Temporal Expression of PR-1 and Enhanced Mature Plant Resistance to Virus Infection Is Controlled by a Single Dominant Gene in a New *Nicotiana* Hybrid

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A new variety of *Nicotiana edwardsonii*, designated *N. edwardsonii* cv. Columbia, expresses pathogenesis-related (PR) proteins in a temporal manner 45 to 49 days postplanting and also exhibits enhanced resistance to *Tobacco mosaic virus*, *Tobacco necrosis virus*, and *Tomato bushy stunt virus*. In contrast, PR proteins were not expressed in the original *N. edwardsonii* variety at comparable ages but were induced after onset of a hypersensitive response to viral infection. The temporal induction of PR proteins in ‘Columbia’ was correlated with increases in salicylic acid and glycosylated salicylic acid. Earlier studies noted that some *Nicotiana* hybrids derived from interspecific crosses constitutively express PR proteins, but the genetic basis of this phenomenon had not been investigated, likely because many interspecific *Nicotiana* crosses are sterile. However, the close genetic relationship between *N. edwardsonii* and ‘Columbia’ indicated that a hybrid between these two plants might be fertile, and this proved to be true. Genetic crosses between ‘Columbia’ and *N. edwardsonii* demonstrated that a single, dominant gene conditioned temporal expression of PR proteins and enhanced resistance. This gene was designated *TPRI* (for temporal expression of PR proteins).

One of the best-characterized host resistance responses to virus infection is conditioned by the *N* gene, a toll interleukin 1 receptor-nucleotide binding site-leucine-rich repeat (TIR-NBS-LRR)-type resistance gene derived from *Nicotiana glutinosa* (Marathe et al. 2002; Whitham et al. 1994). The *N* gene recognizes sequences within the helicase domain of the replicase protein of *Tobacco mosaic virus* (TMV) (Abbink et al. 1998; Erickson et al. 1999; Padgett and Beachy 1993), and this recognition sets in motion a cascade of plant defenses (Baker et al. 1997; Dixon et al. 1994) that limit the TMV infection to an area surrounding a small necrotic lesion in the inoculated leaf (a hypersensitive response [HR]). Host defense responses include production of reactive oxygen species (Dangl et al. 1996), activation of mitogen-activated protein kinases (Zhang and Klessig 2001), localized cell death at the initial infection

site (Mittler et al. 1996), and induction of salicylic acid with a subsequent increase in PR proteins (Bol et al. 1990).

Through the years, at least one anomaly has been noted concerning *N* gene-mediated resistance that has not been widely recognized; some *Nicotiana* species that contain the *N* gene respond with an initial HR but fail to contain the virus, permitting systemic invasion. This systemic movement of TMV occurs at temperatures well below the threshold temperature for inactivation of the *N* gene. Development of systemic necrosis in the presence of the *N* gene was actually noted by Holmes (1954) and has also been investigated by other researchers (Dijkstra et al. 1977; Zaitlin 1962). Zaitlin (1962) characterized systemic movement of TMV in *N. glutinosa* and *N. tabacum* cv. Xanthi-nc and concluded that the necrotic reaction could spread through vascular bundles in two ways, a relatively slow cell-to-cell manner and, more rapidly, through the phloem. This phenomenon is not due to selection of a TMV mutant, as only one isolate has been found to overcome *N* gene-mediated resistance (Padgett and Beachy 1993). Furthermore TMV recovered from systemically infected *N. glutinosa* did not exhibit an enhanced ability to infect *N. glutinosa* in subsequent inoculations (Zaitlin 1962). Consequently, it is considered that systemic movement of TMV in *N*-gene plants is due to effects on the host side of the interaction.

It is not known whether the failure of *N* gene-mediated resistance in some *Nicotiana* species is conditioned by the genetic background of the plant (McKinney and Clayton 1945) or by defects in the *N* gene itself, although experimental evidence indicates either hypothesis is plausible. For example, systemic necrosis due to movement of TMV in *N*-gene plants occurs if some component of the plant defense response is compromised. *N. tabacum* cv. Xanthi-nc tobacco, which expresses a *NahG* transgene, is unable to accumulate salicylic acid and, consequently, PR proteins (Delaney et al. 1994). In addition, antioxidant enzyme activity is decreased in *NahG* tobacco (Király et al. 2002). The impairment in these defenses results in development of necrotic symptoms that extend into the stem. On the other hand, alterations in expression patterns of the *N* gene may also lead to systemic necrosis (Dinesh-Kumar and Baker 2000). Thus, either mechanism could conceivably explain failure of *N* gene-mediated resistance in some *Nicotiana* species.

Another unusual feature of the *Nicotiana* genus is the tendency of some hybrids to constitutively express pathogenesis-related (PR) proteins, a phenomenon first noted in a cross

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between *N. glutinosa* and *N. debneyi* (Ahl and Gianinazzi 1982). The hybrids also exhibited enhanced resistance to virus infection, characterized by reduction in lesion size in response to inoculation with either TMV or *Tobacco necrosis virus* (TNV). Later studies have indicated that virus resistance can be correlated with increases in salicylic acid (Chivasa et al. 1997; Murphy et al. 1999) and is not due to increases in levels of PR proteins themselves (Carr et al. 1989; Cutt et al. 1989; Dumas and Gianinazzi 1986; Linthorst et al. 1989). However, the genetic basis for constitutive expression of PR proteins is unknown, because many *Nicotiana* interspecific crosses are sterile. Presumably though, both parental species contribute one or more genes that result in constitutive expression of PR proteins.

