European Journal of Plant Pathology **101:** 579–583, 1995.

Meeting report

Molecular genetics of fungal plant pathogens and signal perception and transduction in plant-fungus interactions – Workshop of two human capital and mobility networks

Guy Honée[1] and Thorsten Nürnberger[2]
[1] *Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands;* [2] *Institut für Pflanzenbiochemie, Weinberg 3, 06120 Halle, Germany*

Accepted 16 August 1995

A joint workshop of two Human Capital and Mobility networks on 'Molecular Genetics of Fungal Plant Pathogens: Perspectives for Molecular Breeding' and 'Signal Perception and Transduction in Plant-Fungus Interactions' was organized by Pierre Ricci in Antibes, France, from 10 to 13 April, 1995.

Nine research groups associated in the HC&M network 'Molecular Genetics of Fungal Plant Pathogens: Perspectives for Molecular Breeding' reported on their strategies to gain molecular insight in pathogenicity and specificity on fungal plant pathogens. Reverse genetics has been the most successful strategy, so far, to isolate pathogenicity and avirulence genes. In Wolgang Knogge's group (Cologne) three *n*ecrosis *i*nducing *p*roteins (NIP's) have been isolated from the barley pathogen *Rhynchosporium secalis* [Wevelsiep *et al.*, 1991]. Based on the N-terminal amino acid sequence degenerated oligonucleotides were synthesised and were used to PCR-amplify a fragment which was subsequently used as a probe to isolate the *nip1* gene encoding the NIP1 protein [Knogge *et al.*, 1994]. Transformation of a strain virulent on *Rrs1* plants, with the *nip1* gene resulted in an avirulent strain and vice versa disruption of the *nip1* gene resulted in a strain virulent on *Rrs1* plants. This showed that the *nip1* gene is the *avrRrs1* avirulence gene. This finding illustrates the theory that a plant is able to identify the presence of a fungus by a recognition of a virulence factor. Homologs of the *avrRrs1* gene, that differ in three nucleotide residues resulting in proteins containing three different amino acid residues, have been found in races avirulent on *Rrs1* plants. Races virulent on *Rrs1* plants carry either a different base pair substitution leading to a different amino acid residue, or lack the *nip1* gene. These observations are reminiscent of the first fungal avirulence genes cloned, *Avr4* and *Avr9*, from the tomato pathogen *Cladosporium fulvum* in De Wit's lab (Wageningen, The Netherlands) [Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1992]. However, no point mutations have been found in the *Avr4* and *Avr9* genes from races avirulent on tomato genotypes Cf4 and Cf9, respectively. Races virulent on Cf9 tomato plants lack the *Avr9* gene, whereas races virulent on Cf4 plants carry a point mutation in the coding sequence of *Avr4* gene. Although during infection all the avr4 alleles of races virulent on Cf4 genotype are transcribed, only one of the proteins could be detected in apoplastic fluid by western blot analysis (Matthieu Joosten, Wageningen, The Netherlands).

Purification of two extracellular proteins (ECP's) present in all compatible interactions between different *C. fulvum* races and tomato genotypes has resulted in the cloning of the corresponding genes, *ecp1* and *ecp2* [Van den Ackerveken *et al.*, 1993]. To address the question whether these genes play a crucial role during infection, mutants of *C. fulvum* missing either a functional *ecp1* or a functional *ecp2* gene were generated. The two different mutants showed no apparent disturbance of pathogenicity on seedlings. However, on adult plants both disruptants showed clearly altered phenotypes and a reduced spore production, leading to a reduction in their pathogenic abilities (Richard Laugé, Wageningen, The Netherlands). Based on these observations, *ecp1* and *ecp2* can be considered as pathogenicity genes, although their precise role in the infectious life cycle of *C. fulvum* remains to be elucidated.

The *ecp1*, *ecp2*, *Avr4*, and *Avr9* genes are only expressed when the fungus grows *in planta*. *In vitro* expression of the *Avr9* gene can be induced at low

nitrogen concentrations [Van den Ackerveken *et al.*, 1994]. The promoter of the *Avr9* gene contains consensus sequences resembling recognition sites of the *NIT2* protein of *Neurospora crassa* and the *AREA* protein of *Aspergillus nidulans*. Both transcription factors act as positive regulator proteins under nitrogen limitation. In the group of Henk Van Den Broek (Wageningen) the function of these consensus sequences for gene expression in planta are studied by deletion and mutation analyses.

