Dissection of Nonhost Resistance of *Nicotiana* to *Phytophthora infestans*

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Specific recognition events, defined by the perception of pathogen elicitors by plant receptors, trigger defense responses including the hypersensitive response (HR), a form of programmed cell death in plants. *Phytophthora infestans*, an oomycete plant pathogen, causes late blight of potato and tomato, a devastating disease of worldwide economical importance. In contrast to the host plants, nonhosts, such as tobacco and other species of the genus *Nicotiana*, are resistant to *P. infestans*. A family of extracellular protein elicitors, termed elicitins, has been identified in *P. infestans* and other *Phytophthora* species and evidence has accumulated for a role of these molecules in delimiting the host-range of *Phytophthora*. *P. infestans* elicitins, known as INF elicitins, induce the HR in a restricted number of plants, particularly in the genus *Nicotiana* within the Solanaceae. Our studies aim at determining the extent to which nonhost resistance of *Nicotiana* to *P. infestans* is mediated by the recognition of elicitins.

RESISTANCE OF *NICOTIANA* TO *P. INFESTANS* IS ALWAYS ASSOCIATED WITH THE HYPERSENSITIVE RESPONSE

Cytological analyses of leaves of several *Nicotiana* species inoculated with *P. infestans* showed that penetration of an epidermal cell by a germinating cyst always occurred (Kamoun et al. 1998). This was followed by the HR that varied between different *Nicotiana* species in timing, severity and number of affected cells. In *Nicotiana tabacum* (tobacco), *P. infestans* was blocked early in the infection following penetration of the epidermal cell and secondary intercellular hyphae were never observed. In contrast, in *Nicotiana benthamiana*, secondary hyphae with haustoria were formed and some level of mesophyll colonisation

occurred. The plant response reached a climax three days post inoculation; clusters of HR cells engulfing the invading hyphae were formed and this correlated with the cessation of further *P. infestans* ingress. These observations suggest that several layers of resistance to *P. infestans* occur with various degrees of effectiveness in different *Nicotiana* species.

INF ELICITINS FORM A DIVERSE FAMILY OF PROTEINS IN *P. INFESTANS*

In *P. infestans*, in addition to the canonical elicitin INF1, a complex family of elicitin-like proteins has been revealed (Fig. 1). Elicitin-like genes were isolated from *P. infestans* using PCR amplification with degenerate primers, low stringency hybridisation techniques, and random sequencing of cDNAs (Kamoun et al. 1997a, b, 1999b). In total, seven elicitin and elicitin-like genes have now been identified in *P. infestans.* All these genes encode putative extracellular proteins that share the 98 amino-acid elicitin domain corresponding to the mature INF1. Five *inf* genes (*inf2A*, *inf2B*, *inf5*, *inf6*, and *inf7*) encode predicted proteins with a C-terminal domain in addition to the N-terminal elicitin domain. Sequence analysis of these C-terminal domains shows a high frequency of serine, threonine, alanine, and proline. The amino-acid composition and the distribution of these four residues suggest the presence of clusters of O-linked glycosylation sites (Kamoun et al. 1997a). These proteins may form a 'lollipop on a stick' structure in which the O-glycosylated domain forms an extended rod that anchors the protein to the cell wall leaving the extracellular N-terminal domain exposed on the cell surface (Jentoft 1990). Therefore, these atypical INF proteins may be surface or cell wall associated glycoproteins that interact with plant cells during infection.

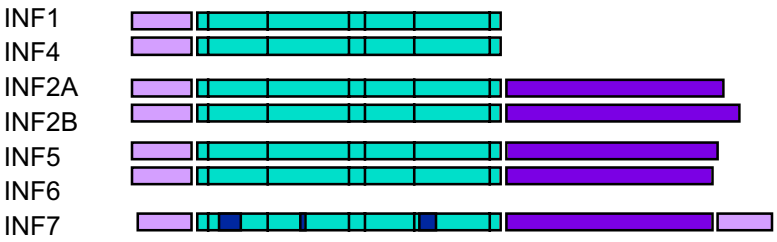

Figure 1. Schematic illustration of the INF elicitin family of *P. infestans*. The four main domains correspond to the signal peptide, elicitin domain, C-terminal domain, and transmembrane domain, respectively. Vertical bars refer to the conserved cysteine residues. Black boxes in the INF7 elicitin domain refer to deletions.

FUNCTIONAL ANALYSES OF INF ELICITOR ACTIVITY USING POTATO VIRUS X

Functional expression of *inf* genes in plants was conducted using a recombinant Potato Virus X (PVX) vector (Kamoun et al. 1999a). A recombinant PVX-*inf1* construct encoding a fusion between the signal peptide of the *PR-1a* gene of tobacco and the mature 98 amino-acid INF1 elicitin induced localized necrotic lesions on inoculated tobacco leaves three days after inoculation and showed no systemic symptoms during the three weeks following inoculation. This indicates that delivery of INF1 in the plant via the PVX vector results in the HR and that *inf1* confers avirulence to PVX on tobacco. Other PVX-*inf* constructs triggered a number of responses on *Nicotiana* including local HR, as well as mild and severe systemic HR lesions, allowing a quantitative evaluation of the response of *Nicotiana* to the various INF elicitins.

