The Plant Gene *CCD1* Selectively Blocks Cell Death During the Hypersensitive Response to Cauliflower Mosaic Virus Infection

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The P6 protein of *Cauliflower mosaic virus* (CaMV) W260 elicits a hypersensitive response (HR) on inoculated leaves of *Nicotiana edwardsonii*. This defense response, common to many plant pathogens, has two key characteristics, cell death within the initially infected tissues and restriction of the pathogen to this area. We present evidence that a plant gene designated *CCD1*, originally identified in *N. bigelovii*, can selectively block the cell death pathway during HR, whereas the resistance pathway against W260 remains intact. Suppression of cell death was evident not only macroscopically but also microscopically. The suppression of HR-mediated cell death was specific to CaMV, as *Tobacco mosaic virus* was able to elicit HR in the plants that contained *CCD1*. *CCD1* also blocks the development of a systemic cell death symptom induced specifically by the P6 protein of W260 in *N. clevelandii*. Introgression of *CCD1* from *N. bigelovii* into *N. clevelandii* blocked the development of systemic cell death in response to W260 infection but could not prevent systemic cell death induced by *Tomato bushy stunt virus*. Thus, *CCD1* blocks both local and systemic cell death induced by P6 of W260 but does not act as a general suppressor of cell death induced by other plant viruses. Furthermore, experiments with *CCD1* provide further evidence that cell death could be uncoupled from resistance in the HR of *Nicotiana edwardsonii* to CaMV W260.

The hypersensitive response (HR) is an active defense response that is triggered in plants when a plant resistance gene product recognizes a specific pathogen avirulence gene product (Baker et al. 1997; Hammond-Kosack and Jones 1996). In this response, an array of plant defense-related pathways is activated that leads to cell death and to the restriction of the pathogen at the initial site of infection (Hammond-Kosack and Jones 1996; Heath 2000). This cell death response has been considered to be a form of programmed cell death that may have similarities with animal apoptosis (Gilchrist 1998; Greenberg and Yao 2004; Xu and Roossinck 2000). Traditionally, the cell death that is the hallmark of HR has been considered to be essential for limitation of the plant pathogen to the site of infection. However, genetic evidence has been accumulating that

HR-associated cell death and disease resistance may not be so tightly coupled as generally perceived. For example, the *Arabidopsis thaliana* mutants *dnd1*, *dnd2*, and *hlm1* lack the capacity to respond to *Pseudomonas syringae* with HR-related cell death but do exhibit typical gene-for-gene resistance (Balague et al. 2003; Clough et al. 2000; Jurkowski et al. 2004; Yu et al. 1998). In addition, several other defense gene products may enhance or repress the development of HR (Greenberg and Yao 2004).

Studies with plant viruses have also indicated that HR can be genetically separated from the plant defense response. We presented genetic evidence that cell death could be uncoupled from resistance in the HR of *Nicotiana edwardsonii* to *Cauliflower mosaic virus* (CaMV) W260 (Cole et al. 2001). *N. edwardsonii* is an amphidiploid derived from *N. clevelandii* and *N. glutinosa*; *N. glutinosa* is resistant to W260, but responds with chlorotic local lesions, whereas *N. clevelandii* responds with systemic cell death. The non-necrotic resistance of *N. glutinosa* to W260 could be converted to a HR through a single cross with *N. clevelandii* (Cole et al. 2001). In addition, cell death and resistance segregated independently in the F2 generation derived from that cross. The responses of F2 plants to W260 infection could be categorized into four distinct classes: HR, chlorotic lesions and resistant, systemic mosaic, or systemic cell death. It was postulated that *N. edwardsonii* plants develop HR in response to CaMV W260 because *N. glutinosa* contributes factors that lead to defense gene activation and *N. clevelandii* contributes the factors that result in cell death (Cole et al. 2001).