In this paper, we describe two varieties of *Nicotiana edwardsonii* that both contain the *N* gene but respond very dif-

ferently to TMV infection as well as to other viruses, such as TNV and *Tomato bushy stunt virus* (TBSV). *N. edwardsonii* is an amphidiploid derived from a cross between *N. glutinosa* and *N. clelandii*. Although the *N. edwardsonii* genome contains a copy of the *N* gene (Christie 1969), this host should be considered susceptible to TMV. In contrast, *N. edwardsonii* cv. Columbia is also derived from *N. glutinosa* and *N. clelandii* (Cole et al. 2001), but it is resistant to TMV infection. Furthermore, 'Columbia' can be distinguished from the original *N. edwardsonii*, because PR proteins are expressed in 'Columbia' in a temporal manner. Because of the close genetic relationship of the two varieties of *N. edwardsonii*, it has been possible to examine, for the first time, inheritance of spontaneous expression of PR proteins and enhancement in virus resistance that occurs in some *Nicotiana* hybrids.

Fig. 1. Comparison of the systemic movement of *Tobacco mosaic virus* (TMV) in *Nicotiana edwardsonii* and *N. edwardsonii* cv. Columbia. **A**, Necrotic local lesions induced by TMV in a *N. edwardsonii* leaf. **B**, Necrotic local lesions induced by TMV in a 'Columbia' leaf. **C**, Necrosis associated with the systemic movement of TMV in *N. edwardsonii* from the petiole of the inoculated leaf into the vascular tissue. **D**, Systemic movement of TMV in *N. edwardsonii* is illustrated by the advancement of necrosis in the vascular tissue of *N. edwardsonii*. At this stage, the necrosis has girdled the stem, resulting in death of the meristem. **E**, Senescence of a 'Columbia' leaf that had been inoculated with TMV.

RESULTS

The resistance response of *N. edwardsonii* to TMV infection is compromised, whereas 'Columbia' exhibits strong resistance.

N. edwardsonii responds to infection by TMV with HR, but the virus does not remain limited to the inoculated leaf. The movement of TMV into the petiole and stem tissues of mature *N. edwardsonii* plants was easily visualized as early as 10 days postinoculation, as TMV killed cells as it advanced (Fig. 1C). Enzyme-linked immunosorbent assays (ELISA) confirmed that upper necrotic tissues contained TMV virions (data not shown). Eventually the stem became girdled, and the top of the plant died (Fig. 1D). It is well-documented that the *N* gene is temperature-sensitive (Samuel 1931; Weststeijn 1981). To investigate whether ambient temperatures had exceeded the threshold for *N*-gene inactivation, greenhouse temperatures were monitored with a data recorder. The average daily high temperature never exceeded 23°C (data not shown), which is well below the 28°C threshold for *N*-gene inactivation.

In contrast to *N. edwardsonii*, mature 'Columbia' plants did not develop systemic TMV infections. TMV induced HR in 'Columbia' leaves and then spread to induce premature leaf senescence. Typically, the spread of the TMV infection could be visualized as yellowing and collapse of the petioles (Fig. 1E). However, virus infections did not spread beyond the petiole, as the infected leaf would senesce and drop from the plant. In contrast, inoculated *N. edwardsonii* leaves tended to remain on the plant, even after the leaf and petiole had turned completely necrotic (Fig. 1C). The premature senescence elicited in 'Columbia' leaves in response to TMV infection may be a part of the explanation why TMV was unable to reach the main stem of the plant.

To investigate whether the *N* gene of *N. edwardsonii* might contain a mutation that would cause it to be defective, total DNA was isolated from *N. edwardsonii* plants, and in a series

of overlapping polymerase chain reactions (PCR), its *N* gene was amplified and sequenced. We found nine differences within the 6,731 nucleotides sequenced that distinguished the *N* gene of *N. edwardsonii* from the published sequence (Whitham et al. 1994) (Table 1). Of these differences, one occurred in exon 1 but was silent, six occurred in introns, and two differences in exon 4 resulted in amino acid changes. To assess the significance of these coding changes, the relevant portions of the *N* genes from *N. glutinosa* and 'Columbia' were also amplified and sequenced. At each of the nine nucleotide positions, the *N*-gene sequences agreed with the *N. edwardsonii* sequence. Since neither *N. glutinosa* nor 'Columbia' exhibit the same degree of susceptibility to TMV as does *N. edwardsonii*, it is unlikely that the systemic movement of TMV in *N. edwardsonii* could be due to a defect in the coding sequence of the *N* gene.

PR proteins are temporally expressed in 'Columbia' plants.

Although *N. edwardsonii* and 'Columbia' are closely related genetically, they responded very differently to TMV infection. A possible explanation for this difference is that plant defenses might be spontaneously turned on in 'Columbia'. For example, some interspecific *Nicotiana* hybrids constitutively express PR proteins and, consequently, are more resistant to TMV infection (Ahl and Gianinazzi 1982). To determine if PR proteins were expressed in 'Columbia' plants, total proteins were extracted from healthy leaves of *N. edwardsonii* and 'Columbia' at weekly intervals, beginning at 7 days postplanting (dpp) and ending at 56 dpp. Protein extracts were then probed with PR-1 antibody by Western blotting.

Neither *N. edwardsonii* nor 'Columbia' plants younger than 42 dpp expressed PR-1 protein. Beginning at 49 dpp, PR-1 protein appeared in healthy 'Columbia' leaves (Fig. 2). In contrast, healthy *N. edwardsonii* leaves did not express PR-1 protein at any timepoint, although *N. edwardsonii* can express PR-1 protein during a HR to *Cauliflower mosaic virus* (CaMV) W260 (Fig. 2, lane 7). This temporal induction of PR-1 protein between 42 and 49 dpp in 'Columbia' plants was very reproducible.

Environmental stresses can also induce PR proteins. However, the *N. edwardsonii* and 'Columbia' plants were grown adjacent to each other, demonstrating that greenhouse conditions were not responsible for the induction of PR proteins in the 'Columbia' plants. Although some *Nicotiana* hybrids constitutively express PR proteins (Ahl and Gianinazzi 1982), to our knowledge, the response of 'Columbia' is the first example of temporal induction of PR proteins in a *Nicotiana* hybrid.

The enhanced resistance of 'Columbia' is correlated with the onset of PR protein induction and is not limited to TMV infections.

To investigate whether 'Columbia' plants become more resistant to TMV infection after the onset of PR protein expression,

Fig. 2. Expression of PR-1 protein in *Nicotiana edwardsonii* cv. Columbia is temporally regulated. Total protein extracts from *N. edwardsonii* (N.e.) and 'Columbia' (N.e.C.) were obtained at 42, 49, and 56 days postplanting (dpp) and were probed with antibodies to PR-1. The positive control for PR-1 protein expression was *N. edwardsonii* inoculated with *Cauliflower mosaic virus* W260, an interaction that results in a hypersensitive response (lane 7).

Table 1. Sequence comparison of the *N* gene derived from different *Nicotiana* species

Base	Location	Published sequence ^a	<i>N. edwardsonii</i> ^b	<i>N. glutinosa</i>	'Columbia'	Line 24	Amino acid change?
513	Exon 1	C	A	A	A	A	No
1963	Intron 3	A	C	C	C	C	
1964	Intron 3	C	A	A	A	A	
2327	Intron 3	A	C	C	C	C	
2457	Intron 3	C	A	A	A	A	
3037	Intron 4	–	+T	+T	+T	+T	
4052	Intron 4	–	+C	+C	+C	+C	
5500	Exon 4	G	T	T	T	T	Yes E to D
5699	Exon 4	T	G	G	G	G	Yes Y to D

^a *N* gene sequence determined from *N. tabacum* by Whitham and associates (1994).

^b The + sign indicates the addition of a nucleotide.

we compared the response of *N. edwardsonii* and 'Columbia' plants inoculated at 35 dpp with those inoculated at 60 dpp. All plants developed necrotic local lesions by 2 days postinoculation (dpi). As illustrated in Figure 3A, there was no significant difference in lesion size between the two varieties, when they were inoculated at 35 dpp. However, in those plants inoculated at 60 dpp, there was a significant difference in lesion size (Fig. 3B). The necrotic local lesions on the 'Columbia' variety were smaller than lesions on the inoculated leaves of the original *N. edwardsonii* (compare Fig. 1A and B). Thus, a reduced lesion size correlated with temporal expression of PR proteins.

To determine if temporal expression of PR proteins correlated with limitations in systemic movement of TMV, the TMV-inoculated plants used in the analysis of lesion size were monitored for 25 days after inoculation. Systemic movement was scored as development of necrosis in the stem at the base

of the petiole of the inoculated leaf. In the case of plants inoculated at an age of 35 dpp, stem necrosis and, thus, virus movement was evident in both varieties at 10 dpi (Fig. 3C), although a greater percentage of *N. edwardsonii* plants developed vascular stem necrosis than 'Columbia' plants. Interestingly, the maximum for 'Columbia' was attained at 15 dpi. At this point in the test, plants were 50 days old and temporal expression of PR proteins would have begun. In the case of plants inoculated at 60 dpp, all *N. edwardsonii* plants exhibited stem necrosis by 25 dpi (Fig. 3D). In contrast, only 4% of *N. edwardsonii* cv. Columbia developed stem necrosis in the same time frame (Fig. 3D). This study indicated that enhanced resistance to systemic movement of TMV correlated with induction of PR proteins in 'Columbia' plants.

Christie (1969) had noted that TMV could kill the apical meristem of *N. edwardsonii*. Of the young *N. edwardsonii*

Fig. 3. Comparison of the development of Tobacco mosaic virus (TMV) infections in *Nicotiana edwardsonii* and *N. edwardsonii* cv. Columbia plants inoculated at either 35 or 60 days postplanting (dpp). The white bars represent TMV infections in *N. edwardsonii*, whereas the black bars represent TMV infections in 'Columbia'. **A, and B,** Comparison of necrotic lesion sizes induced by TMV on the leaves of the two varieties, inoculated at 35 or 60 dpp. **C, and D,** Movement of TMV into the stem of *N. edwardsonii* and 'Columbia' following inoculation at 35 or 60 dpp. The observation at each timepoint represents the percentage of plants that exhibited stem necrosis. **E, and F,** Death of the apical meristem induced by TMV in *N. edwardsonii* and 'Columbia' plants inoculated at 35 or 60 dpp. The observation at each timepoint represents the percentage of plants in which the apical meristem has died.

plants (35 dpp) inoculated with TMV, nearly 28% developed systemic necrosis by 25 dpi that was severe enough to kill the apical meristem (Fig. 3E). Killing of the apical meristem of *N. edwardsonii* was even more pronounced in the older *N. edwardsonii* plants (60 dpp) (Fig. 3F). On average, 80% of the apical meristems of these plants died from girdling induced by systemic necrosis (Figs. 1B and 3F). In contrast, none of the api-

Fig. 4. Comparison of local lesions induced by *Tobacco necrosis virus* and *Tomato bushy stunt virus* in *Nicotiana edwardsonii* and *N. edwardsonii* cv. Columbia. **A.** The *N. edwardsonii* leaf is positioned on the left, and the 'Columbia' leaf is on the right. **B.** The 'Columbia' leaf is positioned on the left, and the *N. edwardsonii* leaf on the right.

cal meristems of either young (35 dpp) or old (60 dpp) 'Columbia' plants died. These results provided further evidence that the Columbia cultivar restricts TMV movement. They also showed that even young 'Columbia' plants were more resistant to TMV infection than young *N. edwardsonii* plants. However, as 'Columbia' plants aged, the difference in susceptibility to TMV between them and the original *N. edwardsonii* increased.

To determine if 'Columbia' plants display enhanced resistance to viruses other than TMV, we inoculated 'Columbia' and *N. edwardsonii* with TNV and TBSV. These plants were 50 to 80 days old at the time of inoculation, an age when 'Columbia' plants express PR proteins. All plants inoculated with TNV developed necrotic local lesions by 3 dpi. As illustrated in Figure 4A, there were significant differences in lesion numbers between the varieties. The number of necrotic local lesions following TNV inoculation was severalfold less on the 'Columbia' leaves than on inoculated leaves of the original *N. edwardsonii*, although the difference became less pronounced by 5 dpi. In addition, lesion size in 'Columbia' plants was about half or less than that in *N. edwardsonii*. Plants were kept for at least 30 dpi, and within this period TNV remained localized in the inoculated leaves (data not shown). A similar enhancement in resistance was observed with TBSV, as lesions were considerably smaller in 'Columbia' leaves relative to *N. edwardsonii* (Fig. 4B). Thus, 'Columbia' displayed generally enhanced resistance to virus infections. The enhancement in resistance to viruses that were unrelated to TMV indicated that this response was not directly mediated by the *N* gene.

Enhanced virus resistance and induction of PR proteins is associated with an increase in salicylic acid.

Salicylic acid (SA) is an endogenous signal involved in resistance to virus infections and induction of *PR-1* gene expression (Klessig and Malamy 1994; Ryals et al. 1996; Sticher et al. 1997). Levels of free SA and its conjugated forms increase during TMV infection of resistant tobacco that contains the *N* gene in parallel with the development of HR. These changes ultimately increase resistance to subsequent infections and induce PR-1 proteins (Hennig et al. 1993; Malamy et al. 1990, 1992).

To determine whether increased levels of salicylic acid are correlated with enhanced virus resistance and PR-1 induction displayed by 'Columbia', we assayed levels of free and conjugated SA in 'Columbia' and *N. edwardsonii* in 90-day-old

Table 2. Comparison of free and conjugated salicylic acid (SA) levels in *Nicotiana edwardsonii* and *N. edwardsonii* cv. Columbia at 90 days postplanting

Form if SA	N.e./mock ^a	N.e.C/mock	N.e./TNV	N.e.C/TNV	N.e./TMV	N.e.C/TMV
Free SA	0.12 ± 0.03	0.21 ± 0.08	0.16 ± 0.02	0.36 ± 0.07	5.76 ± 1.93	11.88 ± 1.57
Conjugated SA	0.15 ± 0.03	1.19 ± 0.75	0.15 ± 0.03	2.36 ± 1.22	2.72 ± 2.12	34.84 ± 2.24

^a N.e. = *N. edwardsonii*; N.e.C = *N. edwardsonii* cv. Columbia; TNV = *Tobacco necrosis virus*; TMV = *Tobacco mosaic virus*; measurement is µg/g fresh weight ± standard deviation.

Table 3. Temporal expression of PR-1 protein in *N. edwardsonii* and *N. edwardsonii* cv. Columbia and their subsequent progeny^a

Plant	PR-1 expression	No PR-1 expression	Expected ratio	Observed ratio	Chi ² (0.05;1)
<i>N. edwardsonii</i>	0	20			
<i>N. edwardsonii</i> cv. Columbia	20	0			
F1 (N.e. × N.e.C.)	47	0	1:0	1:0	0
B1 (F1 × N.e.)	30	6	1:1	5:1	16
B2 (B1 ^b × N.e.)	52	44	1:1	1.2:1	0.67
B2 Self (F2)	17	5	3:1	3.4:1	0.06
B3 (B2 × N.e.)	11	11	1:1	1:1	0
B3 Self (F3)	43	13	3:1	3.6:1	0.36

^a Temporal expression of PR-1 protein was determined at 60 days postplanting by Western blotting, using a PR-1 monoclonal antibody. Plants were scored for expression or nonexpression. N.e. = *N. edwardsonii* and N.e.C. = *N. edwardsonii* cv. Columbia.

^b The backcross 1, 2, and 3 parents (B1, B2, and B3) were selected for temporal expression of PR-1 protein.

plants that were either inoculated with TNV, TMV, or mock-inoculated. In mock-inoculated plants, the level of free SA was slightly higher in 'Columbia' than in *N. edwardsonii*, whereas conjugated SA was nearly eight times higher in 'Columbia' than in *N. edwardsonii* (Table 2). The differences between 'Columbia' and *N. edwardsonii* became more pronounced after infection with either TNV or TMV. Free SA levels were approximately twofold higher in 'Columbia' relative to *N. edwardsonii* after inoculation with either virus. However, the most dramatic differences could be seen in the levels of conjugated SA after inoculation with either virus. After inoculation with TNV, the level of conjugated SA was 15-fold higher in 'Columbia' leaves than in *N. edwardsonii* leaves (Table 2). Similarly, there was a 12-fold difference in conjugated SA levels between 'Columbia' and *N. edwardsonii* leaves after inoculation with TMV. The majority of the conjugated, acid hydrolyzable SA fraction we detected probably represents SA glucoside (SAG) (Hennig et al. 1993; Malamy et al. 1992). Although SAG is considered to be biologically inactive (Hennig et al. 1993), its hydrolysis to SA may require as little as 2 h. Therefore, SAG could be a storage form to be rapidly converted to SA after pathogen infection. The increases in SA and conjugated SA levels in 'Columbia' indicated that these plants were primed to resist pathogen infections before inoculation and, consequently, could respond more strongly after virus infection.

Temporal expression of PR-1 protein is conditioned by a single, dominant gene.

Although earlier papers noted that some *Nicotiana* hybrids derived from interspecific crosses constitutively express PR proteins (Ahl and Gianinazzi 1982), the genetic basis of this phenomenon had not been investigated, likely because many interspecific *Nicotiana* crosses are sterile. However, the close genetic relationship between *N. edwardsonii* and the 'Colum-

bia' variety indicated that a hybrid between these two plants might be fertile. To evaluate inheritance of one or more genes that condition temporal PR protein expression, we crossed 'Columbia' with *N. edwardsonii* and evaluated progeny for PR-1 protein expression in healthy leaves at 60 dpp. All F1 progeny expressed detectable PR-1 protein at 60 dpp (Table 3), which suggested that a dominant gene conditioned temporal expression of PR-1.

To further investigate inheritance of PR protein expression, we backcrossed the F1 plants with the original *N. edwardsonii*. Of the 36 B1 plants examined, 30 expressed PR-1, resulting in a 5:1 segregation of expressors to nonexpressors (Table 3). This atypical ratio might reflect unequal segregation of chromosomes from the two parents, as *N. edwardsonii* has 68 chromosomes (Christie and Hall 1979), while 'Columbia' has 72 chromosomes (Cole et al. 2001). To determine if the segregation ratio could be stabilized, hybrids were backcrossed two additional times with *N. edwardsonii*.

The near 1:1 segregation of PR-1 expression observed in the B2 population as well as the 1:1 ratio observed in the subsequent B3 population supported the hypothesis that temporal expression was a heritable, dominant trait (Table 3). The segregation of temporal expressors to nonexpressors in the F2 and F3 populations at near 3:1 ratios verified that a single, dominant gene conditions temporal expression of PR-1. This gene was designated *TPR1* (for temporal expression of PR proteins).

Identification of homozygous expressors and nonexpressors of PR-1.

From the 43 expressors in the F3 population, 14 plants were selected, in an attempt to identify homozygous temporal expressors of PR-1. In addition, two of the five nonexpressors from the F3 population were also selected to identify homozygous non-expressors. The individual plants were selfed, and 20 plants from each of the subsequent F4 lines were evaluated for expres-

Fig. 5. Identification of F4 lines that are homozygous for the temporal expression of PR-1 protein. Total protein extracts were obtained from selected F4 lines, *Nicotiana edwardsonii* (N.e.) plants, and *N. edwardsonii* cv. Columbia (N.e.C.) plants. The positive control for PR-1 protein expression was *N. edwardsonii* inoculated with *Cauliflower mosaic virus* W260 (N.e. W260), an interaction that results in a hypersensitive response.

Fig. 6. Comparison of the development of *Tobacco mosaic virus* infections in the F4 lines 24 and 58 as well as in *Nicotiana edwardsonii* and *N. edwardsonii* cv. Columbia plants, inoculated at either 35 or 60 days post-planting. The graph illustrates the percentage of plants that exhibited any stem necrosis during the period from 15 to 35 days postinoculation (dpi). *N. edwardsonii* is represented by filled triangles, F4 line 58 (nonexpressor line) by filled squares, 'Columbia' by open circles, and F4 line 24 (temporal expressor line) by Xs.

Table 4. Comparison of the sizes of *Tobacco mosaic virus*-induced lesions on the inoculated leaves of *Nicotiana* species

Host	5 Days postinoculation	8 Days postinoculation	11 Days postinoculation
<i>N. edwardsonii</i>	0.86 ± 0.15 (20) ^a	1.11 ± 0.17 (20)	1.23 ± 0.23 (20)
F4 Line 58	0.89 ± 0.13 (20)	1.09 ± 0.12 (20)	1.17 ± 0.22 (20)
F4 line 24	0.53 ± 0.19 (20)	0.80 ± 0.13 (20)	0.90 ± 0.10 (20)
<i>N. edwardsonii</i> cv. Columbia	0.72 ± 0.17 (20)	0.94 ± 0.10 (20)	0.91 ± 0.09 (20)

^a Values are expressed as lesion diameters in mm. The number of lesions measured is presented in parentheses.

sion of PR-1 at 60 dpp. In this manner, the F4 line 24 was identified as a homozygous expressor of PR-1, and the F4 line 58 was found to be a homozygous nonexpressor (Fig. 5). The identification of several heterozygous lines, represented by the F4 line 21 in Figure 5, validated this method for selection of homozygous lines. Line 24 was determined to have 68 chromosomes (data not shown), which demonstrated that *TPR1* had been introgressed successfully from 'Columbia' to *N. edwardsonii*.

Interestingly, expressors were shorter than nonexpressors (data not shown), a trait that has been associated with expression of SA. In fact, the homozygous nonexpressor lines were comparable in height to the original *N. edwardsonii*, whereas the homozygous expressor line was uniformly shorter than *N. edwardsonii*. Heterozygous lines were a mixture of short and tall plants, and this difference in height correlated with temporal PR-1 expression.

Our sequence analysis of the *N* gene from *N. edwardsonii* and 'Columbia' had revealed nine differences with the published sequence (Table 1). To ensure that the *N* gene of line 24 agreed with the sequence derived from *N. edwardsonii* and 'Columbia', the relevant portions of the *N* gene from line 24 were also amplified and sequenced. At each of the nine nucleotide positions, the *N*-gene sequences agreed with the *N. edwardsonii* sequence. This provided further evidence that the coding sequence of the *N* gene was not responsible for the temporal expression of PR proteins.

The homozygous expressor of PR-1 protein is more resistant to TMV than is the homozygous nonexpressor.

To confirm continued association of PR protein expression and TMV resistance in the F4 lines, 20 plants each of *N. edwardsonii*, 'Columbia', the homozygous expressor F4 line 24, and the homozygous nonexpressor F4 line 58 were inoculated with TMV at 60 dpp. The diameters of 20 lesions per cultivar were measured daily, until individual lesions could no longer be discerned. As shown in Table 4, necrotic lesions induced on the inoculated leaves of F4 line 24 were smaller than those on either F4 line 58 and *N. edwardsonii* but were the same size as those on 'Columbia'.

These plants were maintained for 35 dpi and were monitored daily for evidence of systemic TMV movement. As shown in Figure 6, TMV moved systemically in line 58 but not in line 24. By 35 dpi, all *N. edwardsonii* and F4 line 58 plants had developed vascular necrosis, indicative of systemic TMV movement. Since line 58 responded to TMV infection with HR, it demonstrated that it had retained the *N* gene and that *TPR-1* segregates independently from the *N* gene. In contrast, TMV was unable to move systemically in either 'Columbia' or in F4 line 24 plants.

Fig. 7. Comparison of flowering times of the F3 plants ($n = 43$) that temporally expressed PR-1 (open squares) to the flowering times of *Nicotiana edwardsonii* (X) and *N. edwardsonii* cv. Columbia (open triangles). The graph illustrates the percentage of plants that had begun to flower between 45 and 65 days postplanting.

This test provided further evidence that the enhanced resistance to TMV was linked to temporal PR-1 expression.

Temporal expression of PR-1 is not associated with flowering.

Flowering induces expression of PR proteins in leaves of healthy tobacco plants and in sepals of tobacco flowers (Fraser 1981; Lotan et al. 1989). Since 'Columbia' begins to flower approximately five days earlier than *N. edwardsonii*, the onset of flowering might be responsible for the induction of PR-1 protein expression in 'Columbia' plants. To determine whether the temporal PR-1 expression observed in 'Columbia' and its progeny was associated with flowering, we compared flowering times of *N. edwardsonii*, 'Columbia', and the PR-1 expressors selected from the F3 backcross population. As illustrated in Figure 7, there was no difference in flowering times between the F3 expressors and *N. edwardsonii*, demonstrating that early onset of flowering and PR-1 protein expression in 'Columbia' leaves are likely controlled by different genes.

DISCUSSION

It had previously been found that an interspecific hybrid between *N. glutinosa* and *N. debneyi* constitutively expressed PR-1 protein and had an enhanced level of resistance to TMV (Ahl and Gianinazzi 1982). The genetic basis of this phenomenon was not characterized in that paper, but the authors speculated that "it is possible that the interaction of their two foreign genomes provokes a permanent accumulation of" PR proteins.

In our paper, we found that PR-1 protein is temporally expressed in *N. edwardsonii* cv. Columbia, an interspecific hybrid between *N. glutinosa* and *N. clevelandii*. Interestingly, PR-1 protein was not temporally expressed in the original *N. edwardsonii*, although it could be induced after the onset of HR. The close genetic relationship between *N. edwardsonii* and 'Columbia' allowed us to examine, for the first time, the inheritance of temporal resistance in interspecific crosses, and our evidence indicates that a single dominant gene is responsible for the difference in temporal expression of PR proteins between the two plants. The effects of *TPR-1* can be observed in both inoculated and upper, noninoculated leaves. *TPR-1* contributed to a reduction in lesion size in the inoculated leaves and was also responsible for blocking systemic movement of TMV, when introgressed into *N. edwardsonii* (Fig. 6). At this point, we do not know if *TPR1* is derived from the *N. glutinosa* parent or the *N. clevelandii* parent. It is likely, though, that both *N. glutinosa* and *N. clevelandii* contributed genes to condition temporal expression in 'Columbia' and that the original *N. edwardsonii* received genes from only one of the parents. Since 'Columbia' contains two pairs of chromosomes that are missing in the original *N. edwardsonii* (Cole et al. 2001), it is also likely that *TPR1* can be localized to one of these two pairs.

Ahl and Gianinazzi (1982) reported constitutive PR protein expression, whereas we found that PR-1 expression was temporally regulated in 'Columbia', beginning about 45 days after planting. This discrepancy may reflect differences in experimental analyses rather than true biological differences, as PR-1 protein expression was not examined in the *N. glutinosa* × *N. debneyi* hybrid until two months after planting (Ahl and Gianinazzi 1982), well after the threshold for temporal induction in 'Columbia' plants. Consequently, PR proteins may be temporally expressed in the *N. glutinosa* × *N. debneyi* hybrid as well as in 'Columbia'.

There were no apparent phenotypic changes in 'Columbia' plants to indicate what happens around 45 dpp to induce SA and PR protein expression. There is, however, an intriguing parallel to the induction of SA in *N. edwardsonii*. The *N. edwardsonii*

genome contains multiple copies of the plant pararetrovirus *Tobacco vein clearing virus* (TVCV), and this virus is released into an episomal form 5 to 6 weeks after transplanting (Lockhart et al. 2000). In an analysis of the two parents of *N. edwardsonii*, TVCV appeared as an integrated form only in *N. glutinosa* plants, and it is absent in the *N. clevelandii* genome. It is speculated that the integrated form may be activated by the hybridization of the two parental genomes. Thus, hybridization of *N. glutinosa* with *N. clevelandii* results in mobilization of TVCV as well as induction of SA. The difference between the two phenomena is that SA is only induced in 'Columbia', whereas TVCV is activated in *N. edwardsonii* as well.

Developmental resistance to pathogens is a phenomenon that has not been well-characterized. Leisner and coworkers (1993) characterized a form of developmental resistance in *Arabidopsis* to CaMV that was related to sink-source relationships. As leaves mature, they change from acting as a sink for photoassimilates and virus to serving as a source. Leaves that mature before becoming infected tend to remain uninfected, because the virus movement into that leaf is impeded. Thus, as *Arabidopsis* plants age, an increasing proportion of leaves become resistant to virus infection. This type of developmental resistance would likely differ from *TPR1*, as it would not be based on induction of SA-dependent defense pathways.

Developmental resistance in *N. tabacum* has been examined in response to *Phytophthora parasitica*, and two components in this defense response have been characterized (Hugot et al. 1999). One component targeted infection effectiveness and was SA-independent. A second component, which restricted fungal expansion, was correlated with induction of PR proteins after the onset of flowering at 80 to 85 dpp. In contrast to their study, we found that induction of plant defenses in 'Columbia' was independent of flowering, as there was no correlation between the induction of PR1 and the timing of flowering (Fig. 7). Furthermore, PR1 protein was induced at a much earlier time in 'Columbia' (45 dpp) than in *N. tabacum* (80 to 85 dpp). Finally, the function of *TPR1* was only revealed in an interspecific cross between two *Nicotiana* species. Consequently, it is likely that *TPR1* is distinct from the plant defenses characterized in Hugot and associates (1999).

Several *Arabidopsis* mutant genes have been characterized that condition constitutive PR protein expression. These mutants include *cpr* (Bowling et al. 1994; Yoshioka et al. 2001), *cim* (Ryals et al. 1996), *acd* (Greenberg et al. 1994; Rate et al. 1999), *ssi* (Shah et al. 1999; Shirano et al. 2002), and *lsl* (Dietrich et al. 1994). Interestingly, the mutant *ssi4* has been shown to be a TIR-NBS-LRR class of resistance proteins (Shirano et al. 2002), which shows that mutations within *R* genes can lead to upregulation of SA levels. *TPR1* could be homologous to one of these types of genes, but differences in regulation of expression and associated phenotypes are worth noting. For example, *TPR1* conditions temporal expression of PR1 protein, whereas the other genes condition constitutive expression of PR proteins. It could be that expression of *TPR1* is itself turned on around 45 dpp and that this leads to the induction of SA and PR proteins. Furthermore, many of the *Arabidopsis* mutants spontaneously develop cell death, whereas no lesions are apparent on the 'Columbia' plants. As we learn more about the genome organization of *Nicotiana* spp., it should be possible to locate and clone *TPR1*. This will reveal how it compares with other plant genes that regulate SA levels.

***N. edwardsonii* is susceptible to TMV infection, in spite of the presence of the *N* gene.**

Papers that describe systemic TMV movement in *N* gene-containing *Nicotiana* species generally fall into two categories.

In some cases, systemic movement reflects inactivation of targeted components of the defense signaling pathway (Delaney et al. 1994; Dinesh-Kumar and Baker 2000; Liu et al. 2002; Peart et al. 2002). However in other cases, TMV moves systemically in certain *Nicotiana* species such as *N. edwardsonii*, even in spite of the *N* gene (Christie 1969; Dijkstra et al. 1977; Holmes 1954; Zaitlin 1962). The systemic movement of TMV in these plants and concomitant development of systemic necrosis indicates that the *N* gene by itself may not prevent infection in some cases. Either the genetic background of the plant is lacking some defense component or the *N* gene itself in these *Nicotiana* species is defective.

The differential response of *N. edwardsonii* and 'Columbia' to TMV inoculation allowed us to explore the genetic basis of systemic movement in the presence of the *N* gene. Our nucleotide sequence evidence revealed that the coding sequence of the *N* gene in *N. edwardsonii* matched that of *N. glutinosa* and 'Columbia' (Table 1). This indicates that the *N* gene in *N. edwardsonii* is functional in recognition of TMV, but the genetic background of the original *N. edwardsonii* may compromise its defense response. In contrast, the genetic composition of 'Columbia' actually enhances the defense response of this plant to a range of pathogens, and presumably, *TPR1* would be one component of this enhanced defense response. *TPR1* may function in several ways to enhance mature plant resistance to TMV in 'Columbia'. The most pronounced effect involves upregulation of SA and PR proteins. Older 'Columbia' plants exhibited an enhanced level of conjugated SA, even when they were uninfected; this enhancement in conjugated SA levels became more pronounced after inoculation with TMV. One interpretation of this phenomenon is that older 'Columbia' plants may be primed to resist infections and this allows their defenses to respond even more rapidly after infection by an avirulent pathogen.

SA is an important signal in the induction of virus resistance, likely through the induction of an alternative oxidase (Chivasa et al. 1997; Murphy et al. 1999). This is consistent with enhanced resistance to TBSV and TNV in mature 'Columbia' plants. Further studies have shown that 'Columbia' exhibits enhanced resistance to infection by *Pseudomonas tabaci* and *P. phaseolicola* (data not shown), which would be consistent with a general enhancement of plant defenses due to elevated SA levels. The temporal induction of plant defenses in 'Columbia' may provide a new tool for studying plant defenses in *Nicotiana*. As microarrays for tobacco become available, it will be useful to examine which genes are specifically turned on in response to the temporal synthesis of the SA signal.

Another intriguing potential function of *TPR1* may involve the activation of senescence pathways in the leaf. In the original *N. edwardsonii*, virus infections in the petiole of inoculated leaves could be clearly delimited into necrotic and healthy sectors. In contrast, the petioles of inoculated 'Columbia' leaves would turn yellow, collapse, and abscise prematurely from the stem. The development of an abscission layer may physically prevent the virus from escaping through the petiole of an inoculated leaf to the rest of the plant. Leaf senescence and the HR are both forms of programmed cell death (Dangl et al. 2000). HR may hasten development of leaf senescence in 'Columbia' to stop pathogen infections.

MATERIALS AND METHODS

Viruses and plants.

The synthesis of *N. edwardsonii* is described in Christie (1969), whereas the synthesis of *N. edwardsonii* cv. Columbia is described in Cole and associates (2001). TMV and TBSV inocula were prepared from infected tobacco (*N. tabacum*) and

N. benthamiana leaves, respectively, by grinding infected leaves in a mortar with a pestle and diluting to approximately 1:20 (wt/vol) with inoculation buffer (0.05 M potassium phosphate buffer, pH 7.0). TNV inoculum was prepared similarly, except that dilution was 1:10 (wt/vol) with inoculation buffer, which was 0.1 M potassium phosphate, pH 7.0. CaMV W260 inoculum for induction of PR-1 protein in *N. edwardsonii* was prepared as described in Cole and associates (2001). Inoculated leaves were lightly dusted with carborundum. The *N. edwardsonii* plants, 'Columbia' plants, and their hybrids were inoculated at either 35 or 60 days after planting (in case of TNV inoculations, 50, 80, and 90 days after planting). Seeds were scarified by soaking in 2% (vol/vol) NaOCl for 30 min prior to planting, as described by Burk (1957). Virus-inoculated plants were maintained in the greenhouse during the months of October to April. Greenhouse temperatures were continuously monitored with a datalogger (Campbell Scientific, Logan, UT, U.S.A.). Root tips of line 24 were prepared for cytological analysis according to Cole and associates (2001). Six plants produced mitotic metaphase chromosomes and two to ten cells per plant were counted. All cells contained 68 chromosomes.

Sequencing of the *N* gene from *Nicotiana* species and hybrids.

Total DNA was isolated from *Nicotiana* leaves by the procedure of Dellaporta and associates (1983). The *N* gene was amplified in a series of overlapping PCR, and amplified DNA fragments were sequenced directly at the DNA sequencing core at the University of Missouri-Columbia. Primer sequences were derived from the published *N*-gene sequence (Whitham et al. 1994) and were synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). All nucleotide differences were verified by determining the sequence of the affected region in both directions.

Western blot analysis for PR-1 protein.

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× 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 (phosphate buffered saline [PBS; pH 7.4], 5% [wt/vol] nonfat dry milk, 1% [wt/vol] bovine serum albumin [BSA], 0.01% [vol/vol] Antifoam A) at 4°C.

Membranes were incubated at room temperature for 1.5 h with a 1:10,000 dilution of anti-PR-1 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 step using a 1:2,000 dilution of alkaline phosphatase-labeled rabbit-anti-mouse IgG (SBA, Birmingham, AL, U.S.A.). 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 MgCl₂, 0.4 mM nitro blue tetrazolium chloride, 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt).

Analysis of free and conjugated forms of salicylic acid.

Free and conjugated (acid hydrolyzable) forms of SA were analyzed as described by Meuwly and Métraux (1993), with minor modifications. *Ortho*-anisic acid (2-methoxybenzoic acid, oANI) was used as an internal standard, in order to take account of losses during extraction, and *para*-hydroxybenzoic acid (pHBA) served as an extraction carrier. The initial extract was centrifuged at 8,000 × *g* for 20 min. Following resuspen-

sion in 90% (vol/vol) methanol, samples were recentrifuged as above. Supernatants were combined in a total volume of 2 ml, and the methanolic portion was evaporated at room temperature in a vacuum centrifuge. Trichloroacetic acid (1 ml of 5% [wt/vol]) was added to the remaining aqueous phase (approximately 0.4 ml), and the mixture was centrifuged at 8,000 × *g* for 10 min. The supernatant was gently partitioned twice (10 min each time) against 2.5 ml of a 1:1 (vol/vol) mixture of ethylacetate/cyclohexane. For determination of levels of free SA, top organic layers containing the free phenolic portion were stored at -20°C. For determination of levels of conjugated SA, lower aqueous phases containing the bound phenolic portion were acid hydrolyzed with HCl. The hydrolysis mixture was then centrifuged at 6,000 × *g* for 10 min, the supernatant was partitioned twice, as above, and the organic layers obtained were stored at -20°C.

Prior to HPLC (high performance liquid chromatography) analysis, organic phases were evaporated to dryness under vacuum and were resuspended in 1 ml of HPLC starting mobile phase (discussed below). HPLC separation of SA and oANI was performed on a system equipped with a deactivated reversed-phase column as described by Meuwly and Métraux (1993). Column temperature was 40°C, while samples were maintained at 10°C. Elution began with an isocratic flow of 15% acetonitrile (ACN) in 25 mM KH₂PO₄ adjusted to pH 2.6 (with HCL) for 1 min. The concentration of ACN was then increased to 20% in 2 min and was kept isocratic for another 2 min. The concentration of ACN was then raised successively to 60% in 15 min and to 100% in 2 min. The column was washed in 100% ACN for 5 min, prior to decreasing ACN concentration to 15% in 2 min and to equilibration for another 6 min, before the subsequent sample was injected. Volume of injected samples was 20 and 40 µl for determination of free and conjugated SA, respectively. Levels of SA and oANI were quantified fluorometrically by changing excitation and emission wavelengths to optimize the signal for each compound, according to Meuwly and Métraux (1993).

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