Research Note

Comparison of the Hypersensitive Response Induced by the Tomato *Cf-4* and *Cf-9* Genes in *Nicotiana* spp.

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We have previously shown that tomato *Cf-9* induces an *Avr9*-dependent hypersensitive response (HR) in *Nicotiana tabacum* and potato. We show here that *Cf-4* also induces an *Avr4*-dependent HR in two tobacco species (*N. tabacum* and *N. benthamiana*). The HR induced by *Cf-4* and *Cf-9* was compared in stable tobacco transgenics by a seedling lethal assay and resistance to recombinant *Potato virus X* expressing *Avr4* or *Avr9*. We also compared HR induction with *Agrobacterium*-mediated transient expression. The *Cf-4/Avr4* combination induced a more rapid HR than *Cf-9/Avr9*. Sensitive assays for *Cf-9* and *Cf-4* function should prove useful for structure/function analyses of these resistance proteins in tobacco.

Additional keywords: *Cf* genes, disease resistance.

Tomato *Cf* genes encode extracellular membrane anchored leucine-rich repeat (LRR) glycoproteins that confer resistance to *Cladosporium fulvum* through recognition of distinct fungal avirulence (*Avr*) determinants (Thomas et al. 1998). DNA sequence analysis of homologous genes from a number of chromosomal loci have identified LRR sequences that determine recognition specificity (Dixon et al. 1998; Parniske et al. 1997; Thomas et al. 1997). Functional analysis of these proteins requires the development of robust assays for *Cf* activity. The genes for two *C. fulvum* *Avr* determinants (*Avr4* and *Avr9*) have been cloned (Joosten et al. 1994; Van den Ackerveken et al. 1992) and are useful reagents to study *Cf-4* and *Cf-9* function in tomato. We have previously shown that *Cf-9* induces an *Avr9*-dependent hypersensitive response (HR) in *Nicotiana tabacum* (Hammond-Kosack et al. 1998). Here we describe sensitive assays to analyze the HR-inducing activity of *Cf-9* and *Cf-4* in tobacco.

Progeny from *N. tabacum* *Cf-9* plants crossed to *N. tabacum* 35S:*Avr9* lines show a seedling lethal phenotype (Hammond-Kosack et al. 1998). To compare *Cf-9*- and *Cf-4*-mediated seedling lethality in tobacco, binary vectors were constructed that expressed *Cf-4* or *Cf-9* from the *Cf-4* promoter. This element consisted of 3.0 kb of DNA upstream of *Cf-4* from plasmid p129P6 (Thomas et al. 1997). These con-

structs were used to transform *N. tabacum* cv. Petite Gerard (C. M. Thomas, M. Smoker, and J. D. G. Jones, *manuscript in preparation*). *N. tabacum* lines expressing 35S:*Avr4* (Thomas et al. 1997) were also generated. Six primary *Cf-4* and *Cf-9* transgenics were crossed reciprocally to 35S:*Avr4* or 35S:*Avr9* (Hammond-Kosack et al. 1998) transgenic lines. Four transgenic lines carrying each construct that contained single T-DNA loci were analyzed further.

All progeny from crosses between *Cf-4* primary transformants and a line homozygous for 35S:*Avr4* germinated and segregated 1:1 for wild type and a seedling lethal phenotype irrespective of which plant was used as male or female parent (Fig. 1). PCR (polymerase chain reaction) analysis confirmed that wild-type seedlings inherited only 35S:*Avr4*. Progeny from crosses between *Cf-9* primary transformants crossed as male parent to a 35S:*Avr9* homozygous line as female parent also segregated 1:1 for wild-type and necrotic seedlings, as described previously (Hammond-Kosack et al. 1998). The *Cf-4/Avr4*-dependent seedling lethal phenotype was distinct from that observed in *Cf-9/Avr9* seeds. Seedlings from the latter cross showed cotyledon expansion and appeared fully green before the onset of necrosis that could be observed initially at the margins of the cotyledons (Fig. 1D). In progeny expressing both *Cf-4* and *Avr4*, a more severe seedling lethal phenotype was observed. Cotyledons showed reduced expansion to the extent that the testa remained attached, and were never green (Fig. 1B). Therefore, the seedling lethal phenotype induced by *Cf-4/Avr4* in tobacco appears to act earlier in development than that induced by *Cf-9/Avr9*.

