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Interactions between tobacco mosaic virus and the tobacco *N* gene

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The interaction between tobacco mosaic virus (TMV) and tobacco harbouring the *N* gene is a classical system for studying gene-for-gene interactions in disease resistance. The *N* gene confers resistance to TMV by mediating defence responses that function to limit viral replication and movement. We isolated the *N* gene and determined that *N* belongs to the nucleotide-binding-site–leucine-rich-repeat (NBS–LRR) class of plant disease resistance genes, and encodes both full-length and truncated proteins. Sequence homologies and mutagenesis studies indicated a signalling role for the N protein similar to that seen for proteins involved in defence responses in insects and mammals. The *N* gene confers resistance to TMV in transgenic tomato, demonstrating the use of the NBS–LRR class of disease resistance genes in engineering crop resistance. From the pathogen side of this interaction, the TMV 126 kDa replicase protein has been implicated as the avirulence factor that triggers *N*-mediated defence responses. We employed *Agrobacterium*-mediated expression strategies to demonstrate that expression of the putative helicase region of the replicase protein is sufficient to elicit *N*-mediated defences. The thermosensitivity of the *N*-mediated response to TMV is retained when induced by expression of this replicase fragment. Thus, both components of this gene-for-gene interaction are now available for studies that address the molecular mechanisms involved in *N*-mediated TMV resistance.

Keywords: tobacco mosaic virus; *N* gene; plant disease resistance; gene-for-gene disease resistance; crop genetic engineering; plant–pathogen interactions

1. INTRODUCTION

Plants interact with multitudes of microorganisms, and while many of these interactions are beneficial, some lead to disease. Plants protect themselves from pathogenic microbes with both preformed defences and inducible defences. When pathogens defeat preformed barriers, such as outer coats of wax that resist pathogen penetration, inducible defences are triggered by the recognition of specific pathogen-derived molecules. One well-studied inducible response to pathogen attack is localized cell death at the site of infection, termed the hypersensitive response (HR). This cellular suicide response apparently functions to limit pathogen proliferation and is thought to be similar to programmed cell death observed in mammals, worms, and insects (Greenberg 1997). The HR has been correlated with a variety of cellular changes including an oxidative burst that generates reactive oxygen intermediates, ion fluxes, protein phosphorylation, increases in lipoxygenase activity, production of anti-microbial compounds such as phytoalexins, lignin deposition, and the expression of defence-related genes (Dixon *et al*. 1994). The precise role of these processes in the execution or regulation of the HR is not clear.

Induction of the HR depends on the expression of a dominant or semi-dominant resistance (*R*) gene in the plant and a corresponding dominant avirulence (*Avr*) gene in the microbe (Flor 1971). This 'gene-for-gene' model of plant–pathogen interaction accurately accounts for the outcome of numerous plant–pathogen encounters. *R* genes are postulated to encode receptors that, upon binding to their cognate *Avr* gene products, elicit defence responses (Keen & Dawson 1992). If either the plant or pathogen partner lacks a functional allele of the corresponding gene pair, then resistance is not triggered, and the plant becomes diseased. Plants and pathogens can harbour a number of corresponding pairs of *R* and *Avr* genes that interact to provide signals for the induction of disease resistance (Keen 1990).

A classical model system for the study of gene-for-gene disease resistance is the interplay between tobacco mosaic virus (TMV) and tobacco plants bearing the *N* gene was first described by F. O. Holmes (Holmes 1938). The *N* gene from *Nicotiana glutinosa* confers resistance to all known tobamoviruses except Ob (Culver *et al*. 1991; Dawson & Hilf 1992; Tobias *et al*. 1982; Csillery *et al*. 1983). TMV spreads systemically in tobacco cultivars lacking the *N* gene, causing mosaic disease symptoms characterized by intermingled areas of light- and dark-green leaf tissue. In contrast, TMV infection of *N*-containing tobacco induces the HR within 48 hours of

infection, and virus particles are restricted to the region immediately surrounding the induced necrotic lesions. In addition, TMV-induced HR is accompanied by systemic acquired resistance (SAR) (Ryals *et al.* 1996), which provides non-specific resistance to a wide range of pathogens throughout the plant that can last for days (Hecht & Bateman 1964; Lamb *et al.* 1989; Lamb 1994). The actual mechanisms involved in activating the HR, the induction of SAR, and the prevention of virus spread have not been established.

