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Plant Biology

Developmentally regulated cell death on expression of the fungal avirulence gene *Avr9* in tomato seedlings carrying the disease-resistance gene *Cf-9*

(gene-for-gene interaction/*Cladosporium fulvum*/tomato leaf mold/*Lycopersicon esculentum*)

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ABSTRACT **Plant defense responses are induced when the products of disease-resistance genes and pathogen avirulence genes interact. We report here the effects of expressing the *Cladosporium fulvum* avirulence *Avr9* gene product in a tomato line containing the *Cf-9* disease-resistance gene. A synthetic *Avr9* gene was constructed to produce constitutive high-level expression of AVR9 peptide in the plant apoplast. *Avr9* expression in *Cf-9*-containing tomato lines is lethal, but cell death is developmentally regulated, in that necrosis is not visible until 10 days after planting seed. Plant lines lacking *Cf-9* and expressing *Avr9* remain healthy. The synthetic *Avr9* gene exhibited the same specificity of action as the authentic *C. fulvum Avr9* gene. Our results have significant implications for strategies using the gene combination *Avr9*/*Cf-9* to engineer plants with enhanced disease resistance.**

The resistance or susceptibility of plants to infection by pathogenic fungal, bacterial, viral, or nematode species is often determined by pairs of complementary genes in the two organisms. If dominant resistance genes (*R* genes) in the plant are matched by the corresponding avirulence genes (*Avr* genes) in the pathogen, the plant recognizes the invading pathogen and responds by triggering active defense responses at or near the site of invasion (1, 2). Little is known about how *Avr* and *R* gene products determine this incompatibility. If *R* or *Avr* genes are absent from either partner, the plant does not recognize the pathogen and disease ensues.

The first *Avr* gene isolated was from a bacterial pathogen, and now >30 have been characterized (2, 3). For viral plant pathogens, some avirulence determinants have been shown to reside in the coat protein (4). In fungi, the only avirulence genes that have been cloned are the genes *Avr9* and *Avr4* from the biotrophic tomato pathogen *Cladosporium fulvum* (5, 6). The products of these two genes are secreted from the fungal cells and processed by fungal and plant proteases to produce peptides of 28 aa (AVR9) and 106 aa (AVR4) (6, 7). AVR9 and AVR4 are both cysteine-rich peptides that elicit the hypersensitive response (HR) on tomato cultivars carrying *Cf-9* and *Cf-4*, respectively.

Expression of a viral *Avr* gene in plants containing the corresponding *R* gene can elicit a necrotic resistance-like reaction. Transgenic *Nicotiana sylvestris* plants expressing viral coat protein from an avirulent strain of tobacco mosaic virus developed an HR phenotype characteristic of the *N'* gene (8). Those expressing coat protein from a virulent viral strain did not develop HR.

Here, we report on the lethal consequences of expressing the *C. fulvum Avr9* gene product in a tomato line that contains the *Cf-9* gene. The *Avr9* gene does not cause necrosis in lines lacking *Cf-9*. Cell death specified by *Cf-9* and *Avr9* was restricted to specific tissues and was under strict developmental control. Our data identify some of the plant organs and tissues in which the *Cf-9* gene product is functional. These results are significant for strategies to isolate the *Cf-9* gene and for strategies that deploy this interaction to engineer plants with enhanced disease resistance.

MATERIALS AND METHODS

Construction of a Synthetic *Avr9* Gene for Plant Transformation. *Step 1.* Four overlapping oligonucleotides encoding the signal peptide sequence (SP) of the tobacco Pr1a protein (10) and the mature 28-aa AVR9 peptide of *C. fulvum* (5, 11) were synthesized. The sequences of the oligonucleotides were as follows: primer A, 5'-CTTCTCTTATTCCTAGTAATATCCCACTCTTGCCGTGCCTACTGTAACAGTTCT-3'; primer B, 5'-GCATCTTCCACATTGTCCAAGACAGTCAAAAGCTCTTGTACAAGAACT-3'; primer C, 5'-GGAATCGATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTTCTTGTCTCTACACTTCTCTTA-3'; and primer D, 5'-AAAAGATCTCAATGTACACATTGAAGCTTATGAAAGACGCATCTTCC-3'. These oligonucleotides were constructed so that primers C and D contained *Cla* I and *Bgl* II sites at the 5' termini, respectively. Primers A and B were annealed together, and their complementary strands were synthesized by the Klenow fragment of DNA polymerase (12). The double-stranded DNA generated was gel purified, and 10 pg was used as the template in a PCR involving primers C and D. The PCR was run for 20 cycles of 15 sec at 94°C, 1 min at 52°C, and 30 sec at 72°C. The full-length DNA product was gel purified and blunt end ligated (12) into *Eco*RV-linearized pBluescript to create pSLJ6069. The integrity of the *SP:Avr9* nucleotide sequence was verified by dideoxynucleotide sequencing.

