Uncoupling Resistance from Cell Death in the Hypersensitive Response of *Nicotiana* Species to *Cauliflower mosaic virus* Infection

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Cauliflower mosaic virus strain W260 elicits a hypersensitive response (HR) in leaves of *Nicotiana edwardsonii*, an interspecific hybrid derived from a cross between *N. glutinosa* and *N. clevelandii*. Interestingly, we found that *N. glutinosa* is resistant to W260, but responds with local chlorotic lesions rather than necrotic lesions. In contrast, *N. clevelandii* responds to W260 with systemic cell death. The reactions of the progenitors of *N. edwardsonii* to W260 infection indicated that each contributed a factor toward the development of HR. In this study, we present two lines of evidence to show that the resistance and cell death that comprise the HR elicited by W260 can indeed be uncoupled. First, we showed that the non-necrotic resistance response of *N. glutinosa* could be converted to HR when these plants were crossed with *N. clevelandii*. Second, we found that cell death and resistance segregated independently in the F₂ population of a cross between *N. edwardsonii* and *N. clevelandii*. We concluded that the resistance of *N. edwardsonii* to W260 infection was conditioned by a gene derived from *N. glutinosa*, whereas cell death was conditioned by a gene derived from *N. clevelandii*. An analysis of pathogenesis-related (PR) protein expression in response to W260 infection revealed that elicitation of PR proteins was associated with resistance rather than with the onset of cell death.

One of the most intensely studied plant defense mechanisms is hypersensitive response (HR). The HR is triggered in plants when a plant resistance (*R*) gene product recognizes a specific pathogen gene product called an avirulence (*avr*) gene (Baker et al. 1997; Flor 1971; Hammond-Kosack and Jones 1996). The outcome of this recognition event is a cascade of plant defense responses that includes the production of reactive oxygen species, pathogenesis-related (PR) proteins, systemic acquired resistance, and localized cell death at the initial infection site (Dangl et al. 1996; Dixon et al. 1994; Goodman and Novacky 1994; Ryals et al. 1996).

As resistance genes have been cloned, several studies have shown that they can be moved to other plants and their role in

elicitation of HR against avirulent isolates of bacteria, viruses, and fungi retained. For example, *Pseudomonas syringae* pv. *tabaci* strains that express *avrPto* will elicit HR in tobacco or *N. benthamiana* that has been transformed with the tomato *Pto* gene (Rommens et al. 1995; Thilmony et al. 1995). Similarly, transgenic tomatoes that express the tobacco *N* gene develop HR against *Tobacco mosaic virus* (TMV) (Whitham et al. 1996), and transgenic tobacco and potato plants that express the tomato *Cf-9* gene develop HR when inoculated with avirulent races of *Cladosporium fulvum* (Hammond-Kosack et al. 1998). Collectively, these studies have provided evidence that the machinery required for the elicitation of HR is conserved between certain plant species.

Although it has been shown that a single resistance gene is sufficient to trigger HR, through the years there has been considerable speculation about whether cell death is an absolute requirement for resistance conditioned by HR. As early as 1972, Király and coworkers suggested that the HR response elicited by *Phytophthora infestans* was incidental to disease resistance (Király et al. 1972). Studies of viral *avr* genes have shown that elicitation of the resistance response can become uncoupled from the elicitation of cell death. For example, the coat protein gene of TMV elicits HR in tobacco that carry the *N'* gene. Mutations within the coat protein will alter this *avr* gene product such that the virus will elicit a systemic HR (Culver and Dawson 1989). Similarly, a single amino acid change within the *Cucumber mosaic virus* 2a polymerase results in a systemic HR in cowpea, whereas the wild-type virus elicits HR that restricts the movement of the virus to the inoculated leaf (Kim and Palukaitis 1997). These mutagenesis studies of viral *avr* genes have led to the suggestion that different plant genes might be involved in eliciting HR and the localization response (Kim and Palukaitis 1997).

On the host side, evidence has accumulated that resistance and cell death can be separated genetically. For example, the potato *Rx* gene conditions an extreme form of resistance to *Potato virus X* (PVX) (Adams et al. 1986; Köhm et al. 1993). At first this resistance was not considered to be related to the HR induced by other *R* genes. The basic structure of the *Rx* gene, however, is similar to those of other *R* genes that do elicit HR. Furthermore, it has been shown that if the PVX coat protein (the viral avirulence gene product) is expressed in *Rx* plants in a transient transformation assay, then the coat protein will elicit HR (Bendahmane et al. 1999). Thus, it is thought

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that the resistance conditioned by *Rx* to PVX occurs so rapidly that the PVX coat protein cannot attain a concentration sufficient to elicit HR. A similar model has been proposed to explain the resistance of the *HRT* gene in *Arabidopsis thaliana* to *Turnip crinkle virus* (TCV). In this case, the *A. thaliana* ecotype that carries the *HRT* gene does respond to TCV with HR (Cooley et al. 2000). Transgenic plants that overexpress *HRT*, however, exhibit a micro-HR or no HR and are thought to be resistant to TCV because the plants respond rapidly to the infection (Cooley et al. 2000). Interestingly, transgenic plants that express lower levels of *HRT* respond to TCV with systemic HR, and a second recessive gene, designated *rrt*, has been found to be required for resistance (Kachroo et al. 2000). It also is possible to knock out the cell death pathway and retain resistance. Yu and coworkers (1998) isolated an *A. thaliana* mutant, *dnd1*, that lost the capacity to respond to *Pseudomonas syringae* pv. *glycinea* Race 4 with HR, yet still exhibited resistance to Race 4. Finally, there are many examples of resistance that are not associated with HR (Callaway et al. 1996; Leisner et al. 1993; Mahajan et al. 1998).

