- Bol, J.F. (1988) In: Temporal and Spatial Regulation of Plant Genes. Verma,D.P.S. and Goldberg,R.B. eds. Spring-Verlag Wien, New York pp 201-221.
3.- Pierpoint,W.S. and Shewry,P.R. (1987) In: Oxford Surveys of Plant Mol and Cell Biol. Miflin,B.J. eds. Vol 4, pp 337-342.
4.- Jamet,E. and Fritig,B. (1986) Plant Mol. Biol. 6 , 69-80.
5.- White,R.F.,Dumas,E.,Shaw,P.,Antoniw,J.F (1986) Antiviral Research 6 ,177-185.
6.- Cornelissen,B.J.C.,Hooft van Huijsduijnen,R.A.M.,van Loon,L.C. and Bol,J.F. (1986) EMBO J. 5 , 37-40.
7.- Nasser,W., de Tapia,M., Kauffmann,S., Montasse-Koushari,S. and Buikard,G. (1988) Plant Mol. Biol. 11 , 529-538.
8.- White,R.F.,Rybicki,E.P.,von Wechmar,M.B.,Dekker,J.L. and Antoniw,J.F. (1987) J. Gen Virol. 68 ,2043-2048.
9.- Lucas, J. Camacho Henriquez, A.C., Lottspeich, F., Henschen, A. and Sanger, H.L. (1985) EMBO J. 4 , 2745-2749.

PATHOGENESIS-RELATED PROTEINS IN LEAVES OF CAPSICUM ANNUUM L. SHOWING DIFFERENT TYPES OF REACTION TO INFECTION BY TOBACCO MOSAIC VIRUS

I. Tóbiás [+], R.S.S. Fraser [++] and A. Gerwitz [++]
[+] University of Horticulture and Food Industry, Dept. of Plant Protection, H-1502 Budapest, Pf. 53. HUNGARY
[++] AFRC Institute of Horticultural Research, Biochem. and Molec. Biol. Dept., WELLESBOURNE, Warwick CV35 9EF, U. K.

Pepper (Capsium annuum) plants containing the L^1 or L^3 genes for resistance to tobacco mosaic virus, and susceptible hosts (L^+), were inoculated with TMV strains overcoming neither, one or both resistance genes. Diverse symptoms were produced in the different host-virus combinations. Measurements of virus multiplication comfirmed, in quantitative terms, the operation of gene-for-gene relationships between host resistance and virulence in the virus. Host metabolism was studied during different types host/virus interaction.

Accumulation of pathogenesis-related (PR) proteins was examined on native and denaturing gels. Major PR proteins with molecular weights of 11, 18, 21 and 28 kDa were found, together with several other bands: all were trypsin-resistant. One host protein disappeared after certain types of infection. PR proteins were induced mainly in necrotically-infected inoculated leaves, and in necrotic or non-necrotic, systemically-infected leaves. TMV strain SL, which caused no necrosis on any host, was an efficient inducer of PR proteins.

Little evidence was found for any acquired systemic resistance in the host-virus combinations tested.

The relationships between ethylene synthesis and necrosis and PR protein accumulation were examined. Necrotic infections stimulated ethylene synthesis, but symptomless inoculated leaves with enhanced ethylene production were also found. The non-necrotic strain SL did not significantly enhance ethylene production. Cases were found where ethylene was increased without PR proteins accumulation, and conversely where PR proteins increased without major ethylene increase.

Early Induction and Accumulation of β-1,3-Glucanases, Chitinases and other Pathogenesis-Related (PR- or b-) Proteins in Tobacco Plants Immunized Against Blue Mold (Peronospora tabacina).

S.Tuzun and J.Kuc, Department of Plant Pathology, University of Kentucky, Lexington, Ky, USA.