Based on the finding that several fungal genes, like *Avr9*, hydrophobin genes of *Magnaporthe grisea* [Talbot *et al.*, 1993] and *ipi* genes of *Phytophthora infestans* [Pieterse *et al.*, 1994] are starvation-induced, Richard Oliver *et al.* (Norwich, UK) screened differentially cDNA libraries for starvation-induced genes which may play a role during infection. So far, four fungal genes were isolated encoding an alcohol dehydrogenase, an aldehyde dehydrogenase, an ATP-ADP translocator and a ribosomal protein. Although this is a tempting strategy the feasibility is not clear yet.

The fungal proteins and corresponding genes mentioned above were identified following a non-biased approach. An alternative approach is isolation of fungal proteins and their encoding genes based on their postulated role during infection: for instance, polygalacturonases from *Fusarium oxysporum* (Isabel Roncero *et al.*, Cordoba, Spain) and *Fusarium moniliforme* (Felice Cervone *et al.*, Rome, Italy), or other cell wall degrading enzymes like cellulase and xylanase from *Claviceps purperea* (Paul Tudzynski *et al.*, Münster, Germany), or plant saponin detoxifying enzymes from *Septoria avenae* (Anne Osbourn, Norwich, UK). Recently, it has been demonstrated that detoxification of oat root saponins (avenacins), by avenacinase is a prerequisite for *Gaeumannomyces graminis* var. *avenae* to be able to infect oat roots [Bowyer *et al.*, 1995] and based on this it is anticipated that the leaf pathogen *S. avenae* produces an extracellular protein detoxifying saponins of oat leaves. Initial studies showed that only oat adapted isolates of *S. avenea* were competent to grow on medium containing oat leaf extracts (Jos Wubben, Norwich, UK).

In recent years, the use of transposons has shown to be a successful tool for isolating specific plant genes including resistance genes. In fungi however, the use of transposons is rather unexplored. Several groups reported on the presence of mobile DNA in fungal genomes. In the lab of Marie-Josée Daboussi (Paris, France) four families of transposons have been identified in the genome of *Fusarium oxysporum*. *Fot1* represents one of these families [Daboussi *et al.*, 1992] and contains a transcription unit of 1.6 kb, encoding for a polypeptide of 542 amino acid residues possibly representing a transposase. Studies are under way to acquire autonomously acting *Fot1* copies. From *Aspergillus niger* a 4.8 kb transposon named *Ant-1* has been identified by Richard Oliver *et al.* (Norwich, UK). Since isolation includes a trapping approach, like insertion in a nitrate reductase gene, these transposons might be autonomous. A retroelement 'skippy' (skp) of 8 kb has been isolated from *F. oxysporum* f. sp. *lycopersici* by Isabel Roncero *et al.*, and shows structural features resembling the 'gypsy' family of LTR-retrotransposons [Marlor *et al.* 1986]. When it has been proven that these mobile elements act autonomously in homologous or heterologous systems they will form attractive tools for fungal gene tagging.

In an alternative approach, genes can be tagged by random insertional mutagenesis using an integrative plasmid. In Michel Dron's research group (Paris, France) four out of six hundred *Colletotrichum lindemuthianum* transformants were found that showed a reduced pathogenicity on bean. In three of these four transformants complex integrations were found, whereas in one transformant only one copy of the integrated plasmid was identified. Analysis of the flanking sequences and cloning of the disrupted gene will reveal which gene is important for pathogenicity of *C. lindemuthianum* and will show the feasibility of this strategy. By mutagenesis, the group of Ben Cornelissen (Amsterdam, The Netherlands) will try to isolate the avirulence gene *avr2* from *F. lycopersici* f. sp. *lycopersici*. They have demonstrated that the interaction of this fungus with its host, tomato, complies with the gene-for-gene model. An *avr2*-containing strain will be chemically mutagenized and mutants will be screened for virulence on tomato containing resistance gene *I-2*.

Contributions of the seven research groups associated in the HC&M Network on 'Signal perception and transduction in plant/fungus interactions' revealed significant progress particularly in the field of fine structure analysis of fungal elicitors as well as in the identification and purification of elicitor-binding proteins from plant plasma membranes. High-affinity receptors for either type of fungal elicitor have been reported to reside in plant plasma membranes by essentially all groups within the network. Furthermore, experimental strategies combining cloning and purification approaches aimed at the isolation of yet unavail-

able elicitor receptors and the corresponding genes were presented.