We recently characterized a unique gene-for-gene interaction in which a systemic cell death symptom was conditioned by a single gene of *Cauliflower mosaic virus* (CaMV) and a single gene in *Nicotiana clevelandii* (Király et al. 1999; Palanichelvam et al. 2000). We also found that the same viral gene, gene VI, is responsible for elicitation of HR in a related host, *N. edwardsonii* (Király et al. 1999; Palanichelvam et al. 2000; Schoelz and Shepherd 1988). Here we continue to examine the host side of this interaction. We present evidence that HR conditioned by *N. edwardsonii* can be dissected into resistance and cell death components and that the cell death component is derived from *N. clevelandii*. Furthermore, we found that the induction of PR proteins is associated with the resistance pathway rather than with the cell death pathway.

RESULTS

Reaction of *Nicotiana* spp. to infection by CaMV strains D4 and W260.

N. edwardsonii responds to infection by CaMV strain W260 with the development of HR (Fig. 1A). Necrotic local lesions appear approximately 8 to 10 days postinoculation (dpi), and the virus infection remains limited to the inoculated leaf. W260 virions could be detected in inoculated leaves, but were not found in the upper portions of the plant (Table 1), an indication that the resistance of *N. edwardsonii* to W260 infection involves a limitation of long-distance movement.

N. edwardsonii ($n = 34$) is an interspecific hybrid that originated from a cross between *N. glutinosa* ($n = 12$) and *N.*

clevelandii ($n = 24$) (Christie 1969; Christie and Hall 1979). Interestingly, when the two parent species were evaluated for their response to W260 infection, neither responded with HR development. Previously we have shown that *N. clevelandii* responds to W260 infection with local (Fig. 1C) and systemic cell death (Fig. 1D), and that this trait is conditioned by a single plant gene, designated *ccd1* (Király et al. 1999). Here we found that *N. glutinosa* was resistant to W260, but inoculated leaves developed local chlorotic lesions (Fig. 1B) rather than the necrotic local lesions typically associated with HR. W260 did not induce any symptoms in the upper, noninoculated portions of the plant and, although virion particles were detected in the inoculated leaves, they were not found in the upper leaves (Table 1). The reactions of the progenitors of *N. edwardsonii* to W260 suggested that its HR to W260 infection might be conditioned by two separate plant genes; resistance to W260 is derived from *N. glutinosa*, whereas cell death is derived from *N. clevelandii*.

In contrast to W260, the D4 strain of CaMV did not elicit a resistance response or cell death in *N. edwardsonii*, *N. glutinosa*, or *N. clevelandii*. D4 induced chlorotic primary lesions on the inoculated leaves at 8 to 10 dpi (Fig. 1E) and a chlorotic mottle on the upper portions of the plant at 18 to 22 dpi (Fig. 1F). Furthermore, D4 virions could be detected in inoculated and systemically infected leaves (Table 1), although the concentration in systemically infected *N. glutinosa* leaves was consistently lower than in *N. edwardsonii*.

Resynthesis of *N. edwardsonii*.

Initially we had planned to characterize the inheritance of resistance of *N. edwardsonii* to CaMV infection, but F_1 hybrid plants made between *N. edwardsonii* and *N. clevelandii* (the *Nicotiana* species susceptible to W260) were sterile. The inability to recover fertile hybrid plants might have been the result of an incompatibility between *N. edwardsonii* and our source of *N. clevelandii* or it might have been associated with the loss of chromosomes that occurred during the formation of *N. edwardsonii*. The genome of *N. edwardsonii* was predicted to contain 72 chromosomes: 24 contributed from *N. glutinosa* and 48 contributed from *N. clevelandii* (Christie 1969). Two pairs of chromosomes were lost during the formation of *N. edwardsonii*, however, so its genome consisted of 68 chromosomes instead of the full complement of 72 (Christie 1969).