FUNCTIONAL ANALYSES OF INF ELICITOR ACTIVITY USING *AGROBACTERIUM* TRANSIENT TRANSFORMATION ASSAYS

Functional expression of *inf* genes in plants was also conducted using an *Agrobacterium tumefaciens* transient transformation assay. A chimaeric gene encoding a fusion between the signal peptide of the *PR-1a* gene of tobacco and INF1 elicitin driven by the 35S promoter of the cauliflower mosaic virus was cloned into an *A. tumefaciens* binary vector. Culturing of *A. tumefaciens* (35S-*inf1*) under inducing conditions and injection into tobacco leaves resulted in the HR. Other *inf* genes are also being used in similar assays resulting in an additional evaluation of the response of *Nicotiana* to INF elicitins.

INF1 DEFICIENT STRAINS OF *P. INFESTANS* INDUCE DISEASE LESIONS ON *NICOTIANA BENTHAMIANA* BUT NOT ON OTHER *NICOTIANA* SPECIES

To engineer *P. infestans* strains deficient in the production of INF1, strain 88069 was co-transformed with a construct containing *inf1* in an antisense orientation, and a geneticin resistance plasmid. Six out of the thirty antisense co-transformants failed to produce detectable levels of INF1 protein and *inf1* mRNA indicating that the *inf1* gene was silenced (Kamoun et al. 1998; van West et al. 1999). In contrast to the wild-type and control transformants, the engineered INF1 deficient strains produced disease lesions with profuse sporulation on *N. benthamiana*. Interestingly, these mutants remained unable to infect other *Nicotiana* species such as tobacco. This indicates that resistance to *P. infestans* in *N. benthamiana* is mainly triggered by INF1, whereas the early resistance reaction observed in

tobacco is not. Possibly, prior to recognition of INF1, tobacco responds to additional host-specific elicitors that are not detected by *N. benthamiana*. Functional analyses of INF elicitins as well as construction of additional *P. infestans* strains deficient in INF production will help test this hypothesis.

DISSECTION OF NONHOST RESISTANCE OF *NICOTIANA*

Using a gene silencing approach we demonstrated that a pathogen protein elicitor that induces the HR on nonhost plants can function as an avirulence factor. We showed that INF1 is the main avirulence determinant in *P. infestans* in its interaction with *N. benthamiana*. However, several observations suggest that nonhost resistance of *Nicotiana* to *P. infestans* may be complex and diverse: (i) The timing, severity, and extent of the HR following inoculation with *P. infestans* vary considerably depending on the *Nicotiana* genotype. (ii) *Nicotiana* species other than *N. benthamiana* remain resistant to INF1 deficient *P. infestans.* (iii) A complex family of *inf1*-related genes is expressed by *P. infestans in planta*. (iv) Differences in temporal and spatial patterns of *inf* expression and INF protein distribution *in planta* occur. (v) Evaluation of INF recognition by *Nicotiana* using PVX and *Agrobacterium* expression systems demonstrated that INF elicitins show different specificities and strength in inducing the HR on *Nicotiana*.

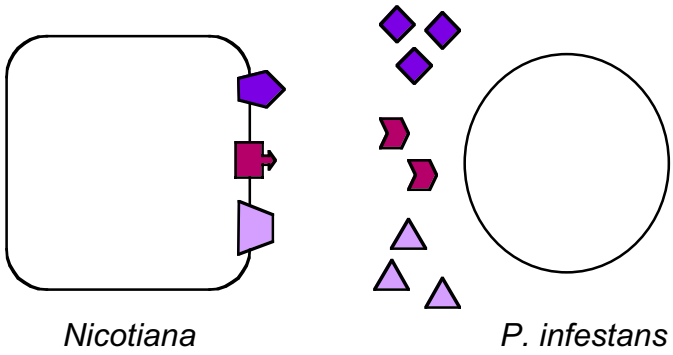

Figure 2. "Genes for genes" model illustrating our current understanding of nonhost resistance of *Nicotiana* to *P. infestans*. This model is based on the hypothesis that multiple *R* genes in *Nicotiana* interact with multiple elicitors produced by *P. infestans* and is sufficient to explain nonhost resistance in this pathosystem. Qualitative and quantitative evaluation of each elicitor interaction with various plant genotypes should allow the dissection of nonhost resistance of *Nicotiana* into specific components.

An improved understanding of the molecular basis of nonhost resistance should lead to novel perspectives for engineering durable disease resistance in crop plants (Kamoun et al. 1999c). Recognition of species-specific elicitors, such as INF elicitins, by an arsenal of *R* genes and/or recognition

of essential pathogen factors could form the basis of nonhost resistance of *Nicotiana* to *P. infestans* (Fig. 2). Our results suggest that multiple layers of INF recognition and late blight resistance occur in *Nicotiana*. Our aim is to achieve a qualitative and quantitative dissection of these layers of nonhost resistance into specific *R* gene-*inf* elicitin interactions by using a combination of *in planta* functional assays of INF elicitor activities, analyses of abundance and localization of *inf* transcripts and INF proteins, and construction of *P. infestans* strains deficient in INF proteins.

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