Step 2. The *SP:Avr9* fragment was excised from pSLJ6069 with *Cla* I and *Bgl* II and ligated into the pUC118-based pSLJ4K1 (13) digested with *Cla* I and *Bam*HI to create the construct pSLJ6071. This placed the *SP:Avr9* sequence 3' of the tobacco mosaic virus Ω leader (14) between a 35S promoter and a *nos* transcription termination sequence.

Step 3. The synthetic *35S:SP:Avr9* gene was excised by digesting pSLJ6071 with *Bgl* II and *Hin*dIII and ligating to the binary vector pSLJ4654A cut with *Bam*HI and *Hin*dIII (13). In the resulting binary vector pSLJ6201, the direction of transcription of the *35S:SP:Avr9* gene is divergent to that of the neomycin phosphotransferase gene (*nptII*) used for selecting transformed plants. Additional details on pSLJ6201 are available upon request.

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Abbreviations: HR, hypersensitive response; SP, signal peptide; IF, intercellular fluid; T-DNA, transferred DNA; GUS, β-glucuronidase; dsp, days after seed planting; SEM, scanning electron microscopic.

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Plant Transformation. Transformations were performed with the kanamycin-sensitive tobacco (*N. tabacum*) cv. Petit Havana and tomato cv. Moneymaker. The binary vector pSLJ6201 was mobilized into *Agrobacterium tumefaciens* LBA4404. Transgenic plants were regenerated (15), and 10 independent transformants of each species were selected for further analysis.

Plant Material and Growth Conditions. The tomato cultivar Moneymaker (Cf0)and a near-isogenic line homozygous for *Cf-9* (Cf9), were used. Detailed analyses of plant development for various F_1 progeny were undertaken in a controlled environment room (16) and a tissue culture room.

To observe individual plant organ growth rates, surface-sterilized tomato seeds were spaced 1 cm apart in a line across the diameter of a 15-cm Petri dish containing Murashige and Skoog medium at pH 5.8 supplemented with 1% (wt/vol) glucose and 0.6% (wt/vol) agarose. The sealed Petri dishes were placed on their sides beneath the light source (70 microeinsteins·m^2·s) with the line of seeds horizontal.

RNA Gel Blot Analysis of Transformants. Total RNA was prepared from leaves and analyzed as described (17).

Intercellular Washing Fluid (IF) Preparation. Authentic *C. fulvum* race-specific elicitors were isolated in IF from Cf0 tomato leaves heavily infected with race 0 (18). IF was also prepared with leaves of various ages from all primary transformants.

Electrophoretic Analysis of the Protein Composition of Leaf IF Under Low-pH Nondenaturing Conditions. Low molecular weight proteins present in 4 ml of IF, obtained from transformed and untransformed plants, were recovered by differential acetone precipitation and centrifugation, a 40% (vol/vol) acetone step followed by an 80% (vol/vol) acetone step, and lyophilized. Proteins present in 250 μl of Cf0-race 0 IF were lyophilized directly. Protein samples were analyzed by electrophoresis on 10% polyacrylamide slab gels (19). To confirm the authenticity of the new peptide present in the IF recovered from active transgenic lines, the proteins present in 40 ml of IF from tobacco line 6201F and tomato line 6201B and 10 ml of Cf0-race 0, prepared as described above, were electrophoresed on separate 10% preparative gels and eluted and recovered according to the procedure of Scholtens-Toma and de Wit (11).