In this study, progeny from crosses between *Cf-9* primary transformants crossed as female parent to a 35S:*Avr9* homozygous line as male parent segregated 1:1 for wild type and a more severe non-germination phenotype (results not shown). This result contrasts with a previous study in which *Cf-9* was expressed from its own promoter (Hammond-Kosack et al. 1998). In that study, nearly 100% of the progeny from crosses with *N. tabacum* *Cf-9* heterozygotes, crossed as female parents to the same 35S:*Avr9* homozygous line used here, failed to germinate (Hammond-Kosack et al. 1998). The seed non-autonomous phenotype was postulated to be due to diffusion of the 28 amino acid *Avr9* peptide from zygotic tissue into surrounding maternal tissue that expressed *Cf-9* and triggered a cellular response mediated by salicylic acid. The different

results may be due to the different promoters used, but DNA sequencing has shown the *Cf-9* and *Cf-4* upstream sequences are highly homologous (Parniske et al. 1997). It is also unlikely that this is due to differences in the binary vectors used since in each case *Cf* transgenes were cloned in near-identical vectors (SLJ 7291 and SLJ 7292; Jones et al. 1992). *Cf-9* expression in the two transgenics studied previously may have been elevated due to additional sequences present on the 4.5-kb sequence upstream of *Cf-9* (Hammond-Kosack et al. 1998), the orientation of *Cf* genes within the T-DNA, or their chromosomal location.

Elevated levels of *Cf-4* expression also result in a seed non-germination phenotype. In progeny from crosses between *N. tabacum* 35S:*Cf-4* heterozygotes and plants heterozygous for 35S:*Avr4*, one quarter of the progeny failed to germinate, indicating the phenotype is seed autonomous (results not shown). This phenotype was not dependent upon which plant was used as male or female parent. The seed autonomy in 35S:*Cf-4* × 35S:*Avr4* crosses may be due to the larger size of *Avr4* (106 amino acids; Thomas et al. 1997), which may be less diffusible than *Avr9*, or alternatively *Avr4* may be more labile.

To test whether *Agrobacterium tumefaciens* could be used to transiently express *Cf-9* to induce an *Avr9*-dependent HR, binary vectors were constructed based on a *Cf-9* cDNA clone (Jones et al. 1994; Tang 1998). In one vector, *Cf-9* expression was under the control of the 35S promoter and the *A. tumefaciens* nopaline synthase 3' terminator (*nos3'*). In a similar vector, which served as a negative control, transcription of *Cf-9* was interrupted by the maize transposon *Activator* inserted between 35S and *Cf-9*. Bacteria were infiltrated into leaves of *N. tabacum* 35S:*Avr9* transgenic plants (Fig. 2). Necrosis was observed in leaf panels infiltrated with *A. tumefaciens* expressing 35S:*Cf-9* at 7 days post infiltration (Fig. 2A). No necrosis was observed in leaf panels expressing empty vector sequences or 35S:*Ac:Cf-9*. These results demonstrate that *Agrobacterium*-mediated transient expression of 35S:*Cf-9* can induce an *Avr9*-dependent HR. In addition, no macroscopic HR could be detected in leaf panels infiltrated with a 35S:*Cf-*

9ΔMA construct that lacks domains E, F, and G of *Cf-9* (Fig. 2A). This shows that *Cf-9* requires a functional juxtamembrane, transmembrane, or cytoplasmic domain, or alternatively all three domains, to induce an *Avr9*-dependent HR.

To compare the HR-inducing activity of *Cf-4* and *Cf-9*, additional vectors were made that co-expressed *Cf* and *Avr* gene cassettes from identical transcription control sequences (Fig. 2; Jones et al. 1995). Binary vectors were mobilized into *A. tumefaciens* GV3101. The HR in *N. benthamiana* was induced more rapidly than in *N. tabacum*. Necrosis was observed in *N. benthamiana* leaf panels expressing *Cf-4* or *Cf-9* only in the presence of the corresponding avirulence determinant (Fig. 2B and C). The necrotic reaction induced by *Cf-4/Avr4* was fully developed after 4 days, compared with the HR induced by *Cf-9/Avr9*, which was fully developed after 7 days. This result is consistent with data from seedling lethal assays that suggested *Cf-4/Avr4* induces a more rapid HR.

Tobacco transgenics expressing *Cf-9* are resistant to *Potato virus X* (PVX) infection when it carries the corresponding *Avr9* avirulence determinant (Kamoun et al. 1999), as also shown for tomato expressing *Pto* after inoculation with PVX:*avrpto* (Tobias et al. 1999). This contrasts with the phenotypes observed in tomato *Cf9* and *Cf4* lines where PVX expressing the corresponding *Avr* gene replicates, resulting in systemic necrosis and plant death (Hammond-Kosack et al. 1995; Thomas et al. 1997). The relative effectiveness of *N. tabacum* *Cf-4* and *Cf-9* transgenic lines in resisting infection to PVX:*Avr4* (Thomas et al. 1997) and PVX:*Avr9* (Hammond-Kosack et al. 1995) was determined.

Equal titers of wild-type PVX, PVX:*Avr4*, and PVX:*Avr9* were inoculated onto *N. tabacum* transgenics at the six- to eight-leaf stage. The three PVX derivatives were all compatible on *N. tabacum* control plants (Fig. 3A) and produced typical symptoms of PVX infection in systemically infected leaves (vein clearing, chlorosis, and mosaic symptoms). The yield of recombinant virus in compatible reactions was reduced, compared with wild-type PVX, particularly PVX:*Avr4* (Fig. 3). In inoculated and upper leaves of PVX:*Avr4*-infected plants, the PVX RNA was heterogeneous and two major

Fig. 1. Seedling lethal phenotype in transgenic *Nicotiana tabacum* expressing *Cf-4* or *Cf-9* from the *Cf-4* promoter. Seedlings were photographed 18 days post sowing (dps) except in **D**. **A**, Progeny from a cross between a plant hemizygous for *Cf-4* (F641) crossed to a 35S:*Avr4* homozygote. Seedlings expressing both transgenes (indicated by arrows) show retarded root development and limited cotyledon expansion and many retain the testa. **B**, In some *Cf-4/Avr4* seedlings expansion of the cotyledons was observed at 18 dps but they were devoid of chlorophyll. **C**, Seedlings from a cross between a transformant hemizygous for *Cf-9* (F797) crossed to a 35S:*Avr9* homozygous line. Seedlings expressing *Cf-9* and *Avr9* were initially green as previously reported. **D**, Tobacco seedling expressing *Cf-9* and *Avr9* at 13 dps. After development of fully green cotyledons, necrotic sectors were observed initially at the margins of the cotyledons.

bands corresponding to recombinant and wild-type virus RNA were detected (Fig. 3). In contrast, PVX:Avr9 appeared more stable (Fig. 3).

Incompatible reactions were observed when recombinant PVX was inoculated onto transgenic *N. tabacum* expressing the corresponding *Cf* gene (Fig. 3B and C). Necrotic lesions

were observed on inoculated leaves 4 to 5 days post inoculation and plants failed to develop typical symptoms of PVX infection. The absence of virus in these plants was confirmed by RNA gel blot analysis (Fig. 3B and C) and by inoculating sap extracted from these leaves onto *N. benthamiana* plants that are highly susceptible to PVX infection. The local lesion

Fig. 2. Expression of *Cf-4* and *Cf-9* in tobacco with *Agrobacterium*-mediated transient expression. Constructs were mobilized into *A. tumefaciens* GV3101 by tri-parental mating. *A. tumefaciens* was resuspended at an OD₆₀₀ of 0.5 in Murashige and Skoog salts, 2% wt/vol sucrose, 500 μ M 2-(*N*-morpholino)ethane sulfonic acid (MES) pH 5.6 and 20 μ M acetosyringone. Bacteria were incubated at 22°C for 3 h without shaking then infiltrated into leaves of *Nicotiana tabacum* 35S:Avr9 with a syringe. Plants were maintained in a glasshouse at 22°C with a 16-h photoperiod supplied at a photon flux density of 300 to 650 μ E s⁻¹ m⁻². **A**, *Cf-9* expression in infiltrated leaf panels of *N. tabacum* 35S:Avr9 at 7 days post infiltration. Leaf panels were infiltrated with *A. tumefaciens* GV3101 containing empty vector (SLJ 7291, panel 1), 35S:*Cf-9* (SLJ 8381, panel 2), 35S:Ac:*Cf-9* (SLJ 8511, panel 3), and 35S:*Cf-9* Δ MA (SLJ 8407, panel 4). **B**, Analysis of *Cf-4*/Avr co-expression constructs in wild-type *N. benthamiana* at 5 days post infiltration. Leaves were injected with *Cf-4*/Avr9 (4/456/Avr9, left hand panel) and *Cf-4*/Avr4 (4/456/Avr4, right hand panel). **C**, Analysis of *Cf-9*/Avr co-expression constructs at 9 days post infiltration; leaves were infiltrated with *Cf-9*/Avr9 (9/456/Avr9, left hand panel) or *Cf-9*/Avr4 (9/456/Avr4, right hand panel). **D**, Binary vector constructs for co-expression of *Cf* and *Avr* genes. *Cf* gene expression cassettes comprised *Cf-4* and *Cf-9* genomic sequences including their identical 3' untranslated regions that contain a small intron (Thomas et al. 1997). Expression was under control of the 35S promoter. *Avr4* and *Avr9* expression cassettes contained the 35S promoter and the *nos3'* terminator. 35S sequences are shown as black boxes and the *nos3'* terminator sequence is shown in gray. *Cf* and *Avr* sequences are shown as white boxes, L and R correspond to the left and right border T-DNA sequences.