To investigate whether the loss of the two pairs of chromosomes might have affected the fertility of hybrids between *N. edwardsonii* and other *Nicotiana* species, we resynthesized *N. edwardsonii* from a cross between *N. glutinosa* and *N. clevelandii* (Fig. 2A). Flowers on the F_1 plants were treated with

Table 1. Comparison of virion concentrations in *Nicotiana* species

Plant	<i>Cauliflower mosaic virus</i> strain			
	D4		W260	
	Inoculated leaf ^a	Upper systemic leaf ^b	Inoculated leaf	Upper systemic leaf
<i>N. edwardsonii</i>	1.0 ± 0.4 (4) ^c	2.0 ± 1.9 (14)	0.3 ± 0.1 (4)	0 (14)
<i>N. clevelandii</i>	2.7 ± 0.3 (2)	3.3 ± 2.6 (12)	1.0 ± 0.4 (5)	2.9 ± 1.0 (10)
<i>N. glutinosa</i>	0.5 ± 0.2 (4)	0.8 ± 0.8 (14)	0.3 ± 0.0 (2)	0 (14)
<i>N. edwardsonii</i> cv. Columbia	1.5 ± 0.9 (4)	4.4 ± 1.0 (14)	0.4 ± 0.1 (4)	0 (14)

^a Inoculated leaves were analyzed by enzyme-linked immunosorbent assay (ELISA) 20 to 25 days postinoculation (dpi).

^b Leaves were located near the inflorescence and were analyzed at 40 to 45 dpi.

^c The number of plants sampled by ELISA appears in parentheses.

Fig. 1. Reactions of *Nicotiana* spp. to inoculation with *Cauliflower mosaic virus* (CaMV) strains W260 and D4. **A**, Hypersensitive response (HR) elicited in *N. edwardsonii* leaves by W260. **B**, Chlorotic primary lesions induced in resistant *N. glutinosa* leaves by W260. **C**, Local cell death induced in W260-inoculated *N. clevelandii* leaves. **D**, Systemic cell death induced in *N. clevelandii* leaves infected with W260. **E**, Chlorotic primary lesions induced in susceptible *N. edwardsonii* leaves by D4. **F**, Systemic mosaic in susceptible *N. edwardsonii* leaves infected with D4. Local symptoms were photographed at 20 to 25 days postinoculation (dpi), whereas systemic symptoms were photographed at 40 to 45 dpi.

colchicine, as described by Gland (1981), and the seed recovered from those flowers was planted to identify plants with restored fertility. Fertile plants were recovered from this treatment, and a chromosome count revealed that the chromosome number of these plants had doubled to 72 (Fig. 2B), an expected consequence of the colchicine treatment.

The plants we recovered from our colchicine treatment differed in appearance from the original *N. edwardsonii*. The leaves and flowers of our hybrid were larger than those of the original *N. edwardsonii*, and its growth habit was more compact than *N. edwardsonii* (Fig. 2C and D). Subsequent generations derived from the resynthesized *N. edwardsonii* did not deviate from the plants derived from the colchicine treatment, demonstrating that the plant characteristics had been stabilized by the colchicine treatment. Because of the distinct morphological differences between the original and the resynthesized *N. edwardsonii* plants, we designated the resynthesized plants made in this study as *N. edwardsonii* cv. Columbia.

Columbia plants did react to CaMV strains in a manner similar to the original *N. edwardsonii*; they were resistant to CaMV strain W260 and susceptible to CaMV strain D4 (Table 1). In addition, the local reaction of Columbia was similar to that of *N. edwardsonii*; both types of plants responded with HR (cf. Figs. 1A and 3A), in contrast with the reaction of *N. glutinosa* (Fig. 1B). Thus, we could show that the nonnecrotic resistance response of *N. glutinosa* could be converted to HR after plants were crossed with *N. clevelandii*. Perhaps most importantly, when Columbia was crossed with *N. clevelandii*, the F₁ plants were fertile so an analysis of the inheritance of resistance and cell death in the F₂ population was possible.

Characterization of the inheritance of HR and cell death in Columbia in response to W260.

F₁ plants derived from a cross between Columbia and *N. clevelandii* responded to inoculation of W260 with primarily necrotic lesions (Fig. 3B). To characterize the resistance of the F₁ hybrids and the two parents to W260 infection, each plant was tagged and the number of leaves that exhibited systemic symptoms was scored on alternate days (Fig. 4). By 47 dpi, W260 had spread to an average of 34.5 leaves per *N. clevelandii* plant, whereas there was no evidence of systemic movement of W260 in Columbia. Two-thirds of the F₁ plants (22 out of 33) developed some systemic symptoms by 47 dpi, but in most cases the infection spread at a much slower rate in the F₁ plants than in *N. clevelandii* (Fig. 4). Four of the thirty-three plants did not appear to be resistant to W260 (Table 2) because virus symptoms were present on an average of 20.5 leaves on these plants. Enzyme-linked immunosorbent assay (ELISA) further confirmed that the four plants were susceptible because the titer of W260 virions in systemically infected leaves was comparable to that measured in *N. clevelandii* (data not shown). In the other F₁ plants, the concentration of W260 virions was significantly lower or undetectable. The majority of F₁ plants indicated that the resistance contributed by Columbia was incompletely dominant. One possible explanation for the four susceptible plants is that the chromosome that carries resistance to W260 was not transmitted to these plants. Alternatively, the genetic background of those four plants might have a negative influence on the incompletely dominant resistance gene.