Analysis of Gene Expression Patterns Conditioned by the 35S Promoter. To ascertain the spatial and temporal pattern of expression of the *35S:SP:Avr9* gene, the expression of a comparable *35S:uidA:nos3'* construct (pSLJ732; ref. 13), hereafter referred to as *35S:GUS*, was examined in tomato. Histochemical localization of β-glucuronidase (GUS) activity in the progeny of two independent transformants, SLJ732A and G, each carrying a single copy of the transferred DNA (T-DNA) construct, was performed with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson (20). Tomato seedlings were fixed in 1% formaldehyde in 50 mM sodium phosphate buffer at pH 7 prior to X-gluc staining. Pollen and both pollinated and unpollinated styles from mature plants of the same transgenic lines were stained after removal from flowers.

Scanning Electron Microscopic (SEM) Analysis of Tomato Cotyledon Surfaces. Specimen casts were prepared by the procedure of Green and Linstead (21) and examined in a CamScan MR4 scanning electron microscope.

RESULTS

Generation and Initial Characterization of the *35S:SP:Avr9* Primary Transformants. A synthetic *Avr9* gene was constructed as shown in Fig. 1. To target the AVR9 peptide product to the apoplast, the SP sequence from the tobacco *Pr1a* gene (10) was placed 5' of the sequence encoding the mature 28-aa AVR9 peptide (5). To ensure constitutive and high level expression *in planta*, the synthetic *Avr9* sequence was placed between the 35S promoter and a *nos* termination sequence and 3' of the tobacco mosaic virus Ω leader (13, 14). Ten tobacco and 10 Cf0 tomato independent primary transformants were generated.

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    ATG GGA TTT GTT CTC TTT TCA CAA TTG CCT TCA TTT CTT CTT GTC
1   M  G  F  V  L  F  S  Q  L  P  S  F  L  L  V
                                                ↓
    TCT ACA CTT CTC TTA TTC CTA GTA ATA TCC CAC TCT TGC CGT GCC
16  S  T  L  L  L  F  L  V  I  S  H  S  C  R  A

    TAC TGT AAC AGT TCT TGT ACA AGA GCT TTT GAC TGT CTT GGA CAA
31  Y  C  N  S  S  C  T  R  A  F  D  C  L  G  Q

    TGT GGA AGA TGC GAC TTT CAT AAG CTT CAA TGT GTA CAT TGA
46  C  G  R  C  D  F  H  K  L  Q  C  V  H  *
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FIG. 1. Nucleotide and amino acid sequences of the synthetic *Avr9* gene (see text for details). Arrow indicates the end of the SP sequence. Codon preference used to generate the entire sequence was based on that of tomato.

To identify active lines and to test the effectiveness of the *35S:SP:Avr9* construct, the biological activity of IF retrieved from the leaves of each transformant was tested. Nine of 10 tobacco transformants and 7 of 10 tomato transformants produced active AVR9 peptide that gave gray necrosis in Cf9 plants within 24 h of injection, as shown in Table 1. In both tobacco and tomato the biological activity of the IF was correlated with mRNA transcript abundance but not T-DNA locus number (Fig. 2*A* and Table 1).

Analysis of the protein composition of IF recovered from active tobacco and tomato transgenic lines using low-pH nondenaturing electrophoresis revealed the presence of a new peptide with a mobility indistinguishable from that of authentic AVR9 as shown in Fig. 2*B*. In this specialized gel system, as shown by de Wit *et al.* (19), authentic AVR9 characteristically migrates ahead of the pathogenesis-related protein P14, in a region depleted of other peptides (figure 1 in ref. 19). Also, when this band was eluted from preparative gels (see *Materials and Methods*), both the peptide produced in transgenic plant and *C. fulvum* race 0-derived peptide specifically induced necrosis on Cf9 but not Cf0 tomato plants (data not shown).

Collectively, the above data indicate that the synthetic *35S:SP:Avr9* gene is capable of directing the synthesis and export of high amounts of active AVR9 peptide.

Table 1. T-DNA loci number and necrosis-inducing activity of IF retrieved from the leaves of tobacco and tomato (pSLJ6201) transformants containing the *Avr9* transgene

	Tobacco		Tomato	
Transformant	T-DNA loci*	IF titer†	T-DNA loci*	IF titer†
SLJ6201A	1	32	1	–
SLJ6201B	1	16	1	4
SLJ6201C	>3	–	2	–
SLJ6201D	1	8	1	8
SLJ6201E	1	4	1	2
SLJ6201F	>3	32	1	4
SLJ6201G	1	16	1	4
SLJ6201H	1	32	2	4
SLJ6201I	1	16	>3	–
SLJ6201J	>3	8	1	2

*Determined by the ratio of susceptible to resistant T_2 seedlings after selection on kanamycin (300 μg/ml)-containing medium.