On the virus side of the CaMV-*Nicotiana* interaction, it has been shown that the P6 protein of CaMV is responsible for triggering systemic cell death in *N. clevelandii*, the non-necrotic resistance response in *N. glutinosa*, and HR in *N. edwardsonii* (Király et al. 1999; Palanichelvam et al. 2000). CaMV P6 is a multifunctional viral protein that interacts with host ribosomes to facilitate translation of the polycistronic 35S RNA (Bonneville et al. 1989; Park et al. 2001). P6 self-aggregates to form the amorphous inclusion bodies seen in individual cells (Li and Leisner 2002), is essential for replication of the virus (Kobayashi and Hohn 2003, 2004), and contributes to long distance movement of the virus (Schoelz et al. 1991). P6 is also a key determinant of pathogenesis in susceptible hosts (Cecchini et al. 1997; Daubert et al. 1984; Stratford and Covey 1989), as well as for avirulence in resistant hosts (Agama et al. 2002; Schoelz et al. 1986). Considerable effort is now being directed towards teasing apart the various functions ascribed to P6. For example, the elicitation

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of HR can be uncoupled from the translational transactivator function of P6 (Palanichelvam and Schoelz 2002), which indicates that the interaction of P6 with ribosomes might not contribute directly to pathogenesis or avirulence. However, it is significant to note that the same N-terminal portion of P6 is responsible for eliciting both HR-mediated cell death in *N. edwardsonii* and systemic cell death in *N. clelandii* (Király et al. 1999; Palanichelvam et al. 2000). Since we have postulated that *N. clelandii* contributes the cell death factor to the HR of *N. edwardsonii* (Cole et al. 2001), it would be expected that common elements within P6 would be responsible for eliciting both types of host response.

In the present study, we have examined whether a host gene might block HR-associated cell death triggered by CaMV P6. We had previously identified a locus, designated *CCD1*, that determines whether hybrids between two *Nicotiana* species respond to CaMV infection with a systemic cell death symptom (Király et al. 1999). Whereas *N. clelandii* responds to W260 with a systemic cell death symptom, *N. bigelovii* responds with a systemic mosaic. The cell death phenotype (*ccd1*) was recessive, which indicated that *N. bigelovii* contains a dominant allele (*CCD1*) that blocks cell death. We now present evidence that *CCD1* blocks both HR-mediated and systemic cell death induced specifically by P6 of CaMV W260. However, *CCD1* failed to block either HR-mediated or systemic cell death induced by Tobacco mosaic virus (TMV) and Tomato bushy stunt virus (TBSV).

RESULTS

Introgression of *CCD1* from *N. bigelovii* to *N. clelandii*.

To test if *CCD1* would suppress cell death after introgression into an *N. clelandii* background, we backcrossed a *N. bigelovii* × *N. clelandii* hybrid that contained *CCD1* to *N. clelandii* and then identified selfed lines that were homozygous for *CCD1*. A previous study had shown that F1 progeny of the cross between *N. clelandii* and *N. bigelovii* exhibited only a systemic mosaic phenotype upon infection with CaMV W260 (Table 1) (Király et al. 1999). A subsequent backcross, B1 (F1 × *N. clelandii*), produced a phenotypic ratio of 18:24 (mosaic/necrotic) in response to W260 infection, whereas further backcrosses (B2 and B3) showed phenotypic ratios of 14:11 and 9:13, respectively. Chi square analysis showed that none of the backcrosses displayed phenotypic ratios with a statistically significant difference ($P = 0.05$) from the expected 1:1 ratio. Self-fertilization of B3 flowers (mosaic phenotype) provided a 25:7 phenotypic ratio, which does not show a statistically significant difference from the expected 3:1 ratio. These results confirmed that *CCD1* acts as a single, dominant gene (Király et al. 1999).

Individuals from the self-fertilized B3 generation (B3 × B3) that responded with a systemic mosaic to W260 infection were themselves selfed to identify a line homozygous for *CCD1*. Out of four lines examined, three segregated for *CCD1*,

whereas one was identified as homozygous for *CCD1*, as all 33 tested individuals of this line responded to W260 infection with a systemic mosaic (Table 1). This line was designated *N. clelandii-SD*, for suppression of cell death. As shown in Figure 1, the growth habit of uninfected *N. clelandii-SD* plants was similar to the original *N. clelandii*. The leaves of young, uninfected *N. clelandii-SD* plants were somewhat narrower than the original (Fig. 1A and B), but this difference was less pronounced as the plants matured. Upon inoculation with CaMV W260, *N. clelandii-SD* responded with mosaic symptoms (Fig. 1D), whereas wild-type *N. clelandii* responded with a pronounced systemic cell death symptom (Fig. 1C). Thus, *CCD1* could block the induction of cell death by W260 in a *N. clelandii* background.