Fig. 3. *Cf-4*- and *Cf-9*-mediated resistance in *Nicotiana tabacum* to recombinant *Potato virus X* (PVX). **A**, Leaf of nontransformed *N. tabacum* cv. Petite Gerard inoculated with PVX at 5 days post inoculation (dpi). Adjacent panel shows an RNA gel blot with ^{32}P -labeled PVX cDNA used as a probe. Ten micrograms of total RNA (equivalent RNA loading was confirmed by ethidium bromide staining) was isolated from plants inoculated with PVX, PVX:*Avr4*, PVX:*Avr9*, or mock-inoculated (M.I.) plants. Total RNA was prepared from inoculated and “systemically infected” leaves (three uppermost leaves) at 10 dpi. In the gel blot, the first lane contains RNA from inoculated leaves; the second lane RNA from “systemically infected” leaves. Only the region of the blot containing full-length PVX RNA is shown. **B**, Local lesion phenotype induced by PVX:*Avr9* at 5 dpi in a transgenic line heterozygous for *Cf-9* transgenic (F797) and corresponding RNA gel blot from siblings inoculated with PVX and PVX:*Avr4*. Local lesions induced by *Cf-9* in incompatible reactions eventually merged to produce a necrotic sector (data not shown). **C**, Local lesion phenotype induced by PVX:*Avr4* in a transformant heterozygous for *Cf-4* (F640) and RNA gel blot of samples isolated at 10 dpi. Lesions induced by *Cf-4* in incompatible reactions were always smaller than those observed in *Cf-9* transformants and no spreading necrosis was observed.

phenotypes in incompatible reactions induced by *Cf-9* and *Cf-4* transgenics were distinct. Larger necrotic lesions were observed in *Cf-9* plants inoculated with PVX:*Avr9* that spread and eventually coalesced to generate a necrotic leaf sector (Fig. 3). The spreading *Cf-9*/*Avr9*-dependent necrosis was reported previously in response to *Avr9* infiltration into *N. tabacum* *Cf-9* leaves and is potentiated by salicylic acid (Hammond-Kosack et al. 1998). The lesions induced by PVX:*Avr4* on *N. tabacum* *Cf-4* leaves were smaller in the four independent transgenics tested and remained discrete. The smaller lesions induced by *Cf-4* are probably due to the slower rate of PVX:*Avr4* replication and the more rapid HR induced by *Cf-4*/*Avr4* in tobacco.

Tobacco is a useful system in which to study the *Cf*/*Avr*-induced HR at biochemical, physiological, and molecular levels (Piedras et al. 1998; Romeis et al. 1999). We have developed a number of sensitive assays to monitor induction of the HR in tobacco. Our analysis showed that *Cf-4*/*Avr4* induces a more rapid HR than *Cf-9*/*Avr9*. These assays, particularly *Agrobacterium*-mediated transient expression, should facili-

tate a structure/function analysis of *Cf-4* and *Cf-9* in tobacco. Characterization of HR induction by specific resistance gene/*Avr* gene combinations is an important criterion for engineering resistance in heterologous species (De Wit 1992). Therefore, in strategies for engineering disease resistance in solanaceous plants (Hammond-Kosack et al. 1998), the *Cf-4*/*Avr4* combination that induces a rapid HR may prove most effective in arresting pathogen ingress.

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