Selected F₁ plants that exhibited resistance to W260 were selfed, and a total of 180 F₂ plants were evaluated in a series of four tests for local lesion type (necrotic versus chlorotic), degree of resistance, and the type of systemic symptoms, if symptoms developed at all. As in the F₁ test, each plant was tagged and the number of leaves that exhibited systemic symptoms was scored on alternate days throughout the duration of the test. One of the tests, which included 54 of the F₂ plants, is illustrated in Figure 5. At 47 dpi, systemic symptoms had developed on an average of 22.4 leaves per plant in F₂ plants that were characterized as susceptible, whereas in plants categorized as resistant, only 1.3 leaves per plant had developed systemic symptoms (Fig. 5). The difference in the rate of development of systemic symptoms allowed us to distinguish resistant plants from susceptible plants. This analysis revealed that the 180 F₂ plants segregated at a ratio of 2.6:1 for resistance versus susceptibility (Table 2), a ratio that is consistent with the hypothesis that resistance is conditioned by a single dominant gene.

In a second genetic test, the F₁ plants were backcrossed to *N. clevelandii* and evaluated for susceptibility to W260. These plants segregated at a ratio of 0.9:1 for resistance versus susceptibility (Table 2), further evidence that resistance is conditioned by a single dominant gene. Given the complex nature of the cross between Columbia (*n* = 36) and *N. clevelandii* (*n* = 24), however, it also is possible that at this point we observed the segregation of whole chromosomes rather than individual genes. To further characterize the inheritance of the W260 resistance factor, we are continuing to introgress this trait from Columbia into *N. clevelandii*.

Plants in both resistant and susceptible categories could be further characterized for the presence of local or systemic cell death. To correlate the local lesion response with the resistance response scored several weeks later, the leaves that developed local lesions on each plant were photographed while still attached to the plant. It is generally considered that cell death segregates with resistance (Baker et al. 1997; Dangl et al. 1996; Dixon et al. 1994), which was the case with many of the F₂ plants. They reacted to W260 with HR development (Fig. 3C) or, alternatively, with a systemic mosaic (Fig. 3F). We also observed two categories of plants in which cell death and resistance had become uncoupled. In one category, a significant number of F₂ plants developed a systemic cell death symptom (Fig. 3E). In fact, of the F₂ plants that were categorized as susceptible, a greater number developed systemic cell death symptoms than systemic mosaic symptoms. A second category consisted of plants that were resistant to W260, but developed chlorotic lesions rather than necrotic lesions (Fig. 3D). Both of these categories provided evidence that resistance and cell death are indeed conditioned by host genes that segregate independently. One gene derived from *N. glutinosa* conditions resistance to CaMV strain W260, whereas a second gene, derived from *N. clevelandii*, triggers cell death. The cell death gene derived from *N. clevelandii* was designated *ccd1* in a previous paper (Király et al. 1999).

CaMV resistance derived from *N. glutinosa* does not cosegregate with resistance to TMV conditioned by the *N* gene.

In addition to carrying a gene that conditions resistance to W260, *N. glutinosa* also is the source for the *N* gene, a single,

Fig. 2. The resynthesis of *Nicotiana edwardsonii* from a cross between *N. glutinosa* and *N. clevelandii*. **A**, Colchicine treatment of flowers of the *N. glutinosa* × *N. clevelandii* hybrid doubles the chromosome number and restores fertility in the F₂ generation. **B**, The 72 chromosomes of resynthesized *N. edwardsonii*, designated *N. edwardsonii* cv. Columbia, in mitotic metaphase. **C**, Growth habit of Columbia. **D**, Growth habit of *N. edwardsonii*.

dominant gene that confers resistance to TMV (Holmes 1960; Whitham et al. 1994). To determine if resistance triggered by W260 cosegregated with the *N* gene, 70 F₂ plants that were scored for their reaction to W260 were subsequently inoculated with TMV strain U1. The 70 plants comprised the members of the first two of the four host range tests involving W260 and the F₂ population derived from Columbia × *N. clevelandii*. To evaluate susceptibility and resistance, the presence or absence of HR was assessed 4 days after inoculation of TMV. Fifty-three of these plants responded to TMV with the development of HR, whereas 17 were susceptible, for a ratio of 3.1:1 (Table 2). This test provided a validation that we could track the segregation of single, dominant genes in the F₂ population derived from the cross of Columbia and *N. clevelandii*.

Of the 53 plants that responded to TMV with HR, 38 were also resistant to CaMV, whereas 15 were susceptible to CaMV. Of the 17 plants that did not develop HR in response to TMV, 13 were resistant and four were scored as susceptible to CaMV. Thus, in this test, we obtained a ratio of 9.5:3.8:3.2:1 ($\chi^2 = 0.349$) for the categories resistant to both viruses, resistant to CaMV and susceptible to TMV, susceptible to CaMV and resistant to TMV, and susceptible to both viruses. This analysis showed that the resistance specified by CaMV is unlinked to the *N* gene.

Induction of PR proteins by CaMV is associated with resistance rather than cell death.