CCD1 might act directly to block the development of cell death or indirectly by affecting some steps of virus accumulation. To determine whether the virion concentration in *N. clelandii-SD* plants was altered relative to wild-type *N. clelandii*, we compared W260 virion concentrations in both types of plants at 25 to 30 days postinoculation (dpi) by enzyme-linked immunosorbance assays (ELISA). This timepoint was chosen because it was possible to find W260-infected leaves in *N. clelandii* that exhibited patches of cell death but were not completely dead. In this analysis, the CaMV concentration in *N. clelandii-SD* was equivalent to that in wild-type *N. clelandii* (Table 2). We also evaluated the effect of *CCD1* on the titer of CaMV D4, a strain that induces a mosaic in *N. clelandii* as well as in *N. clelandii-SD*. As with W260, the introgression of *CCD1* into *N. clelandii* had no effect on the titer of D4 (Table 2). Overall, the titer of D4 was higher than that of W260, but this was not unexpected, as it had been previously shown that D4 can attain higher titers than W260 (Király et al. 1999). Thus, *CCD1* did not attenuate or intensify the replication and spread of either W260 or D4 in *N. clelandii*.

Cell death suppression is specific to CaMV W260.

Several viruses in addition to CaMV elicit systemic cell death in *N. clelandii*. To determine if *CCD1* could block cell death pathways triggered by another plant virus, we inoculated *N. clelandii-SD* with TBSV, which is capable of inducing massive necrosis on wild-type *N. clelandii* plants. As shown in Figure 1E and F, there was no phenotypic difference in the response of *N. clelandii-SD* and wild-type *N. clelandii* to TBSV. Both types of plants developed extensive systemic cell death symptoms. An ELISA was used to compare TBSV virion concentrations in *N. clelandii* and *N. clelandii-SD* at 6 dpi, and it revealed that TBSV viral titers in the two hosts were indistinguishable. The absorbance obtained from TBSV-infected *N. clelandii* plants was 1.684 ± 0.149 ($n = 12$), whereas the absorbance for the TBSV-infected *N. clelandii-SD* plants was 1.609 ± 0.109 ($n = 12$). A *t*-test analysis showed no significant difference between the two means (*t* value = 1.403, $r^* = 2.074$). Therefore, *CCD1* does not have an effect on the titer of TBSV and it does not act as a general suppressor of cell

Table 1. Phenotypic response of interspecific crosses and backcrosses between *Nicotiana bigelovii* and *N. clelandii* after challenge with Cauliflower mosaic virus W260

Plant	Observed ratio (mosaic:necrotic)	Expected ratio (mosaic:necrotic)	χ^2	$P \geq 3.84$
F1 ^a (<i>N. bigelovii</i> × <i>N. clelandii</i>)	195:0	100:0	0.000	$P = 1.0$
B1 ^a (F1 × <i>N. clelandii</i>)	18:24	1:1	0.431	$P = 0.511$
B2 (B1 ^b × <i>N. clelandii</i>)	14:11	1:1	0.181	$P = 0.671$
B3 (B2 ^b × <i>N. clelandii</i>)	9:13	1:1	0.367	$P = 0.545$
B3 ^b Self	25:7	3:1	0.087	$P = 0.768$
<i>N. clelandii-SD</i>	33:0	100:0	0.000	$P = 1.0$

^a F1 and B1 data are from Király and associates 1999.

^b The backcross parent or selfed parent was selected for mosaic symptoms.

death; it appears to specifically inhibit cell death induced by CaMV.

CCD1 blocks HR-related cell death induced by CaMV W260.

CaMV W260 also elicits a HR in *N. edwardsonii*, as well as systemic cell death in *N. clevelandii*. *N. edwardsonii* is an amphidiploid between *N. glutinosa* and *N. clevelandii* (Christie

1969), and a recent study indicated that the *N. clevelandii* parent contributed the cell death component of the HR of *N. edwardsonii* to W260 (Cole et al. 2001). Consequently, it could be possible that the cell death component of HR might be blocked in the W260-*N. edwardsonii* interaction as well.

