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Research Note

Functional Expression of a Fungal Avirulence Gene from a Modified Potato Virus X Genome

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The *Cf-9* gene of tomato controls resistance to races of the fungal pathogen *Cladosporium fulvum* that possess the avirulence gene *Avr9*. When AVR9 elicitor is injected into healthy leaves or cotyledons of *Cf-9* containing genotypes, a gray necrotic response develops within 24 hr. To test whether expression of *Avr9* from an unrelated pathogenic microbe would elicit a *Cf-9* dependent necrotic response, the *Avr9* gene was inserted into a modified potato virus X expression vector capable of infecting many Solanaceous species. Progeny virus, derived from *in vitro* transcripts of the hybrid PVX:Avr9 construct were infectious on *Nicotiana clevelandii* plants and directed high level expression of *Avr9* in systemically infected tissue. When near-isogenic tomato lines of the cv. Moneymaker either containing *Cf-9* or lacking this gene (plants designated Cf9 and Cf0, respectively) were infected with PVX:Avr9, small necrotic flecks developed at the cotyledon inoculation site specifically on the Cf9 plants within 3 days. Subsequently, these necrotic areas expanded, coalesced, spread to other plant organs and the shoot apex, and eventually killed the Cf9 plants. On Cf0 plants only mild mosaic symptoms formed. These data indicate that AVR9 peptide is produced by virus-infected plant cells and that its function and specificity of action is effectively retained. Although the interaction mediated by *Cf-9* and *Avr9* occurred throughout the entire infection this does not result in the permanent cessation of viral movement. Virus particles that express *Avr9* are not avirulent on *Cf-9* tomato. As several explanations could account for the lack of viral containment, it cannot be concluded that *Cf-9* mediated resistance responses were ineffective in restricting the unrelated microbe PVX. The striking and reliable necrotic symptoms produced by PVX:Avr9 on Cf9 plants make this an ideal vector to perform a rapid structure/function analysis of the Avr9 sequence and to identify mutant plant loci that abolish viral multiplication and/or systemic spread.

Avirulence (*Avr*) genes of pathogenic microbes encode products that are either themselves recognized in a host plant that carries the corresponding disease resistance (*R*) gene, or mediate the production of another elicitor molecule that fulfills this role (Flor 1971; Staskawicz and Long 1993). Recognition by the plant of these *Avr*-specific pathogen signals leads to localized induction of an array of plant defense responses around the invading microbe to prevent its subsequent growth (Keen 1992). *R*-gene mediated resistance can occur within minutes of the host recognizing the invading microbe (for review, see Lamb *et al.* 1989). In accordance with Flor's original 'gene-for-gene' hypothesis (1946) these incompatible interactions only occur when both dominant genes are present. If either the *R* or *Avr* gene is absent, the pathogen is not detected by the host plant cells and disease ensues. It is unknown whether *R*-gene mediated plant defense responses triggered by one group of pathogenic microbe (for example, fungal species) could also be triggered by other groups of microbes (for example, either virus or bacterial species) if the microbe synthesized the appropriate AVR signal molecule.

The first two fungal avirulence genes have been cloned from the biotrophic pathogen of tomato, *Cladosporium fulvum* (van Kan *et al.* 1991; Joosten *et al.* 1994). The products of both the *Avr9* and *Avr4* genes are secreted from fungal cells and are processed by fungal and plant proteases to produce peptides of 28 amino acids (aa) AVR9 and 106 aa AVR4 (Van den Ackerveken *et al.* 1992; Joosten *et al.* 1994). Both purified peptides have been shown to elicit plant defense responses when injected into tomato lines containing the corresponding *Cf* gene.

To determine whether a fungal elicitor can activate plant defense responses when expressed by an unrelated pathogenic microbe, we have introduced *Avr9* into a viral expression vector. This vector is based on the potato virus X (PVX) genome and permits high level but unregulated expression of foreign genes in systemically infected whole plants of the family Solanaceae (Chapman *et al.* 1992). The gene of interest can be added to the viral genome by coupling it to a duplicated copy of the viral promoter for the coat protein mRNA. As PVX infects tomato, the PVX vector appeared suitable for the heterologous expression of the *C. fulvum Avr* genes. The *Avr9* gene was chosen for these experiments because it had already been shown that plant cells could synthesize an AVR9 gene product that exhibited the identical specificity of action as the authentic AVR9 peptide produced by *C. fulvum* (Hammond-

Kosack *et al.* 1994). In this paper we report on the symptoms produced and the degree of PVX spread that occurred after tomato lines either containing the fungal disease resistance gene *Cf-9* or lacking this gene were infected with PVX:Avr9.

A synthetic gene encoding for the 28 amino acid product of the *C. fulvum* avirulence gene *Avr9* and fused to the signal peptide sequence of the *Nicotiana tabacum* PR1a gene (Hammond-Kosack *et al.* 1994), was inserted into the intact PVX genome. To achieve this, a small polylinker was placed behind the duplicated promoter sequence of the coat protein gene of a modified PVX vector (pTXΔGC3) (Baulcombe *et al.* 1993), as shown in Figure 1. The *Avr9* gene was inserted at the *Cla*I site in the polylinker, to give the plasmid PVX:Avr9 (Fig. 1A). A summary of the nucleotide sequences of each PVX construct is given in Figure 1B.

To examine the infectivity of the *Avr9* containing PVX vector compared to wild-type PVX, *N. clevelandii* plants were inoculated with RNA transcripts generated *in vitro* from both constructs. Wild-type PVX RNA was produced as tTXS, whereas tPVX:Avr9 represented the recombinant virus containing *Avr9*. The symptoms caused by tPVX:Avr9 were milder than those produced by the wild-type PVX (tTXS) and developed more slowly. The chlorotic mosaic and leaf curl symptoms were attenuated on the leaf initial inoculated and

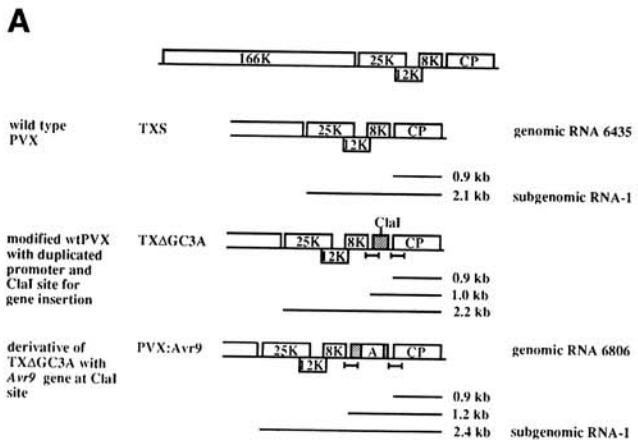

Fig. 1. Insertion of the *Avr9* gene in the genome of PVX. **A,** Schematic representation of hybrid PVX constructs. The first four major ORFs in the PVX sequence are indicated by the size (K = kDa) of their putative products (166K, 25K, 12K and 8K) and the coat protein gene by CP. The position of the polylinker is indicated by the hatched box and the position of the 81bp duplicated region is shown by bars under the pTXΔGC3A and pPVX:Avr9 diagrams. The sizes of predicted mRNAs are given below their progenitor constructs. **B,** Sequence of PVX constructs. To produce a PVX vector suitable for insertion of the Avr9 sequence, a polylinker sequence was substituted for GUS in GC3 (Chapman *et al* 1982, as described, to create the new vector pTXΔGC3A (Baulcombe *et al* 1993). A *Taq*I fragment containing the Avr9 sequence (isolated from plasmid SLJ6082 (Hammond-Kosack *et al* 1994) was ligated into pTXΔGC3A. To achieve this pTXΔGC3A was digested with *Cla*I and the cut ends dephosphorylated. The orientation of the *Avr9* gene in the resulting pPVX:Avr9 construct was verified by polymerase chain reactions involving a primer specific to the Avr9 sequence and a second primer specific to the flanking vector DNA.

symptoms developed 3–5 days later on leaves systemically infected by the PVX:Avr9 hybrid virus compared to those infected by wild-type PVX. A similar level of symptom attenuation was obtained when mRNA transcripts from a PVX:GUS construct was used as the inoculum (Chapman *et al.* 1992).

If the *Avr9* insert was retained in the PVX genome it is predicted that the genomic RNA and the subgenomic RNA-1 of the wild-type construct would be smaller than the RNA of the recombinant virus (Fig. 1). This prediction was confirmed by gel blot analysis of PVX RNA in systemically infected *N. clevelandii*, as shown in Figure 2A and B. These viral RNAs migrated more slowly than the analogous RNAs of tissue infected with wild-type PVX and hybridized with probes specific for the *Avr9* coding sequence (Fig. 2A and B). It was also predicted that the PVX:Avr9 infected tissue would contain one more subgenomic RNA than the tissue infected with wild-type PVX: This additional RNA would be the mRNA for production of the *Avr9* peptide, as shown in Figure 1. This RNA was not detected either with the PVX or the *Avr9* probes. However, the results presented below indicate that the *AVR9* peptide was produced in leaves infected with PVX:Avr9. It is therefore concluded that the additional subgenomic RNA was present at a low level or that the *Avr9* reading frame was translated as an internal open reading frame of a larger subgenomic RNA. In previous work we have found that the duplicated promoter for expression of GUS in the PVX:GUS construct was tenfold less efficient than the copy of the promoter for expression of the coat protein gene: It was necessary to use extracts of infected protoplasts to detect the subgenomic mRNA for GUS (Chapman *et al.* 1992).

Two near-isogenic tomato lines of the cv. Moneymaker either containing the fungal resistance gene *Cf-9* or lacking this gene (Tigchelaar 1984) were inoculated with mRNA isolated from systemically infected leaves of *N. clevelandii* plants. The two tomato lines are here designated Cf9 and Cf0,

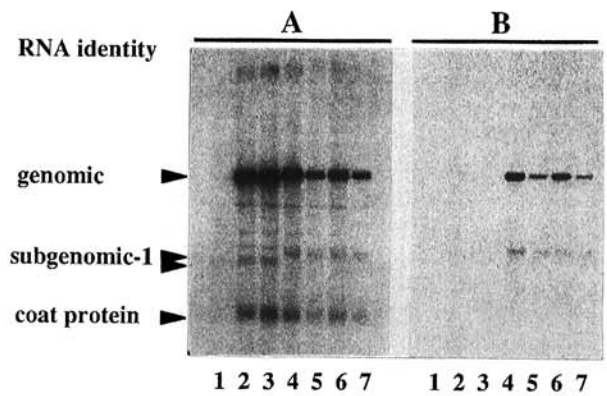

Fig. 2. Northern analysis of mRNA extracted from *Nicotiana clevelandii* plants, 10 days after inoculation with RNA transcripts produced *in vitro* (Chapman *et al* 1992) from each PVX construct. The samples were probed with a riboprobe specific for the positive strand of PVX (A) and with a ^{32}-P labeled DNA probe of the entire Avr9 sequence (B). Lane 1, mock inoculated; lanes 2 and 3, tTXS inoculated; lanes 4 to 7, tPVX:Avr9 inoculated. The position of various mRNA species are indicated in the right-hand margin. Autoradiographs were produced by exposure of panel A for 1 hour and panel B for 50 hr. Infectious RNA transcripts, plant inoculations, RNA isolation, and RNA gel blot analyses were performed as previously described (Chapman *et al* 1992).

respectively. RNA inoculum of either the PVX virus or the PVX:Avr9 hybrid virus was applied to a single cotyledon of 21-day-old seedlings. Four days after inoculation, small gray necrotic flecks were visible specifically on the cotyledon of the Cf9 plants inoculated with PVX:Avr9. Over the subsequent 3 days these gray necrotic areas expanded and coalesced and the entire cotyledon collapsed. By day 10, gray necrosis occurred in the adjoining stem tissue, on the other cotyledon and small gray necrotic flecks were visible on leaf 1. The necrotic areas on leaf 1 also coalesced, expanded into leaves 2 and 3 and finally the shoot apex. By day 14 post-inoculation, the majority of the Cf9 seedlings infected with the PVX:Avr9 virus were either severely stunted and had large patches of gray necrosis or had died. In response to either authentic AVR9 peptide produced by *C. fulvum* or AVR9 peptide produced by transgenic plants, confluent gray necrois develops within 12–24 hr of AVR9 injection into the leaf or cotyledon apoplast (Van Kan *et al.*1991; Hammond-Kosack *et al.* 1994).

On Cf0 plants infected with PVX:Avr9 a gray necrotic reaction was never observed. Instead the typical mild chlorotic mosaic symptoms formed on systemically infected leaves from day 10 onwards. Control Cf0 and Cf9 plants infected with wild-type PVX developed severe chlorotic

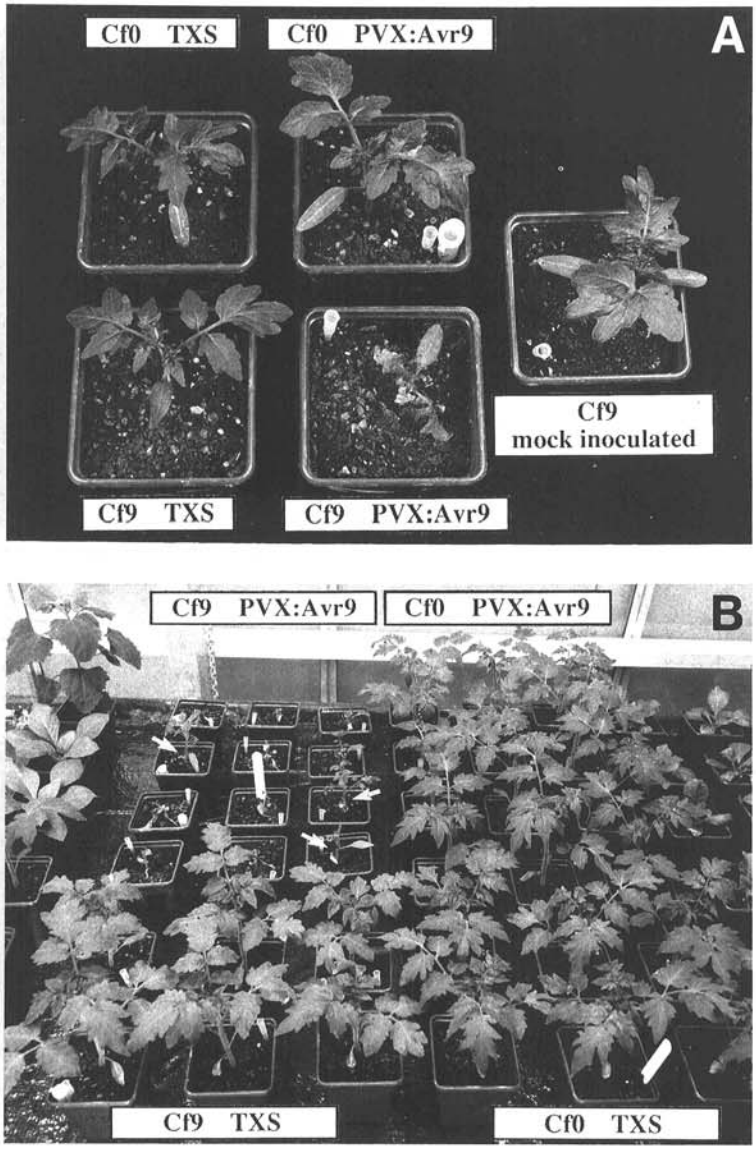

Fig. 3. Symptoms produced on Cf0 and Cf9 tomato plants after inoculation with wild-type TXS and hybrid PVX:Avr9 virus. Plants were photographed 14 days after inoculation (A) and 21 days after inoculation (B). All the TXS inoculated plants display moderate mosaic symtoms, while the Cf0 plants inoculated with PVX:Avr9 displayed milder symptoms. On Cf9 plants infected with PVX:Avr9 the mosaic symptom was replaced by a severe gray necrosis. Nine out of the 12 Cf9 plants inoculated with PVX:Avr 9 shown in panel B, died within 21 days of inoculation. Death was caused because the the gray necrosis symptoms reached the apical meristem. The other three Cf9 plants infected with PVX:Avr9 (marked with an arrow) only displayed gray necrosis on the lower leaves and cotyledons, their apical leaves exhibited a mild mosaic symptom. Using various PVX specific primers in polymerase chain reactions it was revealed the leaves with the mosaic symptoms were infected with a PVX:Avr9 construct that had lost the Avr9 sequence. For the tomato plant inoculations, purified mRNA isolated from systemically infected *N. clevlandii* plants was used. 2 µg RNA was applied to a single cotyledon on each plant by the procedure previously described (Santa Cruz and Baulcombe 1993).

mosaic symptoms on systemically infected leaves from day 8 onwards. The typical appearances of Cf0 and Cf9 seedlings infected with either PVX:Avr9 or wild-type PVX are shown in Figure 3 at 10 days after inoculation (A) and at 21 days after inoculation (B).

Occasionally the spreading gray necrosis on the Cf9 seedlings infected with the PVX:Avr9 stopped before the apical meristem was reached, and mild chlorotic mosaic symptoms developed on all subsequent systemically injected leaves. Three plants exhibiting the phenotype are evident in Figure 3, panel B. Polymerase chain reaction analysis of mRNA isolated from the green infected leaf tissue of these plants revealed the Avr9 sequence had been lost from the PVX genome (data not shown). A similar frequency of Avr9 sequence loss was also found in the Cf0 seedlings infected with PVX:Avr9. These results reveal the inherent instability of the PVX vector which, as previously suggested (Chapman *et al.* 1992), is probably caused by homologous recombination between the duplicated coat protein promoter sequences. As no difference in the frequency of Avr9 loss was apparent in the Cf0 and Cf9 inoculated seedlings (data not shown) there appeared to be no positive selection for loss of the Avr9 sequence in the *Cf-9* containing genetic background.

The development of gray necrosis solely on Cf9 plants following PVX:Avr9 infection shows that the fungal peptide was produced in the virus-infected tissue. This result also shows the function and specificity of the fungal avirulence Avr9 gene product has been effectively retained when its synthesis was directed by pathogenic virus particles inside plant cells. The timing of the necrotic response of Cf9 plants to PVX:Avr9 was earlier than the responses to avirulent *C. fulvum* races expressing *Avr9*. The response to PVX:Avr9 was also more severe. In incompatible *C. fulvum* interactions conferred by *Cf-9*, highly localized mesophyll cell necrosis and death is observed late in the infection, at approximately 6–8 days after stomatal penetration, and only occurs after hyphal growth has been restricted (Hammond-Kosack and Jones 1994). The response of Cf9 plants to PVX:Avr9 more closely resembles the gray necrotic phenotype induced following the injection of AVR9 peptide into leaves or cotyledons. Overall these data indicate that an unrelated pathogenic microbe expressing a foreign AVR signal molecule is capable of inducing plant defense responses specifically in the appropriate *R*-gene containing plant genotype.

The gray necrotic response spread throughout the aerial parts of the Cf9 plant infected with PVX:Avr9 and viral spread was not confined to the gray necrotic regions. Thus the interaction of the Cf-9 and Avr9 gene products occurred throughout the entire infection but this did not result in the cessation of viral movement. Several explanations could account for why the *Cf-9/Avr9*-mediated defense responses did not arrest PVX spread. Possibly, the array of defense responses induced by the fungal resistance gene *Cf-9* were nonfunctional against the viral pathogen PVX and are therefore either *Cladosporium fulvum* or fungal pathogen specific. Other, more likely explanations, are based on the level or timing of AVR9 production. For example, we cannot rule out that the titer of active AVR9 peptide produced by each PVX:Avr9 infected plant cell, may have been too low to elicit the full *Cf-9*-mediated defense response. Also, the need to export the 28 aa peptide product from infected plant cells for it to function, may delay the activation of the defense response in the infected Cf9 plant cells until after the virus had moved into the adjoining plant cells. PVX spread is thought, as with other viruses, to occur solely through the symplast via plasmodesmata connections (Deom *et al.* 1992). Finally, PVX may replicate and spread through a plant cell lineage which is either incapable of expressing the functional Cf-9 gene product or in which a defense response cannot be manifested. The AVR9 peptide produced by these cells could spread to other cell lineages where *Cf-9*-mediated defense responses can be triggered and culminate in gray necrosis. Due to the plethora of plausible explanations that could account for the data obtained, it cannot be concluded that the *Cf-9* mediated resistance responses are ineffective in containing infections by the unrelated microbe PVX.

The gray necrotic symptoms on Cf9 plants infected with PVX-Avr9 were both striking and reliably reproduced. These two features make the vector very suitable for various types of analysis. Firstly, the vector could be used to perform a rapid structure/function analysis of the Avr9 sequence itself. Transformation of *C. fulvum,* although moderately efficient compared to that achieved in other pathogenic fungal species, is slow and homologous reintegration events occur only rarely (Oliver *et al.* 1987; de Wit, personal communication). In addition, the PVX:Avr9 vector could be used as a screening tool to identify mutations in the host that render the plant unable to support viral multiplication and/or systemic spread. Although the normal mosaic associated with wild-type PVX infections would be suitable for this purpose, the gray necrosis on Cf9 plants infected with PVX:Avr9 is significantly easier to score. Plants with interesting mutations would be identified because they either lacked or exhibited an attenuated gray necrotic phenotype.

The PVX vector system is generally applicable to the analysis of heterologous genes when only high level and unregulated expression is required. Currently, the largest gene successfully inserted into the PVX genome that has retained biological activity is the β-glucuronidase gene from *E. coli*, which had an insert size of 1.9 kb (Chapman *et al.* 1992). The stability of the insert in the PVX vector could be problematic in certain analyses. However, the speed with which each new construct can be generated and then assessed in a whole plant over a lengthy period (days to months, if necessary) makes the PVX vector system a highly attractive experimental tool. Also, the ease with which mixed or sequential inoculations with different constructs can be carried out, suggests the PVX vector may be suitable to determine rapidly if functional relationship between two or more gene products exist.

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