†Value given is the dilution endpoint of necrosis-inducing activity of the IF on Cf9 plants. Assessments were done 1 day after injection (16). Response to IF of the eight Cf9 test plants injected was the same. –, No necrosis was visible after injection of the undiluted IF.

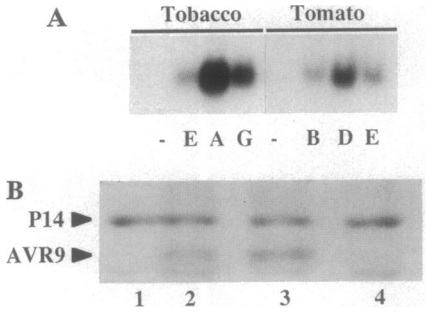

FIG. 2. (*A*) RNA gel blot analysis of *Avr9* mRNA transcript abundance in various tobacco and tomato primary SLJ6201 transformants. (*B*) Protein profiles of purified IF samples retrieved after electrophoretic separation under low-pH nondenaturing conditions. Lanes: 1, untransformed healthy Cf0 tomato after 14 days under the high-humidity conditions used for fungal infections; 2, untransformed Cf0 plants heavily infected with *C. fulvum* (day 14 postinoculation); 3, tomato transformant 6201B; 4, untransformed healthy Cf0 plants. Positions of the AVR9 peptide and the pathogenesis-related protein P14 are indicated.

Tomato Seed Production Was Not Affected by the *Avr9* Transgene in Crosses to Plants Possessing the *Cf-9* Resistance Gene. Three independent tomato transformants SJL6201D, -B, and -E producing high, intermediate, and low levels of AVR9, respectively (Table 1), were selected for further analysis. These transformants each contained a single T-DNA insertion locus, confirmed by a 3:1 segregation of kanamycin-resistant and -sensitive individuals in the self-progeny and by DNA gel blot analysis using T-DNA-specific probes (data not shown).

To assess the effect of the *Avr9* construct on *Cf-9*-containing lines, each transformant was crossed to Cf9 plants as both a male and female parent. Control pollinations involving nontransformed Cf0 were also undertaken. If the *Cf-9* resistance gene is expressed in pollen, pollen tubes, stigma, conducting tissue of the style, or the developing embryo, a successful defense response may be initiated in these tissues, and either no or impaired F_1 seed production would result. An analysis of the pattern of *35S:GUS* gene expression in transgenic tomato plants (construct pSLJ732) revealed high levels of GUS activity in pollen, at the stigma surface, and in developing embryo (Fig. 3 *A* and *B*; data not shown). GUS activity was not found in pollen germ tubes growing in the style or in the style conducting tissue (data not shown). No obvious differences were observed between the rate of fruit development arising from the various *Avr9* (Cf0) × Cf9 crosses and the wild-type Cf0 × Cf9 crosses. Similarly, the seed appearance and total seed yield per fruit were not altered in the presence or absence of the transgene (data not shown).

The *Avr9* Transgene Causes Developmentally Conditioned Lethality in Seedlings Possessing the *Cf-9* Resistance Gene. [*Avr9* (Cf0) × Cf9]F_1 seedlings developed at an identical rate for the first 10 days after sowing (i.e., for the first 5 days after root radicle emergence) even though these seedlings segregate 1:1 for the T-DNA. The first visual effects of the *Avr9* transgene were evident between 10 and 11 days after seed planting (dsp). Tomato hypocotyls, which normally show pink anthocyanin accumulations, began to turn green, and epidermal hair growth was impaired, as shown in Fig. 3*C*. Second, on day 10 the cotyledons of *Avr9*-containing seedlings closed to their normal vertical orientation as the dark photoperiod approached but failed to reopen completely in the next light photoperiod. Subsequently, all cotyledons of seedlings that had inherited the transgene remained constantly at an angle of >45° from the horizontal, as shown in Fig. 3*D*. After 11 days, a gray necrotic reaction of the