To determine if the cell death component of HR could be blocked, *N. edwardsonii* was crossed with *N. bigelovii*, the source of *CCD1*, to generate hybrid plants. After the *N. edward-*

Fig. 1. Phenotypic responses of *Nicotiana clevelandii*-SD to *Cauliflower mosaic virus* (CaMV) W260 and *Tomato bushy stunt virus* (TBSV) in comparison with wild-type *N. clevelandii*. **A**, Uninoculated *N. clevelandii*. **B**, Uninoculated *N. clevelandii*-SD. **C**, *N. clevelandii* illustrating the systemic cell death phenotype in response to CaMV W260. **D**, *N. clevelandii*-SD shows a systemic mosaic phenotype in response to CaMV W260. **E**, *N. clevelandii* leaves inoculated with TBSV. **F**, *N. clevelandii*-SD inoculated with TBSV.

Table 2. Comparison of phenotypic responses and virion concentrations in *Nicotiana* species after inoculation with *Cauliflower mosaic virus* (CaMV)

Host species	CaMV strain	Phenotype		Virion concentration	
		Inoculated leaf ^a	Upper leaf ^b	Inoculated leaf ^c	Upper leaf ^d
<i>N. clevelandii</i>	W260	N	N	nt	1.4 ± 0.3 (10) ^e
	D4	M	M	nt	15.2 ± 6.5 (4)
<i>N. clevelandii</i> -SD	W260	C	M	nt	1.4 ± 0.3 (10)
	D4	C	M	nt	20.4 ± 9.3 (4)
<i>N. edwardsonii</i>	W260	HR	θ	0.3 ± 0.1 (4)	0 (14)
	D4	C	M	1.0 ± 0.4 (4)	2.0 ± 1.9 (14)
<i>N. edwardsonii</i> × <i>N. bigelovii</i>	W260	C	C	0.6 ± 0.1 (4)	0.1 ± 0.3 (14)
	D4	C	M	2.5 ± 0.2 (4)	1.9 ± 3.0 (14)
<i>N. edwardsonii</i> × <i>N. clevelandii</i>	W260	HR	θ	0.7 ± 0.1 (2)	0 (14)
	D4	C	M	1.1 ± 0.0 (2)	2.8 ± 1.0 (14)

^a Local responses, N = necrotic local lesions, C = chlorotic local lesions, HR = hypersensitive response.

^b Systemic symptoms, N = systemic necrosis, M = mosaic, θ = no symptoms, C = chlorotic lesions.

^c The inoculated leaves were analyzed by enzyme-linked immunosorbance assays (ELISA) at 20 to 25 days postinoculation (dpi).

^d Leaves located near the inflorescence were analyzed by ELISA at 25 to 30 dpi for *N. clevelandii* and *N. clevelandii*-SD, or 40 to 45 dpi for *N. edwardsonii* and its hybrids.

^e The number of plants sampled by ELISA.

sonii × *N. bigelovii* hybrid plants were inoculated with CaMV W260, they responded with chlorotic local lesions (Fig. 2B). The hybrid plants remained resistant to systemic infection by strain W260, although the level of resistance was not as complete as in *N. edwardsonii*. Chlorotic spots did develop on upper,

noninoculated leaves of the F1 hybrid, and a low level of virus was detected in some of the upper, noninoculated leaves (Table 2). To demonstrate that the block in cell death was specific to a hybrid with *N. bigelovii*, *N. edwardsonii* was also crossed with *N. clevelandii* and the resultant hybrid was evaluated for its

Fig. 2. Phenotypic responses of *Nicotiana edwardsonii* hybrids to Cauliflower mosaic virus (CaMV) W260 and Tobacco mosaic virus (TMV). **A**, Necrotic lesions on CaMV W260-inoculated leaves of the *N. edwardsonii* × *N. clevelandii* hybrid. **B**, Chlorotic or cell death-suppressed lesions on the W260-inoculated leaves of the *N. edwardsonii* × *N. bigelovii* hybrid. **C** through **E**, Histochemical staining of CaMV-induced lesions and **F**, and **G**, TMV-induced lesions on inoculated leaves of *Nicotiana* spp. **C** and **F**, *N. edwardsonii*; **D**, *N. bigelovii*; **E** and **G**, *N. edwardsonii* × *N. bigelovii* hybrid. The insets in **C** through **G** show corresponding virus-induced lesions. Leaf disks were stained with Evans blue as described by Bowling and associates 1997.

response to W260. As seen in Figure 2A, the *N. edwardsonii* × *N. clevelandii* hybrid responded with a HR indistinguishable from that in wild-type *N. edwardsonii* (Cole et al. 2001). The *N. edwardsonii* × *N. clevelandii* hybrid plants were resistant to systemic movement of W260, as no symptoms developed in upper, noninoculated leaves and no virions were detected in these leaves (Table 2).

Although the degree of resistance of the *N. edwardsonii* × *N. bigelovii* hybrid to W260 was partially compromised, these

Fig. 3. Schematic models depicting genetic interactions in *Nicotiana* species susceptible and resistant to *Cauliflower mosaic virus* (CaMV). **A**, Phenotypic responses to CaMV W260 in the susceptible host, *N. clevelandii* (only homozygous recessive genotype *ccd1* responds with cell death.) **B**, Hypersensitive response (HR) induced in *N. edwardsonii*. **C**, Suppression of HR-related cell death in a *N. edwardsonii* hybrid with at least one copy of *CCD1*.

plants remained much more resistant than other susceptible host-virus combinations included in the test. For example, CaMV D4 was able to systemically infect both the *N. edwardsonii* × *N. bigelovii* hybrid and the *N. edwardsonii* × *N. clevelandii* hybrid as well as the wild-type *N. edwardsonii* (Cole et al. 2001). CaMV D4 induced chlorotic primary lesions and a systemic mosaic in all three hosts. An analysis of viral titers in the hybrids indicated that the D4 titer was significantly higher than that of W260 in both the inoculated and upper, noninoculated leaves (Table 2). However, W260 virion concentrations in *N. bigelovii* and *N. clevelandii* were approximately 14-fold greater than the concentration in the *N. edwardsonii* × *N. bigelovii* hybrid. This study underscored that cell death could be uncoupled from resistance, as the *N. edwardsonii* × *N. bigelovii* hybrid developed chlorotic lesions in response to W260 but retained a level of resistance to systemic movement of W260.

Histochemical staining and microscopic examination further confirmed the absence of cell death in the local lesions induced by W260 in the *N. edwardsonii* × *N. bigelovii* hybrid. Figure 2C shows a necrotic lesion (inset) from a *N. edwardsonii* leaf infected with CaMV W260. Evan's blue stain is retained in the dead cells and, when examined under the microscope, necrotic lesions exhibit a blue appearance. These lesions can be contrasted with the chlorotic lesions induced by W260 in *N. bigelovii* (Fig. 2D, inset), which did not retain the Evan's blue stain. Significantly, there was little difference between the lesions induced by W260 in the *N. edwardsonii* × *N. bigelovii* hybrid and those induced in *N. bigelovii* at either the macro- or microscopic level (Fig. 2E).

To investigate whether *CCD1* could block HR-related cell death associated with other pathogens, the *N. edwardsonii* × *N. bigelovii* hybrid was inoculated with TMV. The *N. edwardsonii* genome contains the *N* gene, a single dominant gene that conditions HR to TMV. Within 48 h of inoculation with TMV, *N. edwardsonii* leaves developed necrotic lesions, and these lesions retained the histochemical stain (Fig. 2F). In contrast to infection with CaMV, the TMV-infected *N. edwardsonii* × *N. bigelovii* hybrid yielded necrotic lesions, which also retained the histochemical stain (Fig. 2G). This study indicated that suppression of cell death associated with HR appeared to be specific to CaMV W260.

DISCUSSION

In this paper, we have shown that *CCD1* can block the development of HR-mediated cell death and systemic cell death induced by CaMV. Interestingly, *CCD1* had no effect on viral titer in systemic infections, as the level of W260 virus detected in *N. clevelandii*-SD was equivalent to that in wild-type *N. clevelandii*. Thus, the development of the systemic cell-death symptom in *N. clevelandii* did not appear to be associated with alterations in multiplication of W260. Similarly, the presence of *CCD1* had only a minor effect on the resistance response in *N. edwardsonii* × *N. bigelovii* hybrids. It appears that CaMV-induced cell death is largely superfluous to the resistance component of HR.

