The Rx Gene from Potato Controls Separate Virus Resistance and Cell Death Responses

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Rx-mediated extreme resistance against potato virus X in potato does not involve a necrotic hypersensitive response at the site of initial infection and thereby differs from the more usual type of disease resistance in plants. However, the Rx protein is structurally similar to products of disease resistance genes conferring the hypersensitive response. We show in both Nicotiana spp and potato that Rx has the potential to initiate a cell death response but that extreme resistance is separate and epistatic to necrosis. These data indicate that cell death and pathogen arrest are separate disease resistance responses in plants.

INTRODUCTION

Rx-mediated resistance against potato virus X (PVX), like that controlled by many disease resistance (R) genes, can be described in terms of an elicitor–receptor model. According to this model, there are separate stages in the process involving pathogen recognition and the plant’s response (Staskawicz et al., 1995). In Rx-mediated resistance, the recognition stage involves an interaction between Rx (Bendahmane et al., 1997), which is thought to encode the receptor, and the PVX coat protein (CP), which is the elicitor (Bendahmane et al., 1995). The response stage consists of mechanisms able to suppress accumulation of viruses, including those that are taxonomically unrelated to PVX (Köhlm et al., 1993; Bendahmane et al., 1995).

Although the Rx-mediated mechanism is consistent with the elicitor–receptor model, the Rx response is distinct from that of other well-characterized R genes. The most striking feature of Rx-mediated resistance is the rapid arrest of PVX accumulation in the infected cell (Köhlm et al., 1993). Unlike other disease resistance responses, this extreme resistance is not associated with a hypersensitive response (HR) at the site of inoculation. In addition, Rx-mediated resistance is active in protoplasts via mechanisms that either suppress virus replication or promote degradation of the viral RNA (Adams et al., 1986; Köhm et al., 1993; Bendahmane et al., 1995). In contrast, the HR type of resistance is not expressed in isolated protoplasts (Otsuki et al., 1972; Adams et al., 1985; Baulcombe et al., 1994). It is thought that expression of the HR type of viral resistance mechanisms requires cell-to-cell contact and is a tissue-related phenomenon (Adams et al., 1986).

With the exception of the Rx response, it is not clear whether an HR is an essential component of disease resistance mechanisms in plants. One view is that cell death removes the substrate for growth of biotrophic pathogens. Alternatively, the dying cells may be able to release signals that are themselves antibiotics or disinfectants (Lamb and Dixon, 1997). This latter view is supported by earlier studies on tobacco N gene-mediated resistance to tobacco mosaic virus (TMV). TMV particles could be found in cells surrounding the necrotic HR lesion, even when lesion expansion had stopped (Da Graça and Martin, 1976). Several candidates have been identified for the putative cell death-associated signals, including reactive oxygen species (Levine et al., 1994; Jabs et al., 1996) and nitric oxide (Deledonne et al., 1998). These signals have also been implicated in apoptotic cell death in animals (Schmidt and Walter, 1994).

In contrast, the phenotype resulting from the dnd1 mutation in Arabidopsis indicates that cell death is separate and independent of the resistance mechanisms leading to suppression of the pathogen. The phenotype of the dnd1 mutant is loss of the HR without loss of disease resistance (Yu et al., 1998). Also consistent with separation of HR and disease resistance are the effects of humidity and temperature that can inhibit the HR response without compromising resistance (Baulcombe et al., 1994; Hammond-Kosack et al., 1996). According to this view of independent resistance and HR, the cell death response may be to reinforce or stimulate the induction of defenses (Dangl et al., 1996; Alvarez et al., 1998) by activation of local and systemic acquired resistance (Staskawicz et al., 1995; Ryals et al., 1996).