Tobacco plants (Burley Ky 14) were immunized against blue mold by stem injection with sporangiaspores of the blue mold pathogen, Peronospora tabacina . Immunization resulted in a drastic reduction (>95%) in lesion area and sporulation following subsequent inoculation (challenge) and this reduction presisted for the life of the plant. β-1, 3-Glucanases , chitinases and other PR-proteins, which may be involved in pathogenesis, were studied in immunized plants and plants stem injected with water (controls). Western blot analyses and enzyme activity assays indicated the presence of chitinases but not β-1,3-glucanases in controls prior to challenge. Increased activities of β-1,3-glucanases and chitinases were observed in immunized plants, but not controls, prior to challenge. Activities of these enzymes increased rapidly after challenge of immunized plants whereas increased levels were detected only after the development of disease in control plants. SDS-PAGE indicated increases in the amounts of several other PR-proteins before challenge in immunized plants; increased levels of these PR-proteins were not detected in controls prior to 6 days after challenge. The increases in β-1,3-glucanases and chitinases as well as other PR-proteins coincided with the onset of immunization, and the further increases in activity after challenge of immunized plants coincided with the period during which the development of P.tabacina was inhibited.

ISOZYME PROFILES ASSOCIATED WITH THE SYSTEMIC INFECTION OF *Nicotiana clevelandii* BY PLUM POX VIRUS. THE ROLE OF THE ETHYLENE IN THE INDUCTION OF THE OBSERVED TRANSFORMATIONS.

Guillermo Visedo[1], José Fernández-Piqueras[1] and Juan Antonio García[2].

(1) Unidad de Genética. Dpto. de Biología. Facultad de Ciencias, C-XV. Universidad Autónoma de Madrid. 28049-Madrid. Spain.
(2) Centro de Biología Molecular (CSIC-UAM). 28049-Madrid. Spain.

Isozyme profiles of peroxidases (POX), esterases (EST) and glutamato oxaloacetate transaminases (GOT) have been determined in healthy and plum pox virus (PPV) infected tissues. The isozyme patterns of expression were different among different tissues and among different developmental stages. IsoPOX profiles increse in complexity and activity with the aging process whereas isoEST and isoGOT ones show a lower number and activity of bands when the leaves are old.

Inoculated leaves develop local lesions around 7 days after PPV inoculation. Local lesions, regions surounding the local lesions and regions far from them into the inoculated leaves have been analyzed separatelly. PPV infection led to transformations in the isozyme profiles, specially marked at the local lesions. When comparing these isozyme profiles with those of healthy tissues, it is possible to postulate that both senescence processes and wounding are implicated in the observed changes at the inoculated leaves and both processes are related with virus infection but not with the inoculation procedure. Systemically infected leaves with symptoms also showed transformations in their isozyme profiles which could be related with senescence. However, leaves without symptoms from infected plants did not show any detectable transformation in their isozyme profiles.

The role of ethylene in the observed changes has been valorated. *In vitro* ethrel treatments of leaves led to senescence and necrotization. The susceptibility to the treatment was variable between and within leaves being found a faster and a more marked response when leaves were older. Necrosed samples showed transformations in their isozyme profiles which corresponded to an extreme senescence situation but, in contrast with viral necrotic lesions, wounding influence was highly minor. We postulate that senescence transformations, mediated by ethylene, play a major role during local lesion formation in *Nicotiana clevelandii* plants infected by PPV. However, the induction of the senescence response could not be effective to localize the virus at the site of infection.

CONCLUSIONS AND PROSPECTS

CONCLUSIONS AND PROSPECTS

PR proteins, their induction in different plant species by different kind of pathogens and elicitors, their cellular and molecular biology and even their action when constitutively expressed in transformed plants have been the concern of more than fifty scientists from fifteen different countries during these days of scientific discussions lived in the "Parador Nacional Luis Vives" near Valencia.

Besides the conventional interchange of scientific information, and discussions held in the programmed, sessions, two objectives have also been of special concern for the organizers of this Workshop:

1. - To promote personal encounters providing oportunities for more informal discussions and generate mutual knowledge and friendship as a basis for cooperative relationships. 2. - To offer the Workshop as a way of introduction into the field of PR proteins to young scientists.

It seems to be the general opinion of the participants that these objectives have been achieved. A quite complete and up to date overview of the state of the art was presented, with specific debates at the end of the six sessions of the Workshop. An interesting and stimulating "Final Discussion", chaired by Dr.Van Loon, on what could be considered as the main open questions for the future was also held. Main topics of the discussion were the following:

- Activity of promoters and factors for transcription of "PRs".
- Inductors. Similarities/Differences between the expression of "PRs" and other inducible defenses.
- Localitation of "PRs" in cells and tissues.
- Biological function. Relationship with resistance.
- Definition of "PRs". Nomenclature.

Finally, there was an unanimous consensus on the necessity to consolidate the "PR group" giving continuity to this type of meetings, and to care about the semantics and nomenclature of PR proteins. In this regard, it was generally agreed to celebrate the next Workshop on PR proteins in 1992. Drs. T.Boller and F.Meins gracefully accepted the proposal to act as organizers of the meeting in Switzerland. A Commitee for Nomenclature of PR proteins was proposed and Drs. T.Boller, V.Conejero and L.C. Van Loon were coopted as menbers.

Finally, the organizers wish to aknowledge the active contribution of all the participants and the continous support of the Juan March Foundation to the success of this Valencian Workshop on Pathogenesis-Related Proteins.

LIST OF PARTICIPANTS

P. Ahl	- Ciba-Geigy. Basel (Switzerland).
T. Bryngelsson	- Department of Crop Genetics and Breeding. The Swedish Agricultural University. Svalöv (Sweden).
G. Burkard	- Institut de Biologie Moléculaire des Plants. CNRS. Strasbourg (France).
P. Carbonero	- E.T.S.I. Agrónomos. Universidad Politécnica. Madrid (Spain).
C. Castresana	- Laboratorium voor Genetica. Rijksuniversiteit Gent. Gent (Belgium).
F. Côte	- Département de Phytologie. Université Laval. Quebec (Canadá).
E. Dumas	- Laboratoire de Phytoparasitologie. Station de Génétique et d'Amélioration des Plantes. INRA. Dijon (France).
G. Faccioli	- Istituto di Patologia Vegetale. Universitá di Bologna. Bologna (Italy).
A.F. Tiburcio	- Unidad de Fisiología Vegetal y Unidad de Bioquímica. Facultad de Farmacia. Universidad de Barcelona. Barcelona (Spain).
M.T. González-Jaén	- Departamento de Genética. Facultad de Biología. Universidad Complutense. Madrid (Spain).
M.H. Harpster	- DNA Plant Technologies Inc. Oakland, Ca. (USA).
M.H.A.J. Joosten	- Department of Phytopathology. Agricultural University. Wageningen (The Netherlands).
J.V. Jorrín Novo	- Departamento de Bioquímica y Biología Molecular. E.T.S.I. Agrónomos. Universidad de Córdoba. Córdoba (Spain).
J. Kallerhoff	- Biosem-Groupe Limagrain. Aubiere (France).
E. Kombrink	- Max-Plank-Institut für Züchtungsforschung. Abteilung Biochemie. Köln (Germany).
H.J.M. Linthorst	- Biochemistry Department. Leiden University. Leiden (The Netherlands).
Y. Meyer	- Laboratoire de Physiologie Végétale. Université de Perpignan. Perpignan (France).
M. Ohshima	- Department of Molecular Biology. National Institute of Agrobiological Resources. Tsukuba, Ibaraki (Japan).
P. Oramas	- Center for Genetic Engineering and Biotechnology. La Habana (Cuba).

T. Palva - Department of Molecular Genetics. Swedish University of Agricultural Sciences. Uppsala (Sweden).

E. Pérez Artés - Departamento de Bioquímica y Biología Molecular. E.T.S.I. Agrónomos. Universidad de Córdoba. Córdoba (Spain).

U.M. Pfitzner - Botanisches Institut der Ludwig-Maximilians Universität and Genzentrum München. München (Germany).

W.S. Pierpoint - Department of Biochemistry. Rothamsted Experimental Station. Harpenden (United Kingdom).

J.A. Pintor Toro - Instituto de Recursos Naturales y Agrobiología. C.S.I.C. Sevilla (Spain).

U. Rasmussen - Agricultural Research Department. Risø National Laboratory. Roskilde (Denmark)

R. Rivera-Bustamante - Centro de Investigaciones y de Estudios Avanzados del I.P.N. Irapuato (Mexico).

D. Roby - Centre de Physiologie Végétale. Université Paul Sabatier. Toulouse (France).

J. Ryals - Ciba-Geigy Biotechnology. Research Triangle Park, N.C. (USA).

J.J. Sánchez-Serrano - Institut für Genbiologische. Forschung Berlin GmbH. Berlin (Germany).

H.L. Sanger - Max-Panck-Institut für Biochemie. Abt. Viroidforschung. Martinsried bei München (Germany).

B. San Segundo - Departamento de Genética Molecular. C.I.D.-C.S.I.C. Barcelona (Spain).

I.E. Somssich - Max-Planck-Institut für Züchtungsforschung. Köln (Germany).

I. Tóbiás - Department of Plant Protection. University of Horticulture Industry. Budapest (Hungary).

S. Tuzun - Department of Plant Pathology. University of Kentucky. Lexington, Ky. (USA).

M.L. Van Buren - Friedrich Kiescher Institute. Basel (Switzerland).

G. Visedo - Unidad de Genética. Facultad de Ciencias. Universidad Autónoma. Madrid (Spain).

K. Wegmann - Institut für Chemische Pilanzenphysiologie. Eberhard-Karls-Universität. Tubingen (Germany)

Fundación Juan March

SERIE UNIVERSITARIA

PUBLISHED TEXTS — Green Series

(Mathematics, Physics, Chemistry, Biology, Medicine)

2 Mulet, A:
Estudio del control y regulación, mediante un calculador numérico, de una operación de rectificación discontinua.

4 Santiuste, J. M.:
Combustión de compuestos oxigenados.

5 Vicent López, J. L.:
Películas ferromagnéticas a baja temperatura.

7 Salvá Lacombe, J. A.:
Mantenimiento del hígado dador in vitro en cirugía experimental.

8 Plá Carrera, J.:
Estructuras algebraicas de los sistemas lógicos deductivos.

11 Drake Moyano, J. M.:
Simulación electrónica del aparato vestibular.

19 Purroy Unanua, A.:
Estudios sobre la hormona Natriurética.

20 Serrano Molina, J. S.:
Análisis de acciones miocárdicas de bloqueantes Beta-adrenérgicos.

22 Pascual Acosta, A.:
Algunos tópicos sobre teoría de la información.

25 I Semana de Biología:
Neurobiología.

26 I Semana de Biología:
Genética.

27 I Semana de Biología:
Genética.

28 Zugasti Arbizu, V.:
Analizador diferencial digital para control en tiempo real.

29 Alonso, J. A.:
Transferencias de carga en aleaciones binarias.

30 Sebastián Franco, J. L.:
Estabilidad de osciladores no sinusoidales en el rango de microondas.

39 Blasco Olcina, J. L.:
Compacidad numerable y pseudocompacidad del producto de dos espacios topológicos.

44 Sánchez Rodríguez, L.:
Estudio de mutantes de saccharomyces cerevisiae.

45 Acha Catalina, J. I.:
Sistema automático para la exploración del campo visual.

47 García-Sancho Martín, F. J.:
Uso del ácido salicílico para la medida del pH intracelular.

48 García García, A.:
Relación entre iones calcio, fármacos ionóforos y liberación de noradrenalina.

49 Trillas, E. y Alsina, C.:
Introducción a los espacios métricos generalizados.

50 Pando Ramos, E.:
Síntesis de antibióticos aminoglicosídicos modificados.

51 Orozco, F. y López-Fanjul, C.:
Utilización óptima de las diferencias genéticas entre razas en la mejora.

52 Gallego Fernández, A.:
Adaptación visual.

55 Castellet Solanas, M.:
Una contribución al estudio de las teorías de cohomología generalizadas.

56 Sánchez Lazo, P.:
Fructosa 1,6 bisfosfatasa de hígado de conejo: modificación por proteasas lisosomales.

57 Carrasco Llamas, L.:
Estudio sobre la expresión genética de virus animales.

59 Alfonso Rodríguez, C. N.:
Efectos magneto-ópticos de simetría par en metales ferromagnéticos.

63 Vidal Costa, F.:
A la escucha de los sonidos cerca de $T\lambda$ en el 4_{He} líquido.

65 Andréu Morales, J. M.:
Una proteína asociada a membrana y sus subunidades.

66 Blázquez Fernández, E.:
Desarrollo ontogénico de los receptores de membrana para insulina y glucagón.

69 Vallejo Vicente, M.:
Razas vacunas autóctonas en vías de extinción.

76 Martín Pérez, R. C.:
Estudio de la susceptibilidad magnetoeléctrica en el Cr_2O_3 policristalino.

80 Guerra Suárez, M.ª D.:
Reacción de amidas con compuestos organoalumínicos.

82 Lamas de León, L.:
Mecanismo de las reacciones de iodación y acoplamiento en el tiroides.

84 Repollés Moliner, J.:
Nitrosación de aminas secundarias como factor de carcinogénesis ambiental.

86 II Semana de Biología:
Flora y fauna acuáticas.

87 II Semana de Biología:
Botánica.

88 II Semana de Biología:
Zoología.

89 II Semana de Biología:
Zoología.

91 Viétez Martín, J. M.:
Ecología comparada de dos playas de las Rías de Pontevedra y Vigo.

92 Cortijo Mérida, M. y García Blanco, F.:
Estudios estructurales de la glucógeno fosforilasa b.

93 Aguilar Benítez de Lugo, E.:
Regulación de la secreción de LH y prolactina en cuadros anovulatorios experimentales.

95 Bueno de las Heras, J. L.:
Empleo de polielectrolitos para la floculación de suspensiones de partículas de carbón.

96 Núñez Álvarez, C. y Ballester Pérez, A.:
Lixiviación del cinabrio mediante el empleo de agentes complejantes.

101 Fernández de Heredia, C.:
Regulación de la expresión genética a nivel de transcripción durante la diferenciación de artemia salina.

103 Guix Pericas, M.:
Estudio morfométrico, óptico y ultraestructural de los inmunocitos en la enfermedad celíaca.

105 Llobera i Sande, M.:
Gluconeogénesis «in vivo» en ratas sometidas a distintos estados tiroideos.

106 Usón Finkenzeller, J. M.:
Estudio clásico de las correcciones radiactivas en el átomo de hidrógeno.

107 Galián Jiménez, R.:
Teoría de la dimensión.

111 Obregón Perea, J. M.ª:
Detección precoz del hiporitoidismo congénito.

115 Cacicedo Egües, L.:
Mecanismos moleculares de acción de hormonas tiroideas sobre la regulación de la hormona tirótropa.

121 Rodríguez García, R.:
Caracterización de lisozimas de diferentes especies.

122 Carravedo Fantova, M.:
Introducción a las orquídeas españolas.

125 Martínez-Almoyna Rullán, C.:
Contribución al estudio de la Manometría ano-rectal en niños normales y con aletraciones de la continencia anal.

127 Marro, J.:
Dinámica de transiciones de fase: Teoría y simulación numérica de la evolución temporal de aleaciones metálicas enfriadas rápidamente.

129 Gracia García, M.:
Estudio de cerámicas de interés arqueológico por espectroscopia Mössbauer.

131 García Sevilla, J. A.:
Receptores opiáceos, endorfinas y regulación de la síntesis de monoaminas en el sistema nervioso central.

132 Rodríguez de Bodas, A.:
Aplicación de la espectroscopia de RPE al estudio conformacional del ribosoma y el tRNA.

136 Aragón Reyes, J. L.:
Interacción del ciclo de los purín nucleóticos con el ciclo del ácido cítrico en músculo esquelético de rata durante el ejercicio.

139 Genís Gálvez, J. M.:
Estudio citológico de la retina del camaleón.

140 Segura Cámara, P. M.:
Las sales de tiazolio ancladas a soporte polimérico insoluble como catalizadores en química orgánica.

141 Vicent López, J. L.:
Efectos anómalos de transporte eléctrico en conductores a baja temperatura.

143 Nieto Vesperinas, M.:
Técnicas de prolongación analítica en el problema de reconstrucción del objeto en óptica.

145 Arias Pérez, J.:
Encefalopatía portosistémica experimental.

147 Palanca Soler, A.:
Aspectos faunísticos y ecológicos de carábidos altoaragoneses.

150 Vioque Cubero, B.:
Estudio de procesos bioquímicos implicados en la abscisión de la aceituna.

151 González López, J.:
La verdadera morfología y fisiología de Azoyobacter: células germinales.

152 Calle García, C.:
Papel modulador de los glucocorticoides en la población de receptores para insulina y glucagón.

154 Alberdi Alonso, M.ª T.:
Paleoecología del yacimiento del Neógeno continental de Los Valles de Fuentidueña (Segovia).

156 Gella Tomás, F. J.:
Estudio de la fosforillasa kinasa de hígado y leucocitos: purificación, características y regulación de su actividad.

157 Margalef Mir, R.:
Distribución de los macrofitos de las aguas dulces y salobres del E. y NE. de España y dependencia de la composición química del medio.

158 Álvarez Fernández-Represa, J.:
Reimplantación experimental de la extremidad posterior en perros.

161 Tomás Ferré, J. M.ª:
Secreción y reutilización de trifosfato de adenosina (ATP) por sinaptosomas colinérgicos.

163 Ferrándiz Leal, J. M.:
Estudio analítico del movimiento de rotación lunar.

164 Rubió Lois, M.; Uriz Lespe, M.ª J. y Bibiloni Rotger, M.ª A.:
Contribución a la fauna de esponjas del litoral catalán. Esponjas córneas.

165 Velasco Rodríguez, V. R.:
Propiedades dinámicas y termodinámicas de superficies de sólidos.

166 Moreno Castillo, I.:
Ciclo anual de zooplancton costero de Gijón.

168 Durán García, S.:
Receptores insulínicos en hipotálamo de rata: localización subcelular y mecanismo(s) de regulación.

169 Martínez Pardo, R.:
Estudio del mecanismo secretor de hormona juvenil en oncopeltus fasciatus.

171 García Jiménez, J.:
Fusariosis del gladiolo: un estudio preliminar.

173 Fernández Aláez, C.:
Análisis estructural en sabinares de la provincia de León.

174 Furio Egea, J.:
Citokinas en agrios. Actividades endógenas, efectos fisiológicos y aplicaciones.

180 Moreno Rodríguez, J. M.:
Estudios ecológicos en jarales (cistion laurofilii): Variación anual de algunos factores del entorno y manifestaciones fenológicas.

182 Pons Vallés, M.:
Estudios espectroscópicos de fosfolípidos polimerizables.

183 Herrero Ruiz de Loizaga, V. J.:
Estudio de reacciones químicas por haces moleculares. Aplicación a la reacción $C_2H_5Br + K$ $Brk + C_2H_5$.

193 Martín García, V. S.:
Utilización sintética en química orgánica de metales pesados como catalizadores. Oxidación asimétrica.

195 Badía Sancho, A.:
Receptores presinápticos en el conducto deferente de rata.

196 Estévez Toranzo, A.:
Supervivencia de patógenos bacterianos y virales de peces en sistemas de cultivo.

197 Lizarbe Iracheta, M.ª A.:
Caracterización molecular de las estructuras de colágeno.

203 López Calderón, I.:
Clonación de genes de «Saccharomyces cerevisiae» implicados en la reparación y la recombinación.

211 Ayala Serrano, J. A.:
Mecanismo de expresión de la PBP-3 de «E. coli»: Obtención de una cepa hiperproductora de la proteína.

240 **Genetic Strategies in Development.**
Symposium in honour of Antonio García Bellido. Lectures by S. Ochoa, S. Brenner, G. S. Stent, E. B. Lewis, D. S. Hogness, E. H. Davidson, J. B. Gurdon y F. Jacob.

244 **Course on Genome Evolution.**
Organized by E. Viñuelas. Lectures by R. F. Doolittle, A. M. Weiner/N. Maizels, G. A. Dover, J. A. Lake, J. E. Walker, J. J. Beintema, A. J. Gibbs, W. M. Fitch, P. Palese, G. Bernardi y J. M. Lowenstein.

246 **Workshop on tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco y P. Marrack.

Established in 1955,
the Juan March Foundation is a non-profit organization
dedicated to cultural,
scientific and charitable objectives.
It is one of the most important in Europe,
considering its endowment and activities.

The Juan March Foundation has collaborated
significantly in the area of science and research by
funding awards, grants and scholarships
for scientific training,
supporting research studies
and publishing research results.

Since 1970, the Foundation
has dedicated special attention to the field of Biology
by means of various programmes,
such as the Plan on Molecular Biology
and its Applications (1981-88)
and at present the Programme of
International Meetings on Biology,
designed to actively promote the relationship
between Spanish biologists and their international colleagues