One of the hallmarks of the HR to plant pathogens is the production of PR proteins (Bol et al. 1990). To determine whether the induction of PR proteins was associated with resistance or cell death, we inoculated selected *Nicotiana* species with CaMV strains W260 and D4. Because CaMV local lesions do not appear on inoculated leaves until 8 to 12 dpi, we first performed a time-course study with *N. edwardsonii* to determine the optimum time for PR protein expression analysis. Total proteins were isolated from inoculated leaves at 15 to 25 dpi, and protein extracts were subsequently probed with antibodies to PR-1 and PR-5. The 25-dpi time points are illustrated in Figure 6. We found that PR proteins were elicited weakly in *N. edwardsonii* leaves by W260 at 15 dpi (data not shown) and PR protein expression increased at 20 and 25 dpi. In contrast with W260, D4 did not elicit PR proteins in *N. edwardsonii* at 15 or 20 dpi, and only weakly elicited PR-1 protein by 25 dpi.

To determine if PR protein induction was associated with resistance or cell death responses, we evaluated the expression of PR-1 and PR-5 proteins in *N. glutinosa* and *N. clevelandii*. Both PR proteins were elicited by W260 in the inoculated leaves of *N. glutinosa*, the resistant host that responds with chlorotic lesions rather than with necrotic lesions (Fig. 6A). To demonstrate that expression of PR proteins was associated with a defense response elicited by the avirulent W260 strain, we showed that the virulent D4 strain did not elicit PR proteins in *N. glutinosa* above background levels. In contrast with *N. glutinosa*, PR-1 protein was not elicited in inoculated *N. clevelandii* leaves in response to W260 and PR-5 protein was elicited weakly only by W260 relative to the D4 strain of CaMV (Fig. 6A). To investigate whether *N. clevelandii* synthesized a protein that could be detected by the PR-1 antibody, we treated *N. clevelandii* leaves with 2,6-dichloroisonicotinic

Fig. 3. Segregation of cell death and Cauliflower mosaic virus (CaMV) resistance in an F₂ population derived from a cross between *Nicotiana clelandii* and *N. edwardsonii* cv. Columbia. **A**, Columbia leaf inoculated with CaMV strain W260. **B**, W260-inoculated leaf of an F₁ plant derived from a cross between Columbia and *N. clelandii*. **C**, W260-inoculated leaf from an F₂ plant that exhibits necrotic local lesions. Plant was scored as resistant at the end of the test. **D**, W260-inoculated leaf from an F₂ plant that exhibits chlorotic local lesions. Plant was scored as resistant at the end of the test. **E**, Leaf from an F₂ plant that was susceptible to W260 and illustrated the systemic cell death phenotype. **F**, Leaf from an F₂ plant that was susceptible to W260 and illustrated the systemic mosaic phenotype. **C to F**, Number of plants in each category is listed below each photograph.

acid (INA), a chemical inducer of PR proteins (Ryals et al. 1996). Protein extracts from these plants were probed with PR-1 antiserum and, as shown in Figure 6B, PR-1 protein was expressed in *N. clevelandii* 4 days after treatment with INA. This study demonstrated that PR protein production was associated with the resistance response to W260 rather than with cell death elicited by W260.

DISCUSSION

Genetic uncoupling of resistance to CaMV from cell death elicited by CaMV.

We have presented two lines of evidence to show that cell death associated with HR to CaMV can be uncoupled genetically from resistance. First, we showed that the nonnecrotic resistance response of *N. glutinosa* to W260 could be converted to HR. Under most environmental conditions, *N. glutinosa* responds to W260 with chlorotic local lesions. The colchicine-treated plants derived from the cross of *N. glutinosa* and *N. clevelandii* (i.e., Columbia), however, responded to W260 with primarily necrotic local lesions, evidence that *N. clevelandii* had contributed a factor that conditioned the local cell death. This is an interesting experiment because there are many forms of virus resistance that are not dependent on HR (Callaway et al. 1996). Our work indicates that some of these resistance responses may be similar to HR, but lack the cell death component.

In a second set of experiments, we showed that, in crosses between Columbia and *N. clevelandii*, resistance segregated independently of cell death in the F₂ population. The occurrence of four different classes of plants, i.e., resistant with necrotic lesions, resistant with chlorotic lesions, susceptible with systemic cell death, and susceptible with systemic mosaic is best explained if the genes that trigger cell death and resistance are unlinked. As a result of the complex nature of the cross between Columbia (*n* = 36) and *N. clevelandii* (*n* = 24), it is possible that the segregation of cell death and resistance might be influenced by the loss of whole chromosomes

in the F₂ progeny. We have shown previously that cell death in response to W260 infection is conditioned by *ccd1*, a single, recessive gene from *N. clevelandii* (Király et al. 1999). Consequently, F₂ plants that did not develop local or systemic cell death probably failed to inherit two copies of the chromosome that carries the cell death gene *ccd1*. Similarly, F₂ plants that were susceptible to W260 did not inherit the chromosome or allele that carries resistance to W260. Here we show that resistance to W260 in Columbia is derived from *N. glutinosa*, and our genetic data is consistent with the possibility that resistance is conditioned by a single, semidominant gene. Further crosses will be necessary, however, to complete the genetic characterization of resistance to W260.

The strategy we used to characterize the resistance of CaMV is similar to that used 75 years ago for the genetic analysis of the *N* gene. Clausen and Goodspeed (1925) introduced the *N* gene from *N. glutinosa* (*n* = 12) to *N. tabacum* cv. Purpurea (*n* = 24) by creating the interspecific hybrid *N. digluta* (*n* = 36). These plants were crossed with *N. tabacum*, and all responded with HR. To prove that resistance to TMV was conditioned by a single, dominant gene, the F₁ plants were selfed and backcrossed to *N. tabacum* (reviewed in Dunigan et al. 1987). In our case, we made the interspecific hybrid Columbia (*n* = 36) from a cross between *N. glutinosa* (*n* = 12) and *N. clevelandii* (*n* = 24). The Columbia plants were subsequently crossed with *N. clevelandii*, which is susceptible to W260, and nearly all responded with resistance and local cell death. The inheritance of resistance to CaMV was then characterized in the F₂ population and in a backcross population to *N. clevelandii*. One important aspect of our work was that we also examined our F₂ progeny for inheritance of the *N* gene. This control with the *N* gene allowed us to confirm that single genes would segregate properly in our crosses.

Association of PR protein induction by CaMV with resistance rather than cell death.

We also investigated whether induction of PR proteins was associated with either the resistance pathway or the cell death

Fig. 4. Development of systemic symptoms in leaves of *Nicotiana clevelandii*, *N. edwardsonii* cv. Columbia, and their F₁ hybrid after inoculation with *Cauliflower mosaic virus* strain W260. The number of leaves that exhibited systemic symptoms was counted on alternate days. Graph presents the average number of leaves per plant that had symptoms at a given time point.

Fig. 5. Development of systemic symptoms in leaves of *Nicotiana clevelandii*, *N. edwardsonii* cv. Columbia, and their F₂ population after inoculation with *Cauliflower mosaic virus* strain W260. The number of leaves that exhibited systemic symptoms was counted on alternate days. Graph presents the average number of leaves per plant that had symptoms at a given time point.

pathway elicited by CaMV gene VI. Interestingly, several studies have shown that PR proteins are not responsible for virus resistance. For example, transgenic plants that constitutively express PR-1 do not exhibit resistance to viruses (Cutt et al. 1989; Linthorst et al. 1989). The induction of PR proteins, however, is dependent upon an increase in salicylic acid (SA), and several studies have shown that SA is a component in the defense response to viruses (Dempsey et al. 1999). For example, resistance to TMV is mediated by an SA-dependent pathway that diverges from the pathway required for PR protein induction (Chivasa et al. 1997; Murphy et al. 1999). The analysis of PR protein expression is useful, however, because it is indicative of the defense pathways that are activated in response to CaMV.

To determine if CaMV could induce PR proteins in *Nicotiana* spp., we initially characterized the response of *N. edwardsonii* to the avirulent W260 strain and the virulent D4 strain. As expected, PR-1 and PR-5 proteins were elicited by W260, whereas the PR proteins were either elicited mildly or not elicited at all by the virulent D4 strain. The analysis of the parents of *N. edwardsonii*, *N. glutinosa* and *N. clevelandii*, indicated that induction of PR proteins by W260 was associated with resistance rather than cell death. Other studies have used biochemical approaches to show that induction of PR proteins can be uncoupled from cell death. For example, peptides that inhibit the function of caspases, a key component in programmed cell death in animals, can block HR-induced cell death in plants (Del Pozo and Lam 1998). The caspase inhibitors, however, have no effect on the induction of defense genes that are associated with HR. Furthermore, it has been shown that HR-induced cell death is inhibited at low oxygen pressure, but plants are still resistant to TMV (Mittler et al. 1996).

A model for the elicitation of HR and plant defenses by CaMV.

The present study has focused on the response of the host to infection by CaMV, but it is important to note that the genetics on the pathogen side of this interaction have been characterized in earlier papers. Evidence has existed since 1984 that CaMV gene VI acts as an avirulence gene in *Datura stramonium* (Daubert et al. 1984). Furthermore, gene VI of W260 was shown to be responsible for eliciting an HR in *N. edwardsonii* (Schoelz and Shepherd 1988), a resistance response in *N. glutinosa* (Schoelz and Wintermantel 1993), and systemic cell death in *N. clevelandii* (Király et al. 1999). Recently, an agroinfiltration assay has proven conclusively that the W260 gene VI protein is an *avr* protein and elicits HR in *N. edward-*

sonii and cell death in *N. clevelandii* (Palanichelvam et al. 2000). Consequently, the interaction between CaMV and *Nicotiana* spp. may represent an example of a unique gene-for-gene system, as illustrated in Figure 7. In this example, HR is conditioned by the interaction of a single viral avirulence gene, CaMV gene VI, with two host genes, one that triggers cell death and a second that triggers resistance. Our model differs from most others because we have placed the cell death pathway in parallel with the induction of plant defenses (Fig. 7), whereas in most other models cell death is placed upstream from plant defenses (Dangl et al. 1996; Ryals et al. 1996).