Many plant viruses induce systemic cell death in their hosts; in extreme cases, they are able to kill the entire plant. In some instances, a genetic relationship has been found between elicitation of systemic cell death and HR, although variations on this theme have emerged. On the virus side, it has been possible to create mutants of *Cucumber mosaic virus* such that one variant elicits HR in cowpea and another elicits systemic cell death in the same host (Kim and Palukaitis 1997). Furthermore, mutations in the coat protein of TMV can alter its recognition by the product of the *N'* gene, converting a HR to a

spreading, systemic cell-death symptom (Culver 2002; Culver and Dawson 1989). On the host side, point mutations within the *N* gene can impair its ability to restrict the spread of TMV, again resulting in systemic cell death (Dinesh-Kumar and Baker 2000). In addition to mutations within *R* genes, variations in *R* gene function and dosage may also influence host response. In the interaction of *Potato virus X* with the potato resistance gene *Rx*, the plant responds to infection so rapidly that there is not sufficient time for a HR to develop (Bendahmane et al. 1999). Furthermore, the product of the *I* gene in bean conditions an incompletely dominant form of resistance to *Bean common mosaic virus* in which the allele dosage and temperature can be manipulated to elicit a continuum of responses from immunity to HR to systemic cell death (Collmer et al. 2000). In each of these instances, the message is that systemic cell death may result from deficiencies in resistance mediated by *R* gene products.

On the other hand, systemic cell death symptoms may also be induced by viruses without the apparent involvement of *R* gene products. For example, silencing of the double-stranded RNA-dependent protein kinase inhibitor P58^{IPK} in *N. benthamiana* leads to death of the plant upon infection by TMV or *Tobacco etch virus* (Bilgin et al. 2003). Similarly, plant virus infections will kill *Arabidopsis* knockouts that lack P58^{IPK}. However, P58^{IPK} is not involved in mediating virus resistance, as silencing of the P58^{IPK} transcript had no effect on *N*-mediated resistance to TMV in transgenic *N. benthamiana* plants that express the *N* gene product. What is intriguing about this example is that P58^{IPK} directly interacts with the helicase domain of the TMV replicase (Bilgin et al. 2003), the same portion of the replicase responsible for *N*-mediated HR (Abbink et al. 1998; Erickson et al. 1999; Padgett and Beachy 1993). Instead of a role in resistance, P58^{IPK} appears to be necessary for maintenance of viral titer in infected plants (Bilgin et al. 2003). Hence, the helicase domain of the TMV replicase must interact in some manner with the *N* gene product to trigger HR, whereas the lack of an interaction of the helicase domain of TMV with host P58^{IPK} results in systemic cell death.

The interaction between CaMV and *Nicotiana* species may represent another variation of a gene-for-gene system in which HR is conditioned by the interaction of a single viral protein, CaMV P6, with two host gene products; one host gene product may trigger resistance, whereas the other, *CCD1*, may regulate the appearance of cell death. If the cell death allele (*ccd1*) is present and the resistance gene product is absent, as with *N. clevelandii*, then the outcome is systemic cell death (Fig. 3A). On the other hand, the presence of both *ccd1* and the resistance gene product results in a HR (Fig. 3B). The *CCD1* product blocks the cell death pathway in *N. clevelandii*-SD, such that the only symptom is a systemic mosaic, and blocks the cell death pathway in the *N. edwardsonii* × *N. bigelovii* hybrid, such that the hybrid responds with chlorotic local lesions and resistance (Fig. 3A and C). It is known that the end result of the CaMV resistance response is the induction of pathogenesis-related (PR) proteins; however, PR proteins are not induced by the cell death pathway (Cole et al. 2001).

Although *CCD1* was effective in blocking local and systemic cell death induced by CaMV, it could not block cell death induced by other viruses. For example, TBSV also induces a systemic cell death symptom in *N. clevelandii* and a HR in *N. edwardsonii* (Scholthof et al. 1995). However, the relationship between TBSV-elicited systemic cell death in *N. clevelandii* and HR in *N. edwardsonii* is not as straightforward as with CaMV, as two separate TBSV proteins are responsible for triggering these plant responses. The TBSV p19 protein elicits a systemic cell death symptom in *N. clevelandii* and HR in *N. tabacum*, whereas the TBSV p22 protein elicits HR in *N.*

edwardsonii (Scholthof et al. 1995). Mutations in the N-terminal portion of the p19 protein abolished the elicitation of cell death in both *N. clevelandii* and *N. tabacum*, but the resistance pathway remained intact in *N. tabacum* against the mutant viruses (Chu et al. 2000). In contrast, mutations in p22 that abolished HR in *N. edwardsonii* also conditioned systemic movement, an indication that cell death was important for effective defense in this host-virus combination (Chu et al. 1999). Our observation that *CCD1* was unable to block the development of cell death induced by TBSV suggests that the systemic cell death pathway elicited by TBSV p19 in *N. clevelandii* must have some steps that are branched out from the CaMV P6-mediated cell death pathway.

In addition to its inability to block the elicitation of systemic cell death by p19 of TBSV, *CCD1* also could not block the development of cell death in the HR that is induced by the interaction of the TMV replicase protein with the *N* gene (Abbink et al. 1998; Erickson et al. 1999; Padgett and Beachy 1993). Recent studies have focused on the identification of plant caspases or caspase-like proteins that are activated during the HR of *N* gene tobacco to TMV (Chichkova et al. 2004; Lam and del Pozo 2000). For example, a vacuolar processing enzyme that has caspase-1 activity has been shown to be essential for TMV-induced HR (Hatsugai et al. 2004). Our results indicate that either *CCD1* blocks the activity of these enzymes specifically in response to CaMV or, alternatively, CaMV induces a HR-mediated cell death pathway that is independent of these enzymes. Although our evidence indicates a divergence in cell death pathways triggered by CaMV and TMV, the signal transduction pathways for defense against CaMV and TMV must converge at some point, as both viruses elicit the expression of PR-1 proteins in *N. edwardsonii* (Bol et al. 1990; Cole et al. 2001). In the case of TMV, it has been shown that several plant genes are required for effective defense against this virus, including such genes as *EDS1*, *Rar1*, *COI1*, *SGT1*, *Hsp-90*, and the *NPR-1/NIM1*-like genes (Liu et al. 2002, 2004a and b; Peart et al. 2002), as well as mitogen-activated protein kinases (Jin et al. 2003; Liu et al. 2004b). It is quite likely that several of these plant genes may also contribute to resistance against CaMV, although this remains to be determined.

The high degree of specificity of *CCD1* distinguishes it from other plant genes that regulate HR-related cell death, such as the *Arabidopsis thaliana* mutants, *dnd1* and *dnd2/hlm1* (Balague et al. 2003; Clough et al. 2000; Jurkowski et al. 2004; Yu et al. 1998). The *DND1* and *DND2/HLM1* genes have been shown to encode a family of cyclic nucleotide-gated channel ion channel proteins (Balague et al. 2003; Clough et al. 2000; Jurkowski et al. 2004). It would be expected that mutations in these gene products would block cell death in any gene-for-gene interaction in *Arabidopsis*. If *CCD1* functioned in a similar manner, then it should have suppressed HR-mediated cell death induced by TMV. Furthermore, the *dnd1* and *dnd2/hlm1* mutants are lesion mimics and, as with many lesion mimics, they exhibit systemic acquired resistance (SAR), an enhanced level of broad-spectrum resistance to pathogens. In contrast, the incorporation of *CCD1* into the *N. clevelandii* genome or into the *N. edwardsonii* genome did not result in a lesion-mimic phenotype or constitutive expression of plant defenses. There were no significant differences in the titers of either TBSV or CaMV in *N. clevelandii* versus *N. clevelandii*-SD. Furthermore, there was no evidence that defenses mediated by salicylic acid were constitutively expressed in the *N. edwardsonii* × *N. bigelovii* hybrid. If SAR had been induced in the *Nicotiana* hybrid, we would have expected to see a significant reduction in the sizes of TMV or CaMV lesions, as was observed in other *Nicotiana* hybrids (Cole et al. 2004). These

observations indicate that the *CCD1* gene likely differs in function from *DND1* and *DND2/HLM1*.

The discovery that the true-to-origin phenotypic interaction of the introgressed *CCD1* with CaMV W260 in *N. clelandii* is important because it illustrates that this gene is fully functional in the *N. clelandii* background. As molecular maps are developed for *Nicotiana* species, it should be possible to employ a map-based approach to identify and clone *CCD1*. Transformation of *CCD1* into *N. clelandii* should constitute the final proof that this gene blocks cell death induced by CaMV.

MATERIALS AND METHODS

Plant materials.

Nicotiana spp. used in these experiments were grown from seed in a greenhouse at approximately 18 to 25°C, with natural light from November to March, which provided a relatively short day length of approximately 9 to 11 h. A commercial peat-based growing medium was used for both seed germination and subsequent cultivation. Nutrients were provided by applications of Osmocote (19-6-12) for long-term growth and supplemented with Peters (15-15-15) water-soluble solution as needed. Both nutrient sources were used in amounts consistent with manufacturer's recommendations for greenhouse-cultivated specimens. Hybridization of *Nicotiana* species was conducted in the greenhouse. The resultant seeds were collected, dried, and treated with a 2% (vol/vol) NaOCl for 30 min prior to planting to break dormancy, as described by Burk (1957).

Virus inoculation.

CaMV inoculum was obtained from CaMV-infected *Brassica rapa* cv. Just Right approximately five weeks postinoculation. Virions were partially purified and were inoculated as in Schoelz and associates (1986). *Nicotiana* species were inoculated with CaMV virions at approximately five to six weeks after germination. TMV U1 was maintained in tobacco plants or as purified virions. Infectious transcripts that were synthesized from a full-length cDNA clone of the TBSV cherry strain (Hearne et al. 1990) were used to infect *N. benthamiana* plants. After TBSV-typical symptoms developed systemically, virus inoculum was made by grinding systemically infected leaf tissues in virus inoculation buffer (0.05 M K₂HPO₄, 1% Celite, pH 7.0) and inoculated onto *Nicotiana* species and hybrids.

Detection of CaMV and TBSV virions in systemically infected leaves.

The level of CaMV virions in leaves was quantified by direct ELISA (Clark and Adams 1977), as described previously (Anderson et al. 1991). Plant tissues were collected between 20 to 25 dpi for inoculated-leaf assays; upper, noninoculated leaves were collected near the inflorescences between 40 to 45 dpi. The level of TBSV virions in leaves was quantified in a direct ELISA. Infected samples were ground in grinding buffer (1× phosphate buffered saline, 2% polyvinylpyrrolidone, molecular weight 40,000, 0.2% bovine serum albumin) and were loaded onto the plates at a dilution of 1:10. Antiserum against TBSV virions, both conjugated to alkaline phosphatase and unconjugated, were purchased from Agdia (Elkhart, IN, U.S.A.) and were used according to their directions. The substrate *p*-nitrophenyl phosphate was purchased from Agdia and was used according to their directions. The average absorbance in the healthy plant samples was 0.091 ± 0.050 (*n* = 6) compared with an average absorbance value greater than 1.6 in the infected samples. Both CaMV and TBSV samples were quantified using a Labsystems Multiskan plate reader (Fisher Scientific, St. Louis).

Histochemical staining and light microscopy.

Characteristic chlorotic and necrotic lesions were excised from the inoculated leaves using a 1.0-cm diameter coring device. The leaf disks were placed singly in microfuge tubes containing 1.0 ml of lactophenol-Evan blue stain solution (10 ml lactic acid, 10 ml glycerol, 10 g of phenol, 10 mg of Evan's blue stain dissolved in 10 ml of distilled water), were placed in a boiling water bath for 1 min, and were vacuum-infiltrated twice for 10 min each at 60°C, allowing the vacuum to return to atmospheric pressure over a 15-min interval. Leaf disks were destained with chloral hydrate solution (2.5 g of chloral hydrate per 1.0 ml of distilled water) for at least 30 min (Baker and Mock 1994; Keogh et al. 1980; Wilson and Coffey 1980). The disks then were mounted on depression-well glass slides and were photographed at 7.5 and 10.0× magnification using ASA 100-color negative film with an Olympus C35AD-4 camera back attached to an Olympus BH2 dissecting scope. Light intensity and exposures were controlled by an integrated Olympus Exposure Control Unit.

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