A very valuable result was presented by Jürgen Ebel *et al.* (München, Germany) who have purified to apparent homogeneity a 70-kDa hepta-ß-glucoside-binding protein from soybean plasma membranes by means of affinity chromatography. The ß-glucans shown to be released from *Phytophthora sojae* zoospores early during germination are elicitors of phytoalexin production in soybean cotyledons. High-affinity binding of both a ß-glucan fraction derived from the fungal cell wall (Kd=37 nM) and a synthetic hepta-ß-glucoside (Kd=3 nM) to soybean membranes has been demonstrated [Cosio *et al.*, 1990]. Furthermore, by photoaffinity labelling, a 70-kDa plasma membrane protein was identified to be a major component of the ß-glucan binding site [Cosio *et al.*, 1992]. Hence, the ligand binding characteristics of the purified 70-kDa protein and its recognition by the photoaffinity label clearly argue for it being the elicitor receptor.

Eric Cosio (Madrid, Spain) reported on the existence of possibly homologous phytoalexin elicitor binding sites within the plant family, *Fabaceae*, which may provide preliminary evidence for evolutionary relationships in pathogen perception mechanisms in plants. Using a radioiodinated aminophenylethylamine conjugate of a glucan fraction derived from mycelial walls of *Phytophthora sojae*, high-affinity binding sites were found in membranes from three legume species, pea, bean and lupin (IC50=5–20 nM). Lower or no binding activity was detected in membranes of chickpea or broad bean. In all species tested, ß-glucan binding activity correlated well with the ability of these elicitors to stimulate accumulation of isoflavonoid phytoalexins. Most importantly, radioligand displacement with unlabeled hepta-ß-glucoside revealed very similar affinities of the pea and lupin binding sites towards this ligand as was found for soybean, indicating recognition of identical structural motifs by all three binding sites. Karl Baureithel (Basel, Swiss) described that suspension-cultured tomato cells possess a very sensitive perception system for chitin fragments, especially for those with a chain length of four and longer, to which they respond by increasing the pH of the culture medium. Using 35S-Met-labeled chitopentaose, saturable high-affinity binding sites for the ligand in both whole cells and microsomal membranes could be demonstrated (Kd=1.4 nM and 23 nM, respectively [Baureithel *et al.*, 1994]). The binding abilities of structural analogs of this ligand, including Nod factor (a lipochitooligosaccharide from *Rhizobium leguminosarum*) closely resembled their abilities to induce the alkalinization response. In an attempt to enrich chitin-binding proteins detergent-solubilized proteins from tomato membranes were incubated with immobilized chitin fragments. The very few proteins specifically eluted from this affinity matrix proved the usefulness of this approach which may soon result in the isolation of an elicitor receptor. Other characteristic fungal molecules such as N-linked glycopeptides with fungal-type mannosyl linkages [Basse *et al.*, 1993] or ergosterol [Granado *et al.*, 1995] are perceived by cultured tomato cells at subnanomolar concentrations. As Thomas Boller (Basel, Swiss) pointed out, some of these perception systems for 'non-self' molecules undergo rapid desensitization, leading to a refractory state reminiscent of smell perception. Furthermore, systemin, a molecule implicated in wound signalling in tomato, stimulated the same physiological responses as the other stimuli, including extracellular alkalinization and ethylene biosynthesis [Felix *et al.*, 1995]. Microbial signals as well as systemin induced similar changes in the pattern of phosphorylated proteins. These changes were sensitive to protein kinase inhibitors. In turn, protein phosphatase inhibitors were shown to induce these changes even in the absence of the stimuli mentioned, indicating that phosphorylation/dephosphorylation events are involved in the transduction of microbial signals as well as systemin.

Pierre Ricci (Antibes, France) described a class of protein elicitors, the elicitins, that are secreted by most *Phytophthora* species and induce a hypersensitive response as well as systemic acquired resistance (SAR) in tobacco [Ricci *et al.*, 1993]. Application of cryptogein to whole plants leads to the formation of large necrotic lesions and renders the plant resistant to subsequent *Phytophthora* infection. Transcriptional activation of a series of SAR-related genes in these plants suggests that activation of these genes may be related to both onset of resistance and necrosis formation (Harald Keller, Antibes, France). Elegant experiments using tobacco plants transformed with a bacterial salicylate hydroxylase gene [Gaffney *et al.*, 1993] revealed that elicitin treatment of these plants did not result in SAR but led to enhanced susceptibility to infection with elicitin-producing *Phytophthora* strains. Lesion formation by cryptogein remained unaffected in these transformants, indicating that salicylic acid is required for SAR but is not essential for lesion formation. Alain Pugin and co-workers (Dijon, France) presented studies on cryptogein signalling in cul-

tured tobacco cells. Using radioiodinated elicitor, they identified a single-class high-affinity binding site in tobacco plasma membrane preparations. Binding was saturable and reversible. The pattern of responses rapidly induced after elicitor treatment includes membrane depolarization, influxes of H+ and Ca2+, K+-efflux, cytosolic acidification [Blein *et al.*, 1991], the production of active oxygen species as well as phytoalexin production. Depletion of extracellularly available Ca2+ as well as use of Ca-channel or protein kinase inhibitors blocked phytoalexin production, indicating the involvement of Ca-dependent and phosphorylation events in cryptogein signalling.

As described before, recognition of the race-specific polypeptide elicitor, AVR9, from *Cladosporium fulvum* leads to a hypersensitive response in tomato cultivars carrying the complementary resistance gene *Cf9* [Scholtens-Toma and De Wit, 1988]. The AVR9 elicitor binds to membrane preparations from different tomato cultivars in an apparently resistance-gene-unspecific manner (Miriam Kooman-Gersmann, Wageningen, The Netherlands). Using radioiodinated AVR9 as ligand, a single-class binding site could be detected in plasma membranes from both resistant and susceptible genotypes. Binding was specific, saturable and reversible. The affinity constant of this binding event was in the low picomolar range. Interestingly, similar binding characteristics of AVR9 to membrane preparations from tobacco, another solanaceous plant were found. 1H-NMR structure analysis of AVR9 revealed a compact, barrel-like structure of the peptide elicitor consisting of three anti-parallel ß-sheets and three disulphide bridges. Inoculation of tomato plants carrying the *Cf9* gene with potato virus X transformed with mutated versions of the *Avr9* gene led to the expression of AVR9 structural analogs with altered elicitor activity (Ralph Vogelsang, Wageningen, The Netherlands). In all but one case, single amino acid exchanges resulted in lowered necrosis-inducing activity of the AVR9 derivative. It will be interesting to see whether the elicitor activity of these analogs parallels their ability to compete for binding of native AVR9 to its receptor.

Dierk Scheel (Halle, Germany) presented the identification of an 13-mer oligopeptide (Pep-13) within a 42-kDa glycoprotein elicitor from *Phytophthora sojae* that was found to be necessary and sufficient to stimulate a complex defense response in parsley cells comprising H+/Ca2+ influxes, K+/Cl-effluxes, an oxidative burst, an increase in the endogenous jasmonic acid concentration, defense-related gene activation and phytoalexin formation. A single-class high-affinity binding site in parsley microsomal membranes and protoplasts has recently been described [Nürnberger *et al.*, 1994]. By chemical crosslinking, a 91-KDa parsley plasma membrane protein was identified to be the receptor of the peptide elicitor [Nürnberger *et al.*, 1995]. Identical structural features of Pep-13 were found to be responsible for specific binding and initiation of all plant responses analyzed, indicating a functional link between the various plant responses to elicitor treatment. Use of a series of ion channel inhibitors as well as inhibitors of the oxidative burst revealed that the observed ion fluxes and active oxygen species were necessary for defense-related gene activation and subsequent phytoalexin formation. Furthermore, the same ion channel inhibitors were found to efficiently inhibit the elicitor-induced oxidative burst. These findings allow the establishment of a sequence of events that may constitute part of a signalling cascade triggering pathogen defense in plants.

Several approaches are followed to dissect fungal pathogenicity and avirulence. Fungal transformation systems are developed and ploidy levels are evaluated to be able to define, by transformation and gene disruption, the role of a particular gene in the infection process. Furthermore, unraveling the molecular mechanisms in the plant underlying the initiation of defense reactions mounted against invading microorganisms is in progress. It can be expected that elucidation of fungal gene functions, and newly identified genes and proteins, together with new insight in how plants sense the presence of potential pathogens and induce defense responses, will be presented in next year's meeting which will be held in Cordoba.

References

Basse CW, Fath A and Boller T (1993) High affinity binding of a glycopeptide elicitor to tomato cells and microsomal membranes and displacement by specific glycan suppressors. J Biol Chem 268: 14724–14731

Baureithel K, Felix G and Boller T (1994) Specific, high affinity binding of chitin fragments to tomato cells and membranes. J Biol Chem 269: 17931–17938

Blein J-P, Milat M-L and Ricci P (1991) Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*: possible plasmalemma involvement. Plant Physiol 95: 486–491

Bowyer P, Clarke BR, Lunness P, Daniels MJ and Osbourn AE (1995) Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science 267: 371–374

Cosio E G, Frey T, Verduyn R, van Boom J and Ebel J (1990) High-affinity of synthetic heptaglucoside and fungal glucan

phytoalexin elicitors to soybean membranes. FEBS Lett 271: 223–226

Cosio E, Frey T and Ebel J (1992) Identification of a high-affinity binding protein for a hepta-ß-glucoside phytoalexin elicitor in soybean. Eur J Biochem 204: 1115–1123

Daboussi MJ, Langin T and Brygoo Y (1992) *Fot1*, a new family of transposable elements. Mol Gen Genet 232: 12–16

Ebel J and Cosio EG (1994) Elicitors of plant defence responses. Int Rev Cytol 148: 1–36

Felix G and Boller T (1995) Systemin induces rapid ion fluxes and ethylene biosynthesis in *Lycopersicon peruvianum* cells. Plant J. 7: 381–189

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H and Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754–756

Granado J, Felix G and Boller T (1995) Perception of sterols in plants: subnanomolar concentrations of ergosterol elicit extracellular alkalinization in tomato cells. Plant Physiol 107: 485–490

Joosten MHAJ, Cozijnsen TJ and De Wit PJGM (1994) Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. Nature 367: 384–386

Knogge W, Gierlich A, Hermann H, Wernert P and Rohe M (1994) Molecular identification and characterization of the *NIP1* gene, an avirulence gene from the barley pathogen *Rhynchosporium secalis*. In: Daniels MJ, Downie JA, Osbourn (eds) Advances in Molecular Genetics of Plant-Microbe Interactions Vol. 3 (pp. 207–214) Kluwer Academic Publishers, Dordrecht

Marlor RL, Parkhurst SM and Corces VG (1986) The *Drosophila melanogaster* gypsy transposable element encodes putative gene products homologous to retroviral proteins. Mol Cell Biol 6: 1129–1134

Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K and Scheel D (1994) High affinity binding of a fungal oligopeptide elicitor to plasma membranes triggers multiple defence responses Cell 78: 449–460

Nürnberger T, Nennstiel D, Hahlbrock K and Scheel D (1995) Covalent cross-linking of the *Phytophthora megasperma* oligopeptide elicitor to its receptor in parsley membranes. Proc Natl Acad Sci USA 92: 2338–2342

Scholtens-Toma IMJ and De Wit PJGM (1988) Purification and primary structure of a necrosis-inducing peptide from the apoplastic fluids of tomato infected with *Cladosporium fulvum* (syn. *Fulvia fulva*). Physiol Mol Plant Pathol 33: 59–67

Pieterse CMJ, Derksen AMCE, Folders J and Govers F (1994) Expression of the *Phytophthora infestans ipiB* and *ipiO* genes in planta and *in vitro*. Mol Gen Genet 244: 269–277

Ricci P, Panabieres F, Bonnet P, Maia M, Ponchet M, Devergne J-C, Marais A, Cardin L, Milat M-L and Blein J-P (1993) Proteinaceous elicitors of plant defence responses. In: Fritig B and Legrand M (eds) Mechanisms of plant defense responses (pp. 121–135) Kluwer Academic Publishers, Dordrecht

Talbot NJ, Ebbole DJ and Hamer JE (1993) Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. The Plant Cell 5: 1575–1590

Van Den Ackerveken GFJM, Van Kan JAL and De Wit PJGM (1992) Molecular analysis of the avirulence gene *avr9* of the tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. Plant J 2: 359–366

Van Den Ackerveken GFJM, Van Kan JAL, Joosten MHAJ, Muisers JM, Verbakel HM and De Wit PJGM (1993) Characterization of two putative pathogenicity genes of the fungal tomato pathogen *Cladosporium fulvum*. Mol Plant-Microbe Interact 6: 21–215

Van den Ackerveken GFJM, Dunn RM, Cozijnsen TJ, Vossen PJ, Van den Broek HWJ and De Wit PJGM (1994) Nitrogen limitation induces expression of the avirulence gene *Avr9* in the tomato pathogen *Cladosporium fulvum*. Mol Gen Genet 243: 243– 277

Wevelsiep L, Kogel KH and Knogge W (1991) Purification and characterization of peptides from *Rhynchosporium secalis* inducing necrosis in barley. Physiol Mol Plant Pathol 39: 471–482