Our model for induction of resistance to CaMV also differs from models proposed for the function of the virus resistance genes *Rx* and *HRT*. These models describe a continuum from extreme resistance through micro-HR to HR (Bendahmane et al. 1999; Cooley et al. 2000). Extreme resistance occurs in the absence of HR through the normal function of the *Rx* gene or through overexpression of *HRT* in transgenic plants. In essence, no HR develops because the plant responds too rapidly for the virus to elicit HR. Micro-HR develops through moderate expression of *HRT* in transgenic plants, whereas a systemic HR develops in transgenic plants that express a low level of *HRT* (Cooley et al. 2000; Kachroo et al. 2000). In the case of extreme resistance against TCV, the plant defense response occurred so rapidly that an insufficient signal was produced for PR protein expression. In addition, PR proteins were weakly induced only in transgenic *HRT* plants that exhibited micro-HR (Cooley et al. 2000).

The nonnecrotic resistance against CaMV exhibited by *N. glutinosa* and within the F₂ population does not have characteristics of extreme resistance. Plants that displayed nonnecrotic resistance to CaMV had prominent chlorotic lesions (Figs. 1B and 3D), and PR proteins were strongly elicited in *N. glutinosa* by W260 (Fig. 6A). This differs from extreme resistance and micro-HR because no visible symptoms are associated with these two forms of resistance, and PR proteins are either not expressed or expressed very weakly. Furthermore, we found that non-necrotic resistance could be genetically combined with a cell death gene and the outcome of this cross was HR. Consequently, the form of resistance to CaMV that we have described may extend the continuum from extreme resistance through HR to the nonnecrotic CaMV resistance.

In addition to the differences in resistance response, the systemic cell death elicited by CaMV differs from TCV-induced systemic cell death, which occurred in transgenic *A. thaliana* plants that expressed relatively low levels of *HRT*

Table 2. Segregation of virus resistance and susceptibility in crosses of *Nicotiana edwardsonii* cv. Columbia and *N. clevelandii*

Host/Virus	Resistant	Susceptible	Observed ratio ^a	Expected ratio ^a	χ^2 (0.95:1)
<i>N. clevelandii</i> /Cauliflower mosaic virus strain W260	0	36	0.0:36		
Columbia/W260	36	0	36.0:0		
F ₁ /W260 ^b	29	4	7.2:1	33:0	0.485
F ₂ /W260 ^c	130	50	2.6:1	3:1	0.741
F ₁ × <i>N. clevelandii</i> /W260	11	12	0.9:1	1:1	0.044
F ₂ /Tobacco mosaic virus	53	17	3.1:1	3:1	0.005

^a Resistance to susceptibility.

^b F₁ = *N. clevelandii* × Columbia.

^c F₂ = selfed F₁(*N. clevelandii* × Columbia).

and lacked the recessive *rrt* gene. This recognition event resulted in a high level of the PR-1 protein. In contrast, neither PR-1 nor PR-5 proteins were strongly elicited in *N. clevelandii* plants that displayed prominent systemic cell death symptoms in response to an interaction between CaMV gene VI and *ccd1* (Fig. 6A). Consequently, it appears that a narrower subset of defense genes are activated by the interaction between CaMV gene VI and *ccd1* than between the TCV coat protein and the *HRT* gene product. The subset of genes activated by the CaMV-*ccd1* interaction may be valuable because

it isolates the cell death pathway from defense pathways.

The role of *ccd1* in activation of cell death may be more related to the *N* gene than to *HRT*. Recently it was found that two transcripts, *N_S* and *N_L*, are produced from the *N* gene (Dinesh-Kumar and Baker 2000). Both transcripts and their protein products appear to be required for resistance to TMV. Interestingly, transgenic plants that expressed only the *N_S* transcript developed a systemic HR upon inoculation with TMV. Consequently, *ccd1* may be a flawed form of the CaMV resistance gene that is present in *N. glutinosa*.

MATERIALS AND METHODS

Host response and virus detection.

The CaMV virus inoculum was prepared from infected turnips (*Brassica rapa* cv. Just Right), as previously described (Schoelz et al. 1986). The *Nicotiana* species and hybrids used in this study were inoculated 5 to 6 weeks after planting. Seeds were scarified by soaking in 2% (vol/vol) NaOCl for 15 to 20 min prior to planting, as described by Burk (1957). Virus-inoculated plants were maintained either in growth chambers set for a 10-h day with an average light intensity of 180 $\mu\text{mol s}^{-1} \text{m}^{-2}$ at 18°C or in the greenhouse during October to April. Symptoms were recorded daily, and the presence of CaMV virions in inoculated and systemically infected leaves was verified by ELISA (Clark and Adams 1977), as previously described (Anderson et al. 1991). All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) or Fisher (St. Louis, MO, U.S.A.), unless stated otherwise.

Resynthesis of *N. edwardsonii*.

An interspecific F_1 hybrid was obtained by crossing *N. clevelandii* (female) with *N. glutinosa* (male). One plant was used for colchicine treatment in order to create a hybrid with a stabilized chromosome number. Colchicine treatment employed a modification of the cotton-plug method described by Gland (1981). Colchicine-soaked cotton plugs were placed on immature flower buds, and the treatment was repeated 24 h later. The cotton plugs were removed 2 days after the start of

Fig. 6. Expression of pathogenesis-related (PR) proteins in *Nicotiana* spp. after inoculation with *Cauliflower mosaic virus* strains W260 and D4. **A**, Western blots that illustrate the expression of PR-1 and PR-5 proteins in healthy and virus-inoculated plants. Total proteins were isolated from inoculated leaves at 20 to 25 days postinoculation. **B**, Induction of PR-1 protein in healthy *N. clevelandii* and *N. glutinosa* plants by 2,4-dichloroisonicotinic acid.