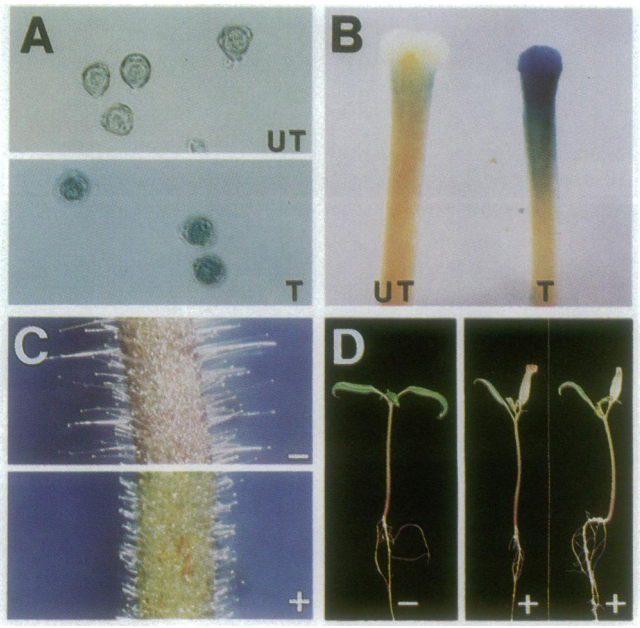

FIG. 3. (*A* and *B*) Histochemical detection of GUS activity in pollen (*A*) and stigma (*B*) of transgenic Cf0 tomato plants harboring a *35S:GUS* T-DNA (T) but not in untransformed Cf0 tomato plant (UT). (*C* and *D*) Appearance of Cf9 hypocotyl (*C*) and cotyledons (*D*) in the presence of the *35S:SP:Avr9* gene in seedlings from the cross Cf0 6201B (*35S:SP:Avr9*) heterozygote × Cf9, 11 days after seed planting. A seedling that had inherited the *Avr9* transgene (+) is compared to a seedling that had not (−). Cf9 seedlings that inherited the *35S:SP:Avr9* gene exhibited a loss of pink anthocyanin pigmentation from their hypocotyl (*C*) and their cotyledons were permanently oriented at an angle of >45° from the horizontal (*D*).

cotyledons developed, first at the cotyledon tips, then gradually spreading down the midvein toward the petiole and laterally to the cotyledon edges. Over the next 4 days, this gray necrotic reaction became progressively more severe, and by 15 dsp the cotyledons abscised. This sequence of events and the growth of sibling progeny that did not inherit the T-DNA are shown sequentially in Fig. 4. The temporal pattern of changes to seedling growth induced by the presence of the *Avr9* transgene was identical whether the Cf0 *Avr9*-expressing plant had been used as the male or female parent (data not shown).

A detailed quantitative analysis of the effects of AVR9 expression *in planta* on Cf9 organ growth rates revealed that the transgene had no effect on primary root growth or hypocotyl elongation up to 15 dsp (Fig. 3*D*; data not shown). Lateral root growth was also unaffected until gray necrosis appeared on the cotyledons but then ceased. Cotyledons did not expand after necrosis appeared at the tip. The maximum cotyledon size attained by $Avr9^+$ cotyledons was 11.8 ± 1.1 mm compared to 21.9 ± 1.9 mm for $Avr9^-$ sibling seedlings. The time of leaf 1 and 2 emergence was unaffected, but subsequent development was impaired (data not shown). The final lengths attained were 16 ± 1.6 mm (leaf 1) and 11.3 ± 2.0 mm (leaf 2). Necrotic flecks on leaf, stem, and petiole tissue were visible from day 14 onward. Essentially identical results were obtained with crosses involving primary transformants SLJ6201B, -D, and -E, which produced different levels of AVR9 peptide. Therefore, it appears unlikely that the onset of the lethal phenotype is determined by the concentration of apoplastic AVR9 peptide.

In transgenic tomato plants containing the *35S:GUS* gene, high levels of GUS activity were found throughout root, hypocotyl, cotyledon, and leaf tissues at all stages of plant development (data not shown). Thus, the response of Cf9 plants was probably not due to major differences in *35S:SP:*

FIG. 4. Development of the necrotic lethal phenotype in seedlings from the cross Cf0 6201B (*35S:SP:Avr9*) heterozygote × Cf9. A time course for the period 10–15 dsp is shown. In each panel, the two seedlings in the right pot inherited the T-DNA, while the two sibling seedlings in the left pot had not.

Avr9 gene expression in the various plant organs. Likewise, comparable levels of active AVR9 peptide could be recovered in soluble protein extracts prepared from root, hypocotyl, cotyledon, and leaf tissues both prior to and coincident with the appearance of the macroscopic response (data not shown).

SEM analysis of cotyledon surfaces revealed responding cells on day 10. Patches of epidermal cells at the cotyledon tip and alongside the midvein had entirely collapsed, but the stomatal guard cells within these areas had retained turgor and were swollen, as shown in Fig. 5*A*. At this stage of cotyledon development, stomata were occluded by wax plugs in both $Avr9^+$ and $Avr9^-$ seedlings (Fig. 5 *A* and *B*). On day 11, wax plugs were lost from stomata over the entire cotyledon surfaces. The sunken tissue patches of the $Avr9^+$ seedlings now contained supraoptimally open stomata, as shown in Fig. 5*C*. Within 3–6 h, all the responding cells in these areas were collapsed and shrunken (Fig. 5*D*). In the remainder of each $Avr9^+$ cotyledon surface, the only microscopic change then evident was a reduction in epidermal cell size, but, on day 12, stomata in other areas of the cotyledon also opened supraoptimally (data not shown). These areas of responding stomata expanded and the intervening epidermal cells and underlying tissue collapsed. By day 13, large areas of collapsed cells were present over the entire upper and lower cotyledon surfaces.

The Seedling Lethal Phenotype Caused by the *Avr9* Transgene is *Cf-9* Specific. When Cf0 plants expressing *Avr9* were crossed to tomato lines containing the *Cf-2*, *Cf-3*, *Cf-4*, *Cf-5*, *Cf-6*, *Cf-7*, *Cf-11*, *Cf-19*, or *Cf-21* resistance genes (16, 22), no major effects on plant growth and development were apparent (data not shown). The plants were assessed until the second fruit truss had set. These results indicate the AVR9 peptide generated *in planta* had the same specificity as the authentic *C. fulvum* AVR9 peptide produced *in planta*.

DISCUSSION

These experiments show the synthetic *35S:SP:Avr9* gene directs the synthesis of active AVR9 peptide in the apoplast.

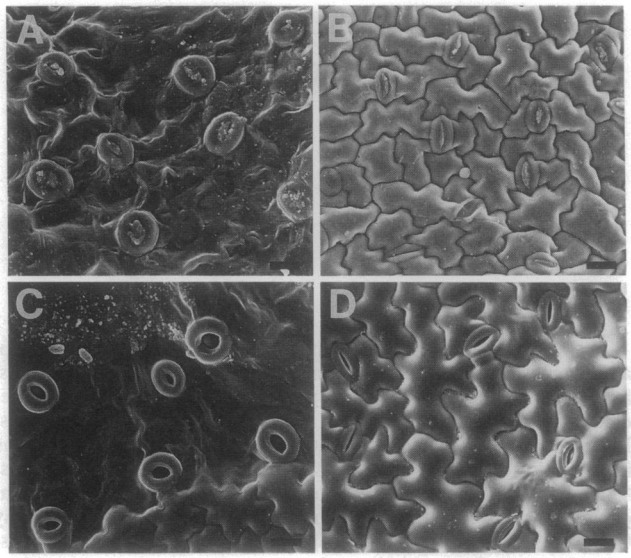

FIG. 5. SEM appearance of Cf9 cotyledon lower surfaces in seedlings from the cross Cf0 6201B (*35S:SP:Avr9*) heterozygote × Cf9. Each panel is representative of the region adjacent to the midvein and near the cotyledon tip. (*A*) $Avr9^+$ seedling with swollen stomata and collapsed epidermal cells (10 dsp). (*B*) A sibling $Avr9^-$ seedling with wax plug-occluded stomatal pores (10 dsp). (*C*) $Avr9^+$ seedling with swollen and now supraoptimally open stomata in the responding area because of wax plug loss (11 dsp). (*D*) A sibling $Avr9^-$ seedling with open stomatal pores (11 dsp). (Bar = 25 μm.)