Our working model for N function proposes that a receptor complex, containing the N protein, binds a TMV-derived ligand (the elicitor). The elicitor-bound receptor then activates the HR to limit viral proliferation and initiate SAR. As described below, the N gene has been isolated and the TMV elicitor has been identified. The availability of these genes facilitates studies that test the ligand-receptor model directly and address the molecular mechanisms involved in N-mediated TMV resistance. Insights derived from these studies may lead to the development of novel disease resistance in crops. Additionally, the apparent similarities between N-mediated defences and mammalian defence responses imply that understanding interactions between TMV and the N gene may further our knowledge of viral defence mechanisms in humans.

2. ISOLATION OF THE TOBACCO *N* GENE

To isolate the N gene, we employed the *Activator* (*Ac*) transposon of maize (McClintock 1948) for insertional mutagenesis in tobacco. The isolation of *Ac*-induced mutations of N required the generation and screening of large populations of F_1 seedlings heterozygous for N to identify potential loss-of-function mutations. Mutants were identified as plants unable to mount a TMV-dependent HR. A positive mutant selection scheme was developed that exploited the reversible temperature sensitivity of the N-mediated HR to TMV. At temperatures below $28\,^\circ C$, plants carrying the N gene can elicit a HR to TMV infection; however, it has been shown that some component of the N-mediated HR is temperature sensitive, and this sensitivity is reversible. Thus, at temperatures above $28\,^\circ C$, plants carrying the N gene do not generate a HR in response to TMV infection, but allow TMV to spread systemically. When systemically infected plants are shifted to a permissive temperature ($<28\,^\circ C$) they develop lethal systemic HR. Plants surviving this temperature shift were thus identified as TMV-sensitive mutant lines. Genetic and restriction-fragment-length-polymorphism analyses were employed to identify lines that potentially contained loss-of-function mutations in N. One line was shown to harbour an unstable mutation that correlated with the presence of a single *Ac* transposon. Mutations caused by *Ac* transposons in maize can revert and lead to an unstable phenotype (Fedoroff 1989); therefore, we postulated that this unstable line bore an *Ac* insertion in the N gene. Using an inverse polymerase chain reaction, genomic DNA sequences flanking the *Ac* element were isolated and used to identify complementary DNA (cDNA) and genomic DNA clones containing N sequences. Confirmation that the N gene
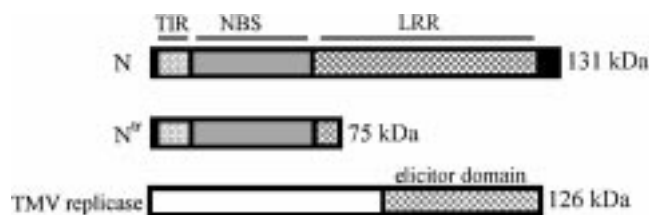
Figure 1. Predicted primary structures of N gene products and TMV 126 kDa replicase protein. TIR: Toll, Interleukin-1 receptor domain. NBS: nucleotide binding site domain. LRR: leucine-rich repeat domain.

had been cloned came from complementation of the TMV-sensitive phenotype with a genomic DNA fragment (Whitham *et al.* 1994).

Sequence analysis of cDNA and genomic DNA clones indicated that the N gene may encode full-length (N) and truncated (N^{tr}) proteins (figure 1). The N gene contains five exons that are spliced together to form a single open reading frame with the potential to encode a protein with a deduced molecular weight of 131.4 kDa. The N^{tr} protein results from alternative splicing of a 70 base pair exon to form an open reading frame that encodes a truncated polypeptide with a deduced molecular weight of 75.3 kDa (N^{tr}, figure 1). N^{tr} protein is identical to the amino portion of the N protein with an additional 36 amino acids at the carboxy-terminus caused by a frameshift (Whitham *et al.* 1994). Interestingly, an alternative transcript encoding a similarly truncated protein has been identified for the *L6* gene, a related *R* gene from flax that confers resistance to a fungal rust pathogen (Lawrence *et al.* 1995). Reverse-genetic analyses performed in our laboratory indicated that the N^{tr} protein is necessary for TMV resistance (S. P. Dinesh-Kumar, unpublished results), and we are currently working towards understanding the regulation and role of this alternative splicing event.

We have divided the full-length N protein into three domains: a TIR domain similar to the cytoplasmic domains of the *Drosophila* and human Toll protein and the interleukin-1 receptor (IL-1R) in mammals (TIR: Toll, IL-1R homology region); a nucleotide-binding site (NBS) domain; and leucine-rich repeat (LRR) domain (see figure 1). The presence of the latter two domains places N in the NBS–LRR class of plant *R* genes (Baker *et al.* 1997). Deletion and site-directed mutagenesis of residues in N predicted by homology to be functionally important indicate that all three putative domains are required for proper N protein function (S. P. Dinesh-Kumar, unpublished results). In addition, some of the constructed N mutations confer interesting partial loss-of-function or dominant change-of-function phenotypes. Some of these mutant alleles are accompanied by a virus-induced, spreading cell death that has been referred to as 'rolling HR'. Further characterization of these mutant N alleles should provide valuable insights into the function of these structural domains.

The products of numerous *R* genes cloned from several plant species share striking structural similarities to N, despite conferring resistance to specific pathogens with radically different modes of pathogenicity (Baker *et al.* 1997). This structural conservation is consistent with the

presence of a common molecular mechanism underlying gene-for-gene mediated disease resistance. The most common feature among these proteins is the LRR domain, a structure that participates in protein–protein and ligand interactions in animal systems (Kobe & Deisenhofer 1994). The *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* genes of tomato encode similar *R* gene products that consist almost entirely of an LRR domain, and each gene confers specific resistance to different races of the fungus *Cladosporium fulvum* (Joosten *et al.* 1994; Dixon *et al.* 1996). This observation led to the hypothesis that LRR structures are direct receptors of corresponding *Avr* gene products (Jones *et al.* 1994), although no biochemical evidence exists supporting this idea. In fact, the only evidence to date supporting a direct interaction hypothesis is the positive interaction observed in the yeast two-hybrid system between the tomato *Pto* gene product, which lacks a LRR domain, and the *P. syringae avrPto* gene product (Scofield *et al.* 1996; Tang *et al.* 1996). Interestingly, an LRR-encoding gene, *Prf*, is required for *Pto* function, although the functional relationship between these genes is not yet known (Salmeron *et al.* 1996).

The *N* gene and two fungal-disease *R* genes, *Arabidopsis RPP5* (Parker *et al.* 1997) and flax *L6*, are NBS–LRR-class genes that also encode TIR domains. The TIR domain in insect and mammalian receptor proteins is necessary for activation of signalling pathways that trigger defence responses (Heguy *et al.* 1992). The similarity in amino acid sequence and the involvement in host defence responses among these proteins raise the interesting possibility that *N*, *RPP5*, and *L6* may trigger signalling pathways similar to those triggered in insects and mammals. Recently, downstream signalling mechanisms and components conserved between the insect and mammalian pathways have been implicated to operate in plant defence signalling (Levin *et al.* 1996; Cao *et al.* 1997). To identify plant proteins that may function in defence signalling, we are using the yeast two-hybrid system (Fields & Sternglanz 1994) and *in vitro* biochemical methods to isolate proteins that interact with N protein. Using the TIR domain from *N* as bait in the two-hybrid system, we have isolated cDNAs that appear to encode proteins that bind specifically to this domain (M. Dutton, unpublished results). Further work is needed to determine if these genes are involved in *N*-mediated signalling.

3. TRANSFER OF THE *N* GENE INTO TOMATO

It has been proposed that cloned plant *R* genes could be transferred from resistant to susceptible plant species to control crop plant diseases. Indeed, the tomato *Pto* gene has been shown to confer resistance to *P. syringae* bearing the *avrPto* gene in transgenic tobacco (Rommens *et al.* 1995; Thilmony *et al.* 1995). To determine if *N* is able to confer TMV resistance in another crop species, the TMV-susceptible tomato cultivar VF36 was transformed with the *N* gene. We found that *N* effectively mediated resistance to TMV by the formation of necrotic local lesions and inhibition of TMV movement in transgenic tomato, as it does in tobacco (Whitham *et al.* 1996). Thus, any signal transduction machinery required for *N*-mediated resistance must also reside in tomato. This result

supports the gene-for-gene hypothesis and demonstrates that *R* genes can be transferred across species barriers that could not be breached by conventional breeding methods, and thereby introducing encoded pathogen resistance into heterologous plant species.

Control of pathogen-induced diseases using natural resistance genes may provide tremendous agricultural benefits for farmers and serve the environment by offering an alternative to pesticide use to prevent disease. In tomato, for example, transfer of the *N* gene may be agriculturally important, since systemic TMV infection of tomato reduces yield by causing mottling of the leaves, stunting growth, and reducing fruit quality (Watterson 1993). Furthermore, none of the known tomato TMV resistance genes alone is effective against as many strains of tobamoviruses as the *N* gene.

From a research standpoint, tomato plants expressing a functional *N* gene provide an excellent genetic system to dissect the signalling pathway leading to the HR, inhibition of viral replication and movement, and SAR. Unlike tetraploid tobacco, tomato is diploid and offers powerful, well developed tools for genetic analysis. In our laboratory, genetic screens using *N*-transgenic tomato have provided numerous mutant lines that display varying degrees of TMV sensitivity and are currently being characterized (C. Ustach, unpublished results). These virus resistance studies complement the genetic analysis being performed in many laboratory using *Arabidopsis* and tomato to isolate mutants that confer altered *R*-gene responses to bacterial and fungal pathogens. Numerous mutants have been isolated and the corresponding genes cloned from studies using *Arabidopsis* (Cao *et al.* 1997; Dietrich *et al.* 1997; Century *et al.* 1997; Parker *et al.* 1996; Greenberg 1997).

4. IDENTIFICATION OF THE TMV ELICITOR

TMV U1, the type member of the tobamovirus group, is one of the most extensively characterized plant pathogens. The genome of TMV consists of a single strand of positive-sense RNA of 6395 nucleotides and encodes at least four proteins. The 126 kDa and 183 kDa replicase proteins are translated from the genomic RNA using the first open reading frame (ORF) and read-through of the stop codon in the first ORF, respectively. The 30 kDa cell-to-cell movement protein and the 17.5 kDa viral coat protein are both translated from subgenomic RNAs. It has been shown that the coat protein is dispensable for the induction of *N*-mediated HR (Culver *et al.* 1991). Transgenic expression of the movement protein alone does not induce *N*-mediated HR, but a role in *N*-mediated resistance has been suggested (Deom *et al.* 1991).

The 126 kDa replicase protein has been implicated as the avirulence factor that triggers *N*-mediated HR. Padgett *et al.* (1997) identified HR-inducing mutations in Ob, the only tobamovirus known to overcome *N*-mediated resistance (Padgett & Beachy 1993). These mutations changed amino acids in the C-terminal region of the 126 kDa replicase protein. Using a modified Ob virus as a vector to express TMV-U1 proteins, Padgett *et al.* (1997) also demonstrated that expression of a C-terminal portion of the TMV 126 kDa replicase elicits *N*-mediated HR. However, because a viral vector was employed in these

expression studies, the possible involvement of viral replication, viral movement, and/or other viral factors in the elicitation of the HR was not excluded.

To determine whether expression of the 126 kDa replicase alone is sufficient to elicit N-mediated HR, we tested the effects of *Agrobacterium*-mediated transient and stable transgenic expression strategies in tobacco plants containing or lacking N. Importantly, these non-viral methods express portions of the TMV genome in the absence of virus particles, replication or movement. The transient method involves pressure-infiltrating *Agrobacterium* cells harbouring a binary vector containing TMV cDNA fragments into the intercellular spaces of mesophyll tissue, allowing for vector transfer and expression. The infiltrated tissue is then monitored for the HR. The stable transgenic method involves constructing transgenic tobacco plants harbouring cDNAs that express TMV protein fragments, crossing these plants with tobacco containing or lacking the N gene, then scoring the F_1 seedlings for the presence of the HR. Both methods demonstrated that expression of the 126 kDa replicase alone elicits the HR in an N-dependent fashion (S. Holzberg, unpublished results). Further analysis showed that the expression of the C-terminal 50 kDa region of the replicase was sufficient to trigger the HR, and we refer to this region of the replicase as the TMV elicitor domain.

We found that the thermosensitivity of the N-mediated response to TMV is retained when induced by expression of the 50 kDa replicase fragment. Seedlings expressing both the TMV elicitor domain and N succumb to systemic HR within a week after germination when incubated at $22\,^{\circ}C$, but grow normally at $32\,^{\circ}C$. When the $32\,^{\circ}C$ germinated seedlings become mature, a temperature shift to $22\,^{\circ}C$ quickly leads to systemic HR. Turgor pressure within these plants is lost one to two days after the temperature shift, necrotic lesions appear on leaves and stem by day two, and spread and destroy the plant by day four (see figure 2). Control plants expressing the TMV elicitor domain but lacking N are unaffected by the temperature shift and appear normal. This lethal phenotype is similar to that seen when tobacco plants, systemically infected with TMV at $32\,^{\circ}C$, are shifted to the HR-permissive temperature. Transgenic plant lines expressing this temperature-sensitive lethality should prove valuable in mutant selection schemes aimed at identifying genes involved in the HR.

The elicitor domain we have identified encompasses the same region identified previously in experiments using the Ob vector (Padgett *et al.* 1997). To further define the elicitor determinants within this region, Padgett *et al.* (1997) constructed and tested six different truncations of the elicitor domain and found that all abolished the ability to induce the HR. Similarly, we tested two elicitor domain truncations, one removing 7 kDa from the N-terminus and the other deleting 4 kDa from the C-terminus, using the *Agrobacterium*-mediated transient method and found that both deletions destroyed elicitor activity. However, immunoblot analysis of epitope-tagged versions of these alleles indicated that the truncated polypeptides were apparently unstable because little, if any, protein was detectable while the 50 kDa elicitor polypeptide accumulates to high levels (F. L. Erickson, unpublished results). Thus, the lack of elicitation by the
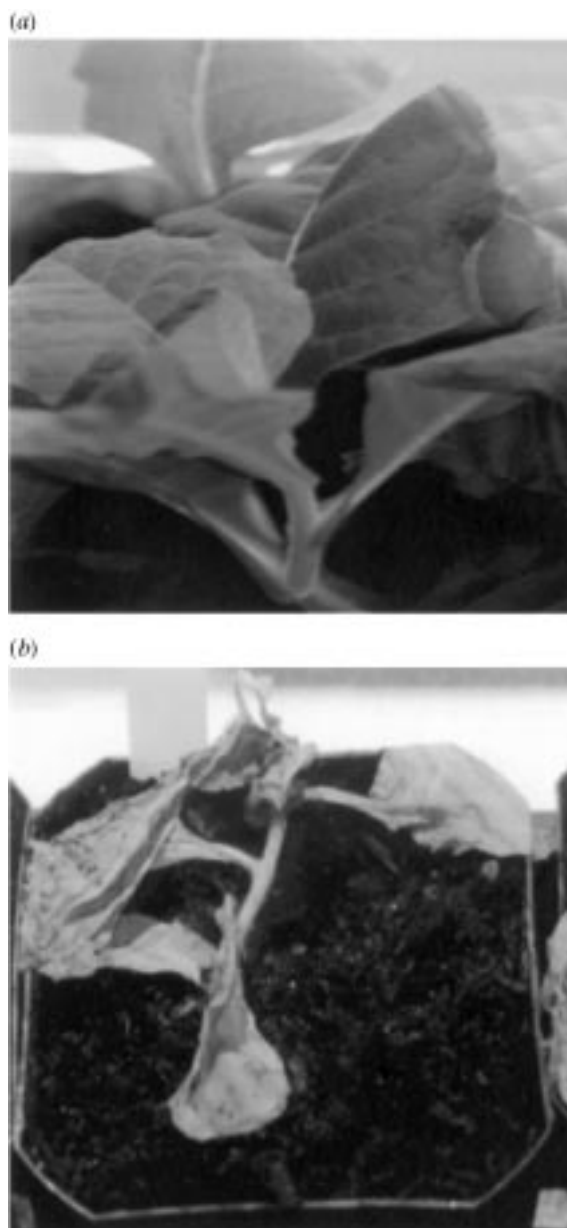
Figure 2. Temperature-sensitive phenotype of tobacco expressing the N gene and the TMV elicitor domain. Transgenic SR1 tobacco expressing (*a*) the TMV elicitor domain or (*b*) TMV elicitor domain and the N gene. Seeds were germinated and grown and $32\,^{\circ}C$. Plants were then shifted from $32\,^{\circ}C$ to $22\,^{\circ}C$. Representative plants are shown four days after temperature shift.

truncated alleles may be due to insufficient expression rather than deletion of amino acids directly involved in elicitation. Since protein levels were not determined in the experiments performed by Padgett *et al.* (1997), it is possible that some or all of their non-eliciting gene constructs also failed to express stable proteins. Additional experiments are necessary to further delimit the molecular determinants involved in elicitation.

The TMV elicitor domain contains the putative helicase region of the 126 kDa and 183 kDa replicases. The six sequence motifs indicative of RNA helicase enzymes (Habili & Symons 1989) are located within the C-terminal two-thirds of the elicitor domain. RNA

helicases use the energy of ATP or GTP hydrolysis to unwind duplex RNA. These enzymes have been characterized in a variety of organisms, from viruses to humans, and function in many different RNA metabolic processes. Most RNA viral genomes encode at least one putative helicase protein, which may function in various capacities, including acting in recombination, replication, transcription, translation, RNA splicing, and cell-to-cell movement of genomic RNA (Habili & Symons 1989; Carrington *et al.* 1998). The function of this putative helicase domain in the TMV replicase protein has not been addressed. To determine whether the elicitor domain has ATPase and helicase activities, biochemical characterization of the elicitor domain is in progress in our laboratory using wild-type and altered recombinant proteins purified from *Escherichia coli* and insect cells. These analyses, coupled with recombinant TMV assays and *Agrobacterium*-mediated expression methods described above should determine if these enzymatic activities are required in the TMV life cycle and for eliciting *N*-mediated defence responses.

5. N PROTEIN AND ELICITOR INTERACTION

Do the N and TMV elicitor domain proteins physically interact? To answer this question, we are employing the yeast two-hybrid system to detect interactions between the elicitor domain and full-length and truncated versions of the N protein. To assay for interactions *in vitro*, recombinant N and elicitor domain proteins are being expressed and purified from *E. coli* and insect cells. Co-immuno-precipitation studies using protein extracts derived from plants expressing both *N* and the elicitor domain are also in progress. The apparent low concentration of N protein in tobacco cells, however, may hinder the use of this latter method. To date, we have failed reproducibly to detect N protein in immunoblot analyses of plant proteins extracted from plants expressing epitope-tagged versions of *N*. We are attempting to increase *N* expression to detectable levels using strong, heterologous promoters to drive *N* gene expression. Low protein levels appear to be a common trait for *R* genes, and may impede biochemical studies *in planta* until further technologies are developed. The fact that an interaction has yet to be reported between an LRR-containing R protein and its cognate pathogen ligand, despite the availability of many of these gene pairs for the past four years, suggests that the simple ligand–receptor model may be difficult to prove, or that the system is more complex than first thought.

6. CONCLUSION

The isolation of the *N* gene and the identification of the TMV replicase as its elicitor completes a chapter in a classical story started by Holmes over 60 years ago. It also launches a new chapter, one that will likely contain fascinating insights into the molecular events underlying *N* and TMV interactions. As this disease-resistance tale unfolds we will see many new pages added to the 100-year-old saga of TMV.

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