Fig. 7. Elicitation of hypersensitive response (HR) in *Nicotiana edwardsonii* by *Cauliflower mosaic virus* (CaMV) strain W260. HR of *N. edwardsonii* to CaMV is determined by the interaction of CaMV gene VI with two plant genes. CaMV gene VI interacts with a resistance gene derived from *N. glutinosa* and triggers a plant defense response, including the production of pathogenesis-related proteins. CaMV gene VI interacts with a cell death gene, designated *ccd1*, that is derived from *N. clevelandii* and triggers a cell death pathway.

treatment. The treatment solution contained 0.05% (wt/vol) colchicine and 5% (vol/vol) ethanol with 0.5% (vol/vol) dimethyl sulfoxide as an adjuvant. Two fertile flowers were obtained and the seeds collected. Five F_2 plants were grown to maturity. The plants were uniform in appearance and vigor and were fertile. Each of the F_2 plants was selfed to obtain an F_3 generation. The F_3 plants and subsequent generations also were fertile and similar in appearance to the F_2 plants.

Cytological analysis.

Columbia seeds were sown in petri dishes on germination paper saturated with a 0.2% solution of Pipracil (piperacillin sodium) antibiotic in distilled H_2O . The petri dishes were wrapped in aluminum foil to eliminate light and maintained at room temperature overnight while seeds imbibed the solution. To synchronize germination and the cell cycle, the seeds were refrigerated for 3 days at $4^\circ C \pm 3^\circ C$, transferred back to room temperature overnight, and refrigerated for 1 day. Seeds were then allowed to germinate at room temperature for 2 days.

When roots attained a length of 10 mm, the root tips were cut, immersed in ice water, and refrigerated on ice for 24 h. Root tips were then fixed in 100% ethanol-glacial acetic acid (3:1 vol/vol) overnight. To visualize mitotic metaphase, the root tips were stained by the Feulgen reaction (Sharma and Sharma 1972) with the following modifications. Hydrolysis in 1 N hydrochloric acid was carried out at $60^\circ C$ for 10 min. Root tips were immersed in Schiff's reagent for 3 h at room temperature or refrigerated overnight. Root-tip meristems were squashed in 45% acetic acid on microscope slides and the chromosomes counted with an oil immersion 100 $\times$ objective photomicroscope III (Zeiss, Thornwood, NY, U.S.A.). Cells were photographed by video capture with a CCD video camera (Optronics, Goleta, CA, U.S.A.) and an Apple video player (Cupertino, CA, U.S.A.), version 1.7.1. Eight plants produced metaphase chromosomes, and three to six cells per plant were counted. All cells contained 72 chromosomes.

Western blot analyses for PR proteins.

Samples were prepared and electrophoresed, as previously described (Király et al. 1999). Proteins were transferred to nitrocellulose membranes for 1.5 h at 300 mA (constant current) in 1 $\times$ Towbin buffer (25 mM Tris, pH 8.2; 192 mM glycine; 0.1% [wt/vol] sodium dodecyl sulfate; 20% [vol/vol] methanol) (Towbin et al. 1979). The membranes were blocked overnight in blocking buffer at $4^\circ C$. The blocking buffer comprises phosphate buffered saline (PBS), pH 7.4; 5% (wt/vol) nonfat dry milk; 1% (wt/vol) bovine serum albumin (BSA); and 0.01% (vol/vol) antifoam A.

For PR-5, membranes were incubated at room temperature for 1.5 h with a 1:250 dilution of anti-PR-5 immunoglobulin G (IgG) in antibody dilution buffer (PBS, pH 7.4; 0.05% [vol/vol] Tween 20; 0.2% [wt/vol] BSA; and 2% [wt/vol] polyvinylpyrrolidone; estimated molecular weight 40,000). This was followed by an incubation for 1.5 h at room temperature with a 1:7,500 dilution of alkaline phosphatase-labeled goat-antirabbit IgG (Fisher). The membranes were washed between each step in PBS, pH 7.4, and 0.05% Tween 20. The same conditions were used for PR-1, with the addition of a 1:10,000 dilution of the anti-PR-1 IgG. This was followed by an incubation step with a 1:2,000 dilution of alkaline phosphatase-labeled rabbit-anti-mouse IgG (Southern Biotechnol-

ogy Associates Inc., Birmingham, AL, U.S.A.). In both cases, protein bands were visualized by the addition of 10 ml of alkaline phosphatase color developer (100 mM Tris, pH 9.1; 100 mM NaCl; 5 mM $MgCl_2$; 0.4 mM nitro blue tetrazolium chloride; and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate D-toluidine salt). PR proteins were induced with INA, as described by Ward et al. (1991). Samples were removed 2 and 4 days after infiltration for analysis of PR protein induction.

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