The AVR9 peptide produced by plants carrying the synthetic *Avr9* gene exhibited a specificity of action identical to that of the authentic AVR9 peptide synthesized by the fungal pathogen *C. fulvum*. When the synthetic *Avr9* gene was crossed into tomato plants containing the *Cf-9* resistance gene, developmentally conditioned seedling lethality occurred. This is consistent with the idea that precocious activation of the defense response in otherwise healthy plants can be lethal. This adds credence to the idea that some necrotic or disease lesion mimic mutants may represent mutants in defense response regulation (23). An HR by plant cells was anticipated because injection of AVR9 peptide into leaves of a Cf9 plant causes a gray necrosis within 24 h (11). The HR was expected to involve numerous plant cells because of the extracellular targeting and high-level expression of the synthetic *Avr9* transgene. However, the exact timing and appearance of the lethality conferred by the combination *Cf-9*/*Avr9* could not have been anticipated. IF injection into tomato cotyledons before seedlings are 12 days old is not possible because of the lack of intercellular air spaces.

The *Avr9* transgene in combination with *Cf-9* did not appear to confer deleterious cellular effects during flower fertilization, embryo and fruit development, seed maturation, root development, and the formation of all young plant organs in germinating seedlings up to 5 days after root radicle emergence. The *35S:SP:Avr9* construct would have been expressed in all tissues and organs throughout this period of plant development except the pollen germ tube and conducting tissue of the style. These results can be interpreted in a number of ways. (*i*) Functional Cf-9 protein could be absent or at too low a concentration in pollen, style, seed embryo, roots, young cotyledons, and young leaves to trigger a discernible response. (*ii*) The level of Cf-9 protein could be adequate but the cells in these organs are physiologically incompetent to respond to the AVR9 peptide. (*iii*) The *Cf-9* gene is expressed and the tissues are physiologically competent, but they have additional mechanisms capable of suppressing the responses triggered. The isolation of the *Cf-9* gene will help resolve which interpretation is correct. Since

the 35S promoter did not appear to confer GUS expression in pollen tubes or conducting tissue of the style, no inference can be made about the interaction between Cf-9 and AVR9 in these tissues.

The exact onset of *Avr9* conditioned lethality of *Cf-9* seedlings was developmentally regulated and invariant. Visible HRs were evident sequentially, from day 5 after root radicle emergence, in the nominal series hypocotyl, cotyledon, leaf, stem, and petiole. Thus, in these tissues one or more of the criteria outlined above presumably no longer apply.

The most plausible explanation to account for the *Avr9*-induced *Cf-9* seedling lethality is the generation of reactive oxygen species combined with the supraoptimal opening of stomata leading to tissue desiccation. Vera-Estrella *et al.* (24) have reported that when Cf5 tomato cells were challenged with low concentrations of AVR5 elicitor, two of the earliest *Cf* gene-dependent-induced events were the generation of superoxide anions and lipid peroxidation. When AVR elicitors were injected into the cotyledons of Cf9 and Cf0 seedlings, lipid peroxidation was induced in Cf9 but not in Cf0 cotyledons within 1–2 h (M. May, K.H.-K., unpublished data). The loss of anthocyanin pigmentation from hypocotyls, arrest of day/night movement in cotyledons, absence of cotyledon expansion after day 11, and impaired growth of epidermal hair cells on hypocotyls may all be symptoms of elevated production of reactive oxygen species (25–28) in the *Cf-9*/*Avr9* seedlings.

The development of functional stomata in cotyledons appears to control the exact timing of the visible response in this organ. Although guard cells responded to the *Avr9* transgene and were swollen, no macroscopic phenotype resulted because each stoma was occluded with a wax plug. The subsequent loss of these wax plugs during normal cotyledon development, between 10 and 11 dsp, generated supraoptimally open stomata in the responding areas, and this caused rapid tissue desiccation and subsequent gray necrosis. However, the eventual tissue necrosis and abscission of cotyledons and young leaves in *Cf-9*/*Avr9* seedlings probably results from the cumulative consequences of reactive oxygen species, lipid peroxidation, and tissue desiccation. Future experiments should attempt to resolve the relative importance of these suspected mechanisms in *Avr9*-induced *Cf-9* seedling lethality.

It has been proposed that placing the *Avr9* gene under the control of a specific pathogen-induced promoter, in a genetic background carrying *Cf-9*, could provide a general mechanism for engineering disease resistance (9). These results show the feasibility of an important component of this scheme—namely, that functional AVR9 race-specific elicitor can be produced in plants. However, to avoid self-perpetuating necrosis, a promoter for the *Avr9* gene would need to be found that was not necrosis induced. Our studies have thus revealed a new problem: how is one to put together a resistance gene and an avirulence gene to activate defense mechanisms without this mechanism causing unacceptable levels of necrosis? It seems reasonable to hope that using cloned *R* genes and *Avr* genes, allelic variation can be engineered in the *R* gene to uncouple these phenomena.

A surprising feature of plants that contain *Cf-9* and *Avr9* is that defense mechanisms do not appear to be elicited in roots, suggesting a difficulty in using this system against root pathogens. With a cloned *Cf-9* gene, it will be possible to evaluate whether this problem is caused by a lack of *Cf-9* expression in roots. Our recent deployment of the *Avr9* gene for transposon tagging of the *Cf-9* gene should permit these ideas to be tested.

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1. Flor, H. H. (1971) *Annu. Rev. Phytopathol.* **9**, 275–296.
2. Keen, N. T. (1992) *Annu. Rev. Genet.* **24**, 447–463.
3. Long, S. R. & Staskawicz, B. J. (1993) *Cell* **73**, 921–935.
4. Chapman, S., Lindbeck, A. G. C. & Dawson, W. O. (1993) *Trends Microbiol.* **1**, 280–283.
5. Van Den Ackerveken, G. F. J. M., van Kan, J. A. L. & de Wit, P. J. G. M. (1992) *Plant J.* **2**, 359–366.
6. Joosten, M. H. A. J., Cozijnsen, T. J. & de Wit, P. J. G. M. (1994) *Nature (London)* **367**, 384–386.
7. Van Den Ackerveken, G. F. J. M., Vossen, P. & de Wit, P. J. G. M. (1993) *Plant Physiol.* **103**, 91–96.
8. Culver, J. N. & Dawson, W. O. (1991) *Mol. Plant Microb. Interact.* **4**, 458–463.
9. de Wit, P. J. G. M. (1992) *Annu. Rev. Phytopathol.* **30**, 391–418.
10. Cornelissen, B. J. C., Horowitz, J., van Kan, J. A. L., Goldberg, R. B. & Bol, J. F. (1987) *Nucleic Acids Res.* **15**, 6799–6811.
11. Scholtens-Toma, I. M. J. & de Wit, P. J. G. M. (1988) *Physiol. Mol. Plant Pathol.* **33**, 59–67.
12. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
13. Jones, J. D. G., Shlumukov, L., Carland, F., English, J., Scofield, S., Bishop, G. & Harrison, K. (1992) *Transgenic Res.* **1**, 285–297.
14. Gallie, D. R., Sleat, D. E., Watts, J. W., Turner, P. C. & Wilson, T. M. A. (1987) *Nucleic Acids. Res.* **15**, 3257–3272.
15. Fillatti, J. J., Kiser, J., Rose, R. & Comai, L. (1987) *Bio/Technology* **5**, 726–730.
16. Hammond-Kosack, K. E. & Jones, J. D. G. (1994) *Mol. Plant Microb. Interact.* **7**, 58–70.
17. Ashfield, T., Hammond-Kosack, K. E., Harison, K. & Jones, J. D. G. (1994) *Mol. Plant Microb. Interact.*, in press.
18. de Wit, P. J. G. M. & Spikman, G. (1982) *Physiol. Plant Pathol.* **21**, 1–11.
19. de Wit, P. J. G. M., Buurlage, M. B. & Hammond, K. E. (1986) *Physiol. Mol. Plant Pathol.* **29**, 159–172.
20. Jefferson, R. A. (1987) *Plant Mol. Biol. Rep.* **5**, 387–405.
21. Green, P. B. & Linstead, P. (1990) *Protoplasma* **158**, 33–38.
22. Higgins, V. J. & de Wit, P. J. G. M. (1985) *Phytopathology* **75**, 695–699.
23. Pryor, T. (1987) *Trends Genet.* **3**, 157–161.
24. Vera-Estrella, R., Blumwald, E. & Higgins, V. J. (1992) *Plant Physiol.* **99**, 1208–1215.
25. Harborne, J. B. (1986) *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships* (Liss, New York), pp. 15–24.
26. Halliwell, B. & Gutteridge, J. M. C. (1990) *Methods Enzymol.* **186**, 1–85.
27. Elstner, E. F. (1987) in *The Biochemistry of Plants*, eds. Stump, P. K. & Conn, E. E. (Academic, San Diego), Vol. 11, pp. 253–317.
28. Bradley, D. J., Kjellbom, P. & Lamb, C. J. (1992) *Cell* **70**, 21–30.