To gain further insight into the relationship of virus resistance and cell death, we isolated Rx from tetraploid potato by using a map-based cloning strategy. We demonstrate that Rx of potato expressed as a transgene either in potato or in Nicotiana spp confers extreme resistance against PVX.
and that this resistance is not associated with cell death. However, Rx shows similarities with the nucleotide binding site, leucine-rich repeat (NBS-LRR) class of plant R genes conferring an HR. Using transient expression tests, we show that Rx has a potential to initiate cell death if the viral elicitor is expressed constitutively as a transgene rather than from the PVX genome. We further show that cell death does not normally occur when plants carrying Rx are challenged with PVX because the extreme resistance in the Rx response is epistatic to the HR.

RESULTS

Molecular Cloning of the Rx Gene

The Rx locus in potato cultivar Cara was genetically delimited within a bacterial artificial chromosome (BAC) clone (BAC77; Kanyuka et al., 1999) (Figure 1A). To confirm the presence of a functional Rx gene within BAC77, we developed a transient expression assay based on microprojectile bombardment (McCabe et al., 1988). The constructs used in this assay comprised a PVX vector under the control of the cauliflower mosaic virus (CaMV) 35S promoter that was inserted into the unique Srfl site of BAC77. This PVX vector was modified to carry a β-glucuronidase (GUS) reporter gene. It was also mutated in the open reading frame of the 25-kD movement protein so that GUS expression would report virus replication only in the bombarded cells expressing the candidate Rx. In the PVX-TK/BAC77 construct (Figure 1B), the PVX cDNA was from an Rx-avirulent strain. In PVX-KR/BAC77, the viral cDNA was from an Rx resistance-breaking (i.e., virulent) strain (Figure 1B). These two constructs were separately coated onto gold particles and then bombarded into the leaves of susceptible potato (rx genotype) and N. benthamiana. The GUS activity in virus-infected cells as a reporter of virus accumulation was assayed by histochemical staining of leaves 48 hr after bombardment.

In this assay, we expected to detect blue spots in leaves bombarded with the PVX-KR/BAC77 construct, which is indicative of high levels of GUS activity and PVX accumulation. However, if BAC77 contains Rx, the PVX-TK/BAC77 construct should have activated resistance so that GUS levels would be low or undetectable. The results of these biolistic transient assay tests for potato (data not shown) and N. benthamiana (Figure 1C) were completely consistent with these predictions: blue spots of GUS activity were obtained with the PVX-KR/BAC77 construct but not with PVX-TK/BAC77. Therefore, we concluded that BAC77 contains Rx and that Rx of potato is functional in heterologous plant species. In many of the subsequent biolistic tests, we exploited this second finding and used leaves of N. benthamiana plants because the microprojectile bombardment was more efficient than in potato.

To delimit Rx more precisely, we prepared five subclones of BAC77 containing overlapping DNA fragments (Figure 1A). These DNA fragments subsequently were cloned into plasmids carrying Rx-virulent or Rx-avirulent PVX constructs and subjected to biolistic assay, as described above for the full-length PVX/BAC77 constructs. Results of these tests showed that the functional Rx was in an 11-kb PvuII DNA fragment present in the BAC6 and BAC9 derivatives of BAC77 (Figures 1A and 1C).

The Rx Transgene Confers Extreme Resistance in Potato

To confirm the transient expression data, the insert DNA from BAC9 was stably introduced into potato cultivar Maris Bard (rx genotype) by Agrobacterium-mediated transformation. This 18-kb BAC9 insert was chosen for plant transformation instead of the smaller DNA insert in BAC6 to ensure that complete transcription regulatory sequences were present. The transgenic plants were tested for resistance to virulent and avirulent strains of PVX by mechanical inoculation. Seven of eight independent transgenic lines carrying insert DNA from BAC9 were resistant to Rx-avirulent PVX-TK and susceptible to virulent PVX-KR. This resistance was manifested as the absence of PVX-TK symptoms and complete lack of PVX-TK accumulation in inoculated and systemic leaves (data not shown).

We also tested the effect of the Rx transgene on PVX accumulation in protoplasts from two of these independent transgenic lines, M4 and M7, transformed with BAC9. For comparison, we also tested protoplasts from cultivar Cara (Rx genotype) and from cultivar Maris Bard (rx genotype). These protoplasts were inoculated with PVX-TK or PVX-KR, and the RNA was sampled at 24 hr after inoculation. Gel blot analysis of these RNAs confirmed that there was no accumulation of PVX-TK in protoplasts of cultivar Cara and of the two Rx-transformed lines. In contrast, PVX-KR accumulated at a high level in all protoplasts tested, as did PVX-TK in the protoplasts of cultivar Maris Bard (rx genotype; Figure 2). Thus, the viral resistance tests in plants and protoplasts confirmed that the transgenic Rx-mediated resistance was indistinguishable from the Rx-mediated phenotype in cultivar Cara. There was early arrest of PVX accumulation in single cells and an absence of the HR.

The Rx Transgene Confers Extreme Resistance in Heterologous Plant Species

Although we had established Rx function in N. benthamiana by using a transient assay (Figure 1C), this method would not have distinguished between extreme resistance and an HR. It remained possible that extreme resistance was a particular characteristic of Rx in potato. To investigate further the function of Rx in heterologous species, we produced
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transgenic N. benthamiana and N. tabacum carrying insert DNA of BAC9. Plants of 12 transgenic lines (six of each species) were rub inoculated with Rx-virulent and Rx-avirulent strains of PVX. RNA samples were taken from the inoculated and upper noninoculated leaves at 2 weeks after inoculation. RNA gel blot analysis of these samples revealed that PVX accumulation occurred only when a resistance-breaking strain of PVX was used as an inoculum. There was no detectable accumulation of the Rx-avirulent strain PVX-TK and no HR on the inoculated or systemic leaves (data not shown).

A more rigorous test for an HR associated with Rx-mediated resistance is to use graft inoculation (Figure 3A). All graft inoculations described below were reproduced in two independent experiments. Scions (upper part) of N. benthamiana carrying Rx were grafted to a stock (lower part) of a nontransgenic N. benthamiana plant that had been preinoculated with PVX-TK (10 independent grafts). As a control, we used N. benthamiana plants carrying the TMV resistance gene N. These plants were resistant against a TMV vector expressing the jellyfish green fluorescent protein (TMV-GFP). This N-mediated resistance was manifested at 4 days after inoculation as a localized HR in leaves inoculated with TMV-GFP (data not shown). We never observed systemic symptoms in these plants, indicating that N-mediated resistance restricted TMV to the inoculated leaf. In TMV-GFP graft–inoculated plants (10 independent grafts) from 10 days

Figure 1. Identification of the Rx Gene by Using a Biolistic Transient Expression Assay.

(A) Genetic and physical map of the potato genomic region containing Rx, showing the BAC77 restriction map and the BAC subclones used in the transient expression assay. Left and right ends of BAC77 are indicated as 77L and 77R, respectively.

(B) Schematic structure of the BAC vectors used in the transient expression assay. PVX-TK/BAC* constructs carry insert DNA from BAC77 or from subclone derivatives described in (A) (BAC*) and the avirulent strain of PVX (PVX-TK) expressed from a CaMV 35S promoter. To monitor virus replication, we expressed the GUS gene from a duplicated PVX CP promoter. PVX-KR/BAC* is the same as PVX-TK/BAC*, except that PVX-KR/BAC* carries the Rx resistance-breaking strain of PVX (PVX-KR). Nos indicates the transcriptional terminator.

(C) Transient expression assay for Rx function. N. benthamiana leaf discs were bombarded with gold particles coated with either PVX-TK/BAC* or PVX-KR/BAC*. Virus replication was revealed by staining for GUS activity 2 days after bombardment. The potato insert DNA used in the bombardment is indicated at left. The lack of blue spots indicates Rx activity preventing virus accumulation.
There was no evidence of an HR, even when leaves of the scions that did not carry the transgene were symptomless, even after several weeks (Figure 3B). Graft union. However, the scions of transgenic plants inoculated with 2 μg of total RNA equivalent to 50,000 protoplasts. Virus RNA accumulation was measured 24 hr after inoculation. Each lane of the gel was loaded with 2 μg of total RNA equivalent to 50,000 protoplasts. The hybridization probe was specific for the positive-strand RNA of PVX.

Figure 2. Gel Blot Analysis of PVX RNA Accumulation in Potato Protoplasts.

Protoplasts of potato cultivar Maris Bard (rx genotype; lanes MARIS BARD), potato cultivar Cara (Rx genotype; lanes CARA), or from two independent transgenic lines (M4 and M7) of potato Maris Bard containing the Rx transgene (BAC9 insert) were inoculated with either the virulent (KR) or the avirulent (TK) strains of PVX. M indicates mock-inoculated protoplast samples. Virus RNA accumulation was measured 24 hr after inoculation. Each lane of the gel was loaded with 2 μg of total RNA equivalent to 50,000 protoplasts. The hybridization probe was specific for the positive-strand RNA of PVX.

after grafting, the HR was manifested as a systemic HR (Figure 3B). Eventually this HR spread to cause death of the scion. The spreading HR was not observed in TMV-GFP graft-inoculated scions, as occurs in rub-inoculated leaves. Thus, graft inoculation exaggerates the HR.

In the graft-inoculated scions of a nontransgenic N. benthamiana, there were high levels (Figure 3C) and mosaic symptoms of PVX-TK, indicating that PVX could cross the graft union. However, the scions of transgenic Rx plants were symptomless, even after several weeks (Figure 3B). There was no evidence of an HR, even when leaves of the Rx scions were examined under a dissecting microscope or by trypan blue staining (Parker et al., 1993) for cell death (data not shown), and there was no accumulation of PVX-TK (Figure 3C).

We also conducted a double-graft experiment in which a scion of N. benthamiana (transgenic Rx genotype) was grafted between a healthy scion and one preinoculated with a PVX-TK stock of nontransgenic N. benthamiana (Figure 3A). We wanted to determine whether PVX-TK was able to cross a graft union and to pass through the vascular tissue of an Rx genotype plant. In 10 independent double grafts, RNA gel blot analysis showed PVX-TK accumulation in the inoculated stock and in the upper (nontransgenic) scion but not in the intermediate scion (Figure 3C). From these results, we conclude that Rx-mediated resistance did not suppress PVX-TK translocation through the phloem. These results also confirm that in the single-grafted plants, there would have been translocation of PVX-TK into the transgenic Rx scion and the potential for activation of an Rx-mediated HR.

From the absence of an HR in these graft-inoculated plants, we conclude that there is no cell death associated with Rx-mediated resistance in Nicotiana spp.

The Rx Protein Is Similar to Products of R Genes Conferring the HR

The sequence of Rx was determined from an Fsp1-PvuII fragment of BAC77 (BAC9; 18,285 bp) and from analysis of Rx cDNA (see Methods and EMBL accession number AJ 011801). Analysis of these sequences revealed that Rx has three exons and two introns of 234 and 111 bp (Figure 4A). The largest first exon contains the 5′ noncoding region of the mRNA and most of the coding region. The third exon contains only two nucleotides of coding sequence plus the TAG stop codon and the 3′ noncoding region of mRNA. The biolistic transient expression of this cDNA in N. benthamiana conferred resistance to PVX (data not shown).

Based on the cDNA sequence of Rx, we found a single open reading frame encoding a protein of 937 amino acid residues with a molecular weight of 107.5 kD (Figure 4B). Surprisingly, the primary structure of Rx is similar to that of the NBS-LRR (Jones and Jones, 1997) class of R proteins, in which the resistance is associated with an HR. The highest degree of similarity is between Rx and a subclass of NBS-LRR resistance proteins represented by RPS2, RPM1, and Prf (Jones and Jones, 1997). These Arabidopsis and tomato proteins contain a putative four-to-six heptad amphipathic leucine zipper motif at the N terminus (Jones and Jones, 1997). A putative leucine zipper is also present in the N-terminal region of Rx (domain I; Figure 4B). However, this domain in Rx does not fit the leucine zipper consensus as well as the corresponding motif in RPS2 (Mindrinos et al., 1994).

As in the other R gene products, the putative NBS domain (domain II; Figure 4B) of Rx comprises three motifs: kinase 1A or “P loop” (residues 168 to 180), kinase 2 (residues 237 to 247), and kinase 3a (residues 265 to 273). In Rx, the putative NBS is followed by a domain with unknown function that includes GLPL, CFLY, and MHD motifs. These motifs are characteristic of all NBS-LRR R gene products thus far identified (Hammond-Kosack and Jones, 1997; van der Biezen and Jones, 1998). The putative LRR domain of Rx (residues 473 to 868) comprises 14 to 16 imperfect copies of the LRR motif (Figure 4B). This motif shows a good match to the cytoplasmic LRR consensus sequence motif (Jones and Jones, 1997) and most closely resembles the LRR domain of the tomato Prf protein (Salmeron et al., 1996).

The C-terminal part of Rx (Figure 4B) contains three unique motifs with unknown functions: an amide-rich region (residues 869 to 893), a short basic region (residues 894 to 902), and an “acidic tail” region (residues 903 to 937). The acidic tail is encoded entirely within the short second exon of Rx. This domain is rich in aspartic and glutamic acid resi-
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dues and contains two copies of a 10-amino acid direct repeat (Figure 4B). These features are not present in any previously described products of R genes.

Constitutive Expression of the CP Induces Rx-Dependent Cell Death

From sequence analysis, Rx is structurally similar to R genes conferring an HR. In contrast, phenotypic analysis showed that Rx-mediated resistance is independent of an HR. These conflicting findings might indicate that subtleties in the Rx primary structure are required for the extreme resistance pathway. Alternatively, these findings could indicate that the Rx-mediated extreme resistance is a variation of the resistance mechanisms leading to HR and cell death. To address these possibilities, we examined the effects on Rx-mediated resistance when the expression of the viral elicitor (the PVX CP) (Bendahmane et al., 1995) was uncoupled from virus replication.

To uncouple CP expression from PVX replication, we expressed the CP gene from either the virulent or the avirulent strain of PVX under the control of the CaMV 35S constitutive promoter. To deliver these CP constructs into plants, we introduced them into binary pBin19 plasmid vector (Bevan, 1984) and transformed them into Agrobacterium (Figure 5A). The Agrobacterium cultures then were infiltrated into leaves of Rx-transformed N. benthamiana (line B18) in a procedure we refer to as agroinfiltration (Vaucheret, 1994).

When constructs encoding the elicitor CP (pBIN35-TK; Figure 5B) were agroinfiltrated into Rx-transformed N. benthamiana line B18, there was an HR. The necrosis first appeared within 48 hr after agroinfiltration and caused complete death of the infiltrated region by 72 hr (Figure 5B). We could rule out that this Rx-mediated HR was related to the Agrobacterium or to the infiltration method, because there was no HR when we used the construct pBIN35-KR (Figure 5B). This construct encoded the CP of an Rx resistance-breaking strain of PVX. Also, there was no HR when non-transgenic N. benthamiana plants were agroinfiltrated with either construct. When Rx genotype potato cultivar Cara or Rx-transformed potato and N. tabacum plants were used for

Figure 3. Cell Death Is Not Required for Rx-Mediated Resistance.

(A) Schematic representation of the grafting experiments indicating the virus-infected stock and the tested scion in the single and the double grafts, respectively.

(B) Grafted plants 4 weeks after grafting. The genotype of the root stock was wild-type N. benthamiana. The genotypes of the scions were wild-type N. benthamiana (indicated by a dash) or N. benthamiana transformed with Rx or the N gene, as indicated above the photographs. The viruses used in the infection of the root stocks were PVX-TK (referred to as PVX) or TMV-GFP (referred to as TMV), as indicated at bottom. The positions of the graft unions are indicated by red arrows. The TMV-induced systemic HR in the scion with N genotype is indicated by a white arrow.

(C) Analysis of PVX-TK accumulation in the root stock and the scion of single and double grafts. T and S indicate RNA extracted from the root stock and the scion, respectively. The genotype of the root stock and the scion is indicated by (±) for wild-type N. benthamiana or by Rx for Rx-transformed N. benthamiana. Each lane of the gel was loaded with 2 µg of total RNA. The hybridization probe was specific for the positive strand RNA of PVX.
similar experiments, the outcome was the same as when N. benthamiana line B18 was used; there was an HR, but only when the PVX-TK CP was expressed under control of the CaMV 35S promoter (data not shown). From these experiments, we conclude that there is the potential for an Rx-mediated HR. However, this potential is not realized when the PVX CP is expressed from the PVX genome during the viral infection cycle.

**Extreme Resistance Is Epistatic to the HR**

To further explore the relationship between extreme resistance and the HR, we analyzed the effect of Rx-mediated resistance on the N gene-mediated, HR-type resistance against TMV. These experiments used tobacco plants carrying the N gene either alone or in combination with transgenic Rx. The plants were challenged with recombinant isolates of TMV expressing the CP gene from either the Rx-virulent (PVX-KR) or the avirulent (PVX-TK) strain of PVX (Bendahmane et al., 1995; Figure 6A). The TMV-TK construct encodes the elicitors of both N- and Rx-mediated resistance (Padgett and Beachy, 1993; Padgett et al., 1997), whereas TMV-KR does not encode the elicitor of Rx-mediated resistance. Transcripts of the TMV-TK and TMV-KR cDNAs initially were inoculated onto N. benthamiana, and
sap extracts were produced from the inoculated leaves at 8 days after inoculation. Tobacco plants carrying the \( N \) gene either alone or in combination with \( Rx \) were inoculated with these extracts containing high concentrations of the virus without dilution (Figure 6B). After 3 to 4 days, TMV-KR and TMV-TK induced an HR on \( N. tabacum \) expressing the \( N \) gene. There was also an HR when TMV-KR was inoculated onto plants of the \( N, Rx \) genotype. However, when these plants were inoculated with TMV-TK, there was no HR. These data indicate that Rx-mediated extreme resistance was activated before \( N \)-mediated resistance and therefore that extreme resistance is epistatic to an HR.

**DISCUSSION**

Here, we provide a molecular characterization of the Rx gene of potato and demonstrate that this gene controls separate virus resistance and cell death responses. The sequence of Rx revealed that the encoded protein has structures including those of the NBS, LRR, and other domains that are common to many R genes of plants (Jones and Jones, 1997; van der Biezen and Jones, 1998). It is surprising that Rx is similar to the leucine zipper class of NBS-LRR proteins. The other proteins in this class control resistance against bacteria (Mindrinos et al., 1994; Grant et al., 1995; Warren et al., 1998), nematodes (Milligan et al., 1998), and insects (Rossi et al., 1998). The product of \( N \), which is the other known virus R gene, belongs to a separate class of NBS-LRR proteins in which the N-terminal domain has similarity to the Toll protein and the interleukin receptor (Whitham et al., 1994).

Although Rx has various R protein motifs, there are also acidic and amide motifs in the C-terminal domain (Figure 4) that are distinguishing features. These motifs are also present in paralogs of Rx on potato chromosome V as well as on chromosome XII adjacent to the cloned Rx locus (A. Bendahmane and K. Kanyuka, unpublished data). One of these paralogs is the Rx2 locus present on chromosome V DNA introgressed from \( S. acaule \) (A. Bendahmane, unpublished results). This gene is functionally identical to but independent of the Rx locus described here (Querci et al., 1995). The Gpa2 nematode resistance gene that is tightly linked to the Rx locus on chromosome XII (Roupp van der Voort et al., 1997) may also be an Rx paralog.

The nature of the Rx response, as either extreme resistance or HR, was determined by the mode of expression of the CP elicitor. Transgenic CP elicited an HR, whereas CP produced from a virus caused extreme resistance (Figure 5). Our interpretation of this result is presented as a model in Figure 7. According to this model, when the elicitor CP was
expressed from a viral genome, there was early and rapid elicitation of extreme resistance. The consequent arrest of PVX accumulation would also have been associated with a block on other processes associated with the viral replication cycle, including further production of the CP (Figure 7). In contrast, when the CP was expressed from a transgene, the initial activation of extreme resistance would have had no effect on continued production of the CP. The continuing buildup of the elicitor CP would then lead to activation of the HR as a secondary resistance response (Figure 7).

Because Rx shares sequence similarity to many R genes (Figure 4), it is likely that variations of the model presented in Figure 7 are relevant to other disease resistance interactions in plants. For example, in N-mediated resistance against TMV, a situation could be envisaged that is intermediate between the extremes shown in Figure 7. The initial activation of N-mediated resistance may be slower than with Rx, and there would be delayed activation of the primary extreme resistance. In this example, the virus would continue to accumulate because it would have reached a high level and be replicating rapidly by the time the extreme resistance was activated. The continued accumulation of TMV would lead to an HR and necrotic lesions. This HR, being upstream of secondary defense induction, would reinforce the extreme resistance and eventually lead to a block on continued TMV accumulation.

A prediction of this model is that the same early responses would be activated in N- and Rx-mediated resistance but that they would be activated more rapidly, or be more pronounced, in the Rx background. These early responses will likely include an oxidative burst (Doke and Ohashi, 1988). Analysis of the Rx response in the background of superoxide dismutase overexpression would indicate whether production of active oxygen species is required for extreme resistance. It is unlikely that new gene expression is required for extreme resistance because high levels of actinomycin D do not block Rx-mediated resistance in protoplasts (Gilbert et al., 1998).

Other types of R gene responses may also have HR-dependent and HR-independent (similar to extreme resistance) components, as shown in Figure 7. The HR-independent response would be evident if resistance is activated early in the infection cycle. An HR would be produced if there was later activation of resistance. The resistance without the HR phenotype of the dnd1 mutation could be explained in terms of this model. The mutation leads to activation of a generalized defense mechanism so that, in an R gene background, elicitation of the resistance mechanism may occur earlier in the infection cycle than in the wild-type plants. In a second example, the Cf-9-mediated resistance against the fungus Cladosporium fulvum in tomato is normally manifested as HR-independent arrest of hyphal growth (Jones et al., 1994). This phenotype is consistent with early activation of resistance in the fungal infection cycle. However, if the elicitor of Cf-9-mediated resistance is expressed from a virus vector, there is a systemic HR (Hammond-Kosack et al., 1995), indicating activation of the resistance late in the viral infection cycle.

The important general point from these various examples is the dynamic nature of resistance interactions in plants. Clearly, there is potential for quantitative and qualitative variation in resistance responses depending on the elicitor-receptor interaction. Factors affecting the response include timing, the level of interacting components, and the affinity between the elicitor and its receptor. According to this view, the outcomes of interactions between plants and pathogens represent variable points in a continuum of responses. At one end of the continuum, as with Rx, there is extreme resistance or HR-independent resistance when there is a high affinity between the elicitor and its receptor and when elicitor production is early in the infection cycle. HR-associated resistance may represent an intermediate response when the elicitor has a lower affinity for the receptor or when elicitor production is later in the infection cycle. A systemic HR may occur when there is a very-low-affinity interaction between the elicitor and the receptor. This response, representing the other end of the continuum of responses, may also occur when elicitor production is at a late stage in the infection cycle. In natural situations, the different types of responses likely represent stages in the oscillating evolutionary battle between disease-resistant plants and their pathogens.

METHODS

Viral cDNA Clones and in Vitro Transcription

The PVX-TK and PVX-KR constructs are cDNAs of potato virus X (PVX) isolate CP4. PVX-TK has codons ACA (specifying T) and AAA (specifying K) at positions 121 and 127 of the coat protein (CP) gene and is the same as wild-type CP4. The construct PVX-KR has AAA (K) and AGG (R) at the CP codons 121 and 127, respectively. These constructs were described previously (Goulden et al., 1993). The tobacco mosaic virus (TMV) constructs were derived from the TMV vector (TMV/odontoglossum ringspot virus; construct TB2) described previously (Donson et al., 1991). The TMV-TK and TMV-KR constructs were made by cloning PVX CP from PVX-TK and PVX-KR, respectively, in the XhoI site of TB2. Schematic structure of these constructs is described in Figure 6A. The TMV-GFP was made by cloning the green fluorescent protein (GFP) open reading frame in the Xhol site of TB2.

Plasmid Constructs for Biolistic Transient Expression Assay

PVX-TK/BAC77 and PVX-KR/BAC77 were made by digestion of the unique SrfI site in bacterial artificial chromosome BAC77 and ligation with the cDNAs of the Rx-avirulent or the Rx-virulent strain of PVX expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter. PVX in these constructs was defective in cell-to-cell movement and modified to express a β-glucuronidase (GUS) reporter gene. The cell-to-cell movement defect was due to a sequence deletion between nucleotides 4588 and 4945 of the 25-kD protein open reading frame (Angell et al., 1996). All the clones
derived from PVX-TK/BAC77 and PVX-KR/BAC77 were designated PVX-TK/BAC* and PVX-KR/BAC*, where the asterisks are used depending on the insert derivatives of BAC77 (see Figure 1).

**Biolistic Transient Expression Assay**

BAC plasmid DNAs were isolated using an alkaline lysis method (Leonardo and Sedivy, 1990) and purified on cesium chloride gradients (Sambrook et al., 1989). One hundred micrograms of plasmid DNA was precipitated onto 10 mg of 0.95-m-diameter gold particles, as described previously (McCabe et al., 1988). Leaf discs from 6-week-old plants, which were placed onto Murashige and Skoog broth medium (50 mL) supplemented with 50 μg/mL kanamycin and 5 μg/mL tetracycline, were bombarded with the gold particles coated with plasmid DNAs, as described previously (McCabe et al., 1988). The leaf discs were incubated at room temperature in the dark for 48 hr and then stained for GUS activity as described previously (Angell and Baulcombe, 1997).

**Agrobacterium tumefaciens-Mediated Transient Expression (Agroinfiltration)**

The CP genes from the avirulent or the virulent strain of PVX were inserted between the 35S promoter and the transcriptional terminator of CaMV and transferred into the binary vector pBin19 (Bevan, 1984) to create pBIN35S-TK and pBIN35S-KR, respectively (see Figure 5). These constructs were transformed into Agrobacterium strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al., 1990) and purified on cesium chloride gradients of CaMV and transferred into the binary vector pBin19 (Bevan, 1984). Transformation of potato cultivar Maris Bard was performed as described previously (Gilbert et al., 1998). The transformation of N. benthamiana with the N disease resistance (R) gene was conducted by using Agrobacterium strain AGL1, which carries the binary vector pTG34 and was kindly provided by B. Baker (University of California, Berkeley; Whitham et al., 1994).

**DNA Sequencing and Analysis**

A shotgun cloning strategy was used for sequencing BAC9. Three aliquots of 15 μg of CsCl-purified DNA were sonicated for 30, 60, and 120 sec, respectively, by using Soniprep 150 (MSE, UK). DNA fragments in the range of 1 and 4 kb were gel purified, blunt ended using T4 DNA polymerase, and cloned into pGEM3Zf+ (Promega) digested with SmaI. Clones carrying potato insert DