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Research Note

The Fungal Gene *Avr9* and the Oomycete Gene *inf1* Confer Avirulence to Potato Virus X on Tobacco

Sophien Kamoun, Guy Honée, Rob Weide, Richard Laugé, Miriam Kooman-Gersmann, Koen de Groot, Francine Govers, and Pierre J. G. M. de Wit

Laboratory of Phytopathology and Graduate School Experimental Plant Sciences, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands
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The AVR9 peptide of the fungal pathogen *Cladosporium fulvum* and the INF1 protein of the oomycete pathogen *Phytophthora infestans* elicit the hypersensitive response (HR) on Cf9 tomato or *Cf-9* transgenic tobacco and on all cultivars of tobacco, respectively. Expression of either the functional *Avr9* or *inf1* genes from engineered potato virus X (PVX) genomes resulted in localized HR lesions on tobacco plants responsive to the elicitors and inhibited spread of the recombinant virus. In contrast, PVX derivatives producing mutant forms of AVR9 and INF1 with reduced elicitor activity caused systemic necrotic and/or mosaic symptoms, and were unable to inhibit PVX spread. These results demonstrate that HR is a highly versatile defense mechanism active against unrelated pathogens irrespective of the HR-inducing agent, and that resistance to recombinant PVX in tobacco is correlated with the strength of the transgene-encoded elicitor.

A central paradigm in the study of plant-microbe interactions is that specificity resides in recognition events but not in the expression of the hypersensitive response (HR) and other defense responses (Staskawicz et al. 1995). Despite a body of circumstantial evidence, this hypothesis has not been demonstrated directly through the expression of an avirulence gene in a totally unrelated pathogen. Here we used potato virus X (PVX), a virus pathogenic on many solanaceous plants, as a gene expression vector to test whether the avirulence genes *Avr9* and *inf1* from the fungal pathogen *Cladosporium fulvum* and the oomycete pathogen *Phytophthora infestans,* respectively, could confer avirulence to PVX on tobacco. The *Avr9* gene encodes the race-specific AVR9 elicitor peptide (van Kan et al. 1991; van den Ackerveken et al. 1992), and is present in all *C. fulvum* races that are avirulent on Cf9 tomato genotypes. Upon injection, AVR9 induces HR in leaves of Cf9 tomato genotypes. The *inf1* elicitin gene of *P. infestans* encodes the species-specific elicitor INF1 (Kamoun et al. 1997). Injection of INF1 into tobacco also results in HR (Kamoun et al. 1997). Engineered *P. infestans* strains deficient in the production of INF1 induce disease lesions when inoculated onto *Nicotiana benthamiana,* suggesting that INF1 elicitin is a species-specific avirulence factor (Kamoun et al. 1998). We examined whether the *Avr9* and *inf1* genes could confer avirulence to PVX on tobacco (*Nicotiana tabacum*), and whether mutant alleles of the genes encoding less active elicitors would be less potent in inhibiting PVX spread.

The PVX expression system allows transient expression of heterologous genes in solanaceous plants, as has been shown previously for the expression of the *Avr9* gene of *C. fulvum* in Cf9 tomato genotypes (Hammond-Kosack et al. 1995; Kooman-Gersmann et al. 1997). *Cf-9* transgenic tobacco responds with HR to AVR9 injection (Hammond-Kosack et al. 1998). To determine the response of *Cf-9* transgenic tobacco to PVX::*Avr9,* we used tobacco (cv. Petite Havana SR1) transformed with a cosmid carrying the *Cf-9* gene. Ten plants originating from two independent transgenic Cf9 lines, 6A3 and 17A3, were inoculated with PVX::*Avr9.* Three days after inoculation, small necrotic lesions were visible on the inoculated leaves that did not increase in size (Fig. 1A) for the following 3 weeks. In addition, no symptoms were observed on the upper (systemic) leaves of these plants (Fig. 1B). Tobacco plants without the *Cf-9* transgene were readily infected by PVX::*Avr9* and showed systemic mosaic symptoms typical of PVX infection (Table 1).

To test the effect of other protein elicitors on PVX infection of tobacco, we constructed a PVX::*inf1* derivative containing a chimeric gene encoding the mature 98-amino-acid INF1 elicitin of *P. infestans* (Kamoun et al. 1997, 1998) fused to the *PR-1a* signal peptide for extracellular targeting in a manner similar to that described for PVX::*Avr9* (Kooman-Gersmann et al. 1997). A fragment containing the *PR-1a* sequence was amplified by polymerase chain reaction (PCR) from PVX::*Avr9* with primers OX10 (5′-CAATCACAGTGT TGGCTTGC-3′) and PR1-INF1A (5′-GTGGTGCACGTGG

Corresponding author: Pierre J. G. M. de Wit; Laboratory of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands; Telephone: +31 317-483130; Fax: +31 317-483412; E-mail: Pierre.deWit@medew.fyto.wau.nl

Present address of S. Kamoun: Department of Plant Pathology, The Ohio State University, Ohio Agricultural Research and Development Center, 1680 Madison Ave., Wooster, OH 44691, USA; E-mail: Kamoun.1@osu.edu.

Present address of M. Kooman-Gersmann: Novartis Seeds BV, P.O. Box 26, 1600 AA Enkhuizen, The Netherlands.

S. Kamoun and G. Honée contributed equally to this paper.

TGGCACGGCAAGAGTGGGATATTAC-3′) and a fragment containing the *inf1* sequence was amplified from the *inf1* cDNA clone pFB7 (Kamoun et al. 1997) with primers PR1-INF1B (5′-CTTGCCGTGCCACCACGTGCACCACCTCG-3′) and SK-R2 (5′-GCGAAGCTTATCGATCATAGCGACG CACACGTAGA-3′). One recombinant plasmid, pPVX::*inf1*, was confirmed by DNA sequencing to have a PR1a-*inf1* fusion inserted in sense orientation between the duplicated PVX coat protein promoters. Infectious PVX::*inf1* virions were prepared as described by Kooman-Gersmann et al. (1997) and inoculated onto leaves of tobacco lines (Table 1). Similarly to PVX::*Avr9* on Cf9 tobacco, localized necrotic lesions were visible on tobacco leaves 3 days after inoculation (Fig. 1C) and no systemic symptoms developed later during infection (Fig. 1D).

To determine whether PVX::*Avr9* and PVX::*inf1* remained localized to the inoculated leaves, systemic leaves from Cf9 tobacco plants inoculated with PVX::*Avr9* and tobacco plants inoculated with PVX::*inf1* were harvested, and leaf homogenates were prepared as described by Kooman-Gersmann et al. (1997). The homogenates were inoculated onto MM-Cf9 tomato for PVX::*Avr9* and onto tobacco for PVX::*inf1*. No necrotic or mosaic symptoms were observed on either plant (data not shown), suggesting that no viable virions had spread systemically in the initially inoculated plants.

The observed local lesions and the absence of systemic spread of PVX derivatives encoding the two active elicitors on tobacco are reminiscent of HR-mediated resistances induced in plants against viruses, such as the resistance of *N* gene-containing tobacco to TMV (Whitham et al. 1994). Apparently, the *Avr9* gene expressed in PVX can confer avirulence to PVX on tobacco plants expressing the *Cf-9* gene and the *inf1* gene expressed in PVX can confer avirulence to PVX on tobacco plants.

If resistance to PVX is correlated with HR, it can be hypothesized that PVX derivatives expressing mutants of *Avr9* and *inf1* encoding less active elicitors would allow larger lesions and systemic spread of PVX. A number of PVX::*Avr9* derivatives expressing *Avr9* mutants with altered elicitor activity have been described by Kooman-Gersmann et al. (1997). To test the relationship between the strength of elicitor activity and the ability to confer avirulence to PVX on Cf9 tobacco, we inoculated five PVX::*Avr9* mutants onto Cf9 tobacco (Table 1). Two mutants, PVX::*Avr9*F10S and PVX::*Avr9*L24S, with lowest elicitor activity (Kooman-Gersmann et al. 1997), showed altered symptoms upon inoculation onto Cf9 tobacco when compared with PVX::*Avr9* (Table 1). On the inoculated leaves, both mutants produced necrotic spots similar to those produced by PVX::*Avr9* (Fig. 1A,E). However, in contrast to PVX::*Avr9*, symptoms extended to the systemic leaves. PVX::*Avr9*F10S produced systemic necrosis similar to that obtained on Cf9 tomato with PVX::*Avr9* (data not shown), while PVX::*Avr9*L24S, which induces no necrosis on Cf9 tomato, produced systemic mosaic symptoms accompanied by minute necrotic spots (Fig. 1F). Apparently, the low elicitor activity of these two mutant AVR9 peptides was not sufficient to confer avirulence to PVX, and systemic spread of the recombinant virions occurred throughout the plant. Three mutants, PVX::*Avr9*R08K, PVX::*Avr9*H22L and PVX::*Avr9*N03A, which still have significant elicitor activity on MM-Cf9 tomato (Kooman-Gersmann et al. 1997), produced local necrotic lesions on Cf9 tobacco similar to those produced by PVX::*Avr9* (Table 1).

Elicitins, such as INF1, are globular proteins with three highly conserved disulfide bridges (Boissy et al. 1996). Exchange of one of the cysteine residues in INF1 results in strong reduction of elicitor activity (Kamoun et al. 1997). In order to test whether a PVX::*inf1* mutant with reduced elicitor activity can or cannot confer avirulence to PVX on tobacco, we constructed PVX::*inf1*C95S, a mutant derivative in which cysteine residue 95 has been exchanged by serine. Recombinant PVX::*inf1*C95S virions were obtained as described above for PVX::*inf1*, except that the PCR-mediated overlap extension involved the mutagenic primer SK-R2S (5′-GCGAAGCTTATCGATCATAGCGACGCACTCGTAGA-3′) instead of SK-R2, and were inoculated onto tobacco lines (Table 1). In contrast to the small local necrotic lesions ob-

Table 1. Summary of symptoms observed on Cf9 tobacco and wild-type tobacco after inoculation with potato virus X (PVX) and PVX expressing *Avr9* or *inf1* wild-type and mutant genes

Insert gene in PVX[a]	Elicitor activity[b]	PVX inoculation			
		Symptoms on Cf9 tobacco[c]		Symptoms on tobacco[d]	
		Inoculated leaves	Systemic leaves	Inoculated leaves	Systemic leaves
None	. . .	No visible symptoms	Mosaic symptoms	No visible symptoms	Mosaic symptoms
Avr9 (wild type)	++++	Necrotic lesions	No visible symptoms	No visible symptoms	Mosaic symptoms
*Avr9*N03A	++	Necrotic lesions	No visible symptoms	ND	ND
*Avr9*R08K	+++++	Necrotic lesions	No visible symptoms	ND	ND
*Avr9*F10S	+	Necrotic lesions	Necrosis and chlorosis	ND	ND
*Avr9*H22L	+++	Necrotic lesions	No visible symptoms	ND	ND
*Avr9*L24S	–	Necrotic lesions	Mosaic and small necrotic lesions	ND	ND
inf1 (wild type)	++++	Necrotic lesions	No visible symptoms	Necrotic lesions	No visible symptoms
*inf1*C95S	+	Large necrotic lesions	Necrosis	Large necrotic lesions	Necrosis

[a] Gene constructs inserted into the *Cla*I site of the PVX vector pTX(GC3A (Baulcombe et al. 1993). *Avr9* corresponds to the *PR-1a* signal sequence fused to the sequence corresponding to the mature AVR9 peptide (Hammond-Kosack et al. 1995). *inf1* corresponds to the *PR-1a* signal sequence fused to the sequence corresponding to the mature INF1 protein (Kamoun et al. 1997).

[b] Relative elicitor or necrosis-inducing activity of AVR9 mutants based on injection assays of Cf9 tomato described by Kooman-Gersman et al. (1997). Relative elicitor activity of INF1 and INF1*C95S* based on infiltration experiments of tobacco described by Kamoun et al. (1997) and experiments described in this study. – = not active; +++++ = most active.

[c] *Nicotiana tabacum* cv. Petite Havana SR1 transformed with *Cf-9* (line 1096).

[d] *N. tabacum* cv. Petite Havana SR1. ND = not determined.

served with PVX::*inf1* (Fig. 1C), large necrotic lesions were observed on leaves inoculated with PVX::*inf1*C95S 3 to 6 days after inoculation (Fig. 1G), which later extended to the stem and systemic leaves (Fig. 1H). These results indicate that the *inf1*C95S gene is unable to confer avirulence to PVX in tobacco and allows spread of PVX, resulting in systemic necrosis.

In this study, we have demonstrated that the fungal elicitor AVR9 and the oomycete elicitor INF1 confer specific avirulence to PVX on tobacco. Our results support the view that HR-based disease resistance is a conserved mechanism in plants that is effective against unrelated pathogens. Clearly, HR and other defense responses triggered by particular elicitor molecules can be equally effective against fungal, oomycete, and viral plant pathogens.

Our results show that gene-for-gene interactions involving fungal pathogens and plants can be transferred to a different pathosystem, i.e., from *C. fulvum*-tomato or *P. infestans*-tobacco to PVX-tobacco. As predicted by the gene-for-gene model for the *Avr9*/*Cf-9* gene pair (van den Ackerveken 1992;

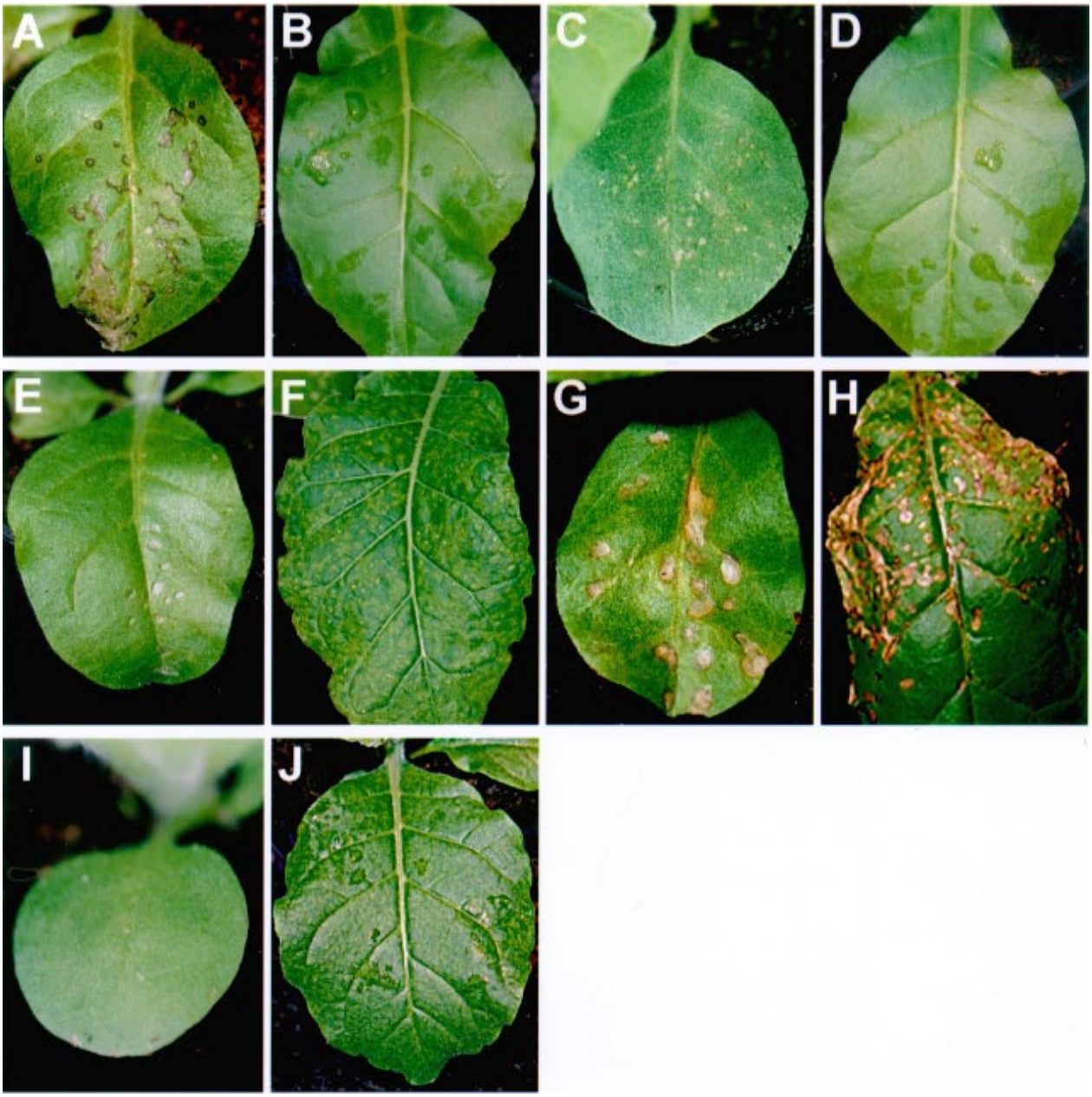

Fig. 1. Symptoms observed on Cf9 tobacco plants after inoculation with potato virus X derivatives expressing *Avr9* or *inf1* wild-type and mutant genes. Inoculated leaves from (**A, E,** and **I**) *Nicotiana tabacum* cv. Petite Havana SR1 line 1096 (containing a cosmid with a genomic copy of *Cf-9*) and (**C** and **G**) *N. tabacum* cv. Petite Havana SR1 were photographed 6 days after inoculation. Representative systemic leaves of the inoculated (**B, F,** and **J**) Petite Havana SR1 1096 and (**D** and **H**) Petite Havana SR1 plants were photographed 21 days after inoculation. **A** and **B,** PVX::*Avr9*. **C** and **D,** PVX::*inf1*. **E** and **E,** PVX::*Avr9*L24S. **G** and **H,** PVX::*inf1*C95S. **I** and **J,** PVX wild type. **A, C, E,** and **G,** Leaves show local necrotic lesions. **B, D,** and **I,** Leaves show no visible symptoms. **F,** Leaf shows mosaic symptoms and small necrotic lesions. **H,** Leaf shows systemic necrosis. **J,** Leaf shows mosaic symptoms.

de Wit 1992, 1997), the absence of either *Avr9* or *Cf-9* leads to mosaic disease symptoms and systemic spread of the virus (Table 1). Similarly, *inf1*, an avirulence gene that confers avirulence to *P. infestans* in solanaceous plants at the species level (Kamoun et al. 1993, 1998), also confers avirulence to PVX on tobacco but not on tomato, a plant species not responding to INF1 (Table 1 and Fig. 1C,D; and data not shown).

Mutants of AVR9 and INF1 with reduced elicitor activity lost the ability to inhibit PVX spread in tobacco. These results strongly support the hypothesis that the effectiveness of the HR-mediated resistance against PVX derivatives in tobacco depends on the strength of the elicitor encoded by the recombinant virus.

The incompatible interaction between PVX::*Avr9* and Cf9 tobacco noted in this study sharply contrasts with the previously examined interaction between PVX::*Avr9* and Cf9 tomato genotypes (Hammond-Kosack et al. 1995; Kooman-Gersmann et al. 1997). On Cf9 tomato PVX::*Avr9* does not remain localized to the site of infection but spreads and causes systemic HR. This discrepancy could result from different levels of sensitivity of these two plant species to the AVR9 peptide, as has been reported by Hammond-Kosack et al. (1998), which could originate from a more efficient *Cf-9*–mediated defense signaling pathway in tobacco than in tomato. Alternatively, differences in leaf structure between the two host plants might have different implications for virus replication and spread, leading to localization in tobacco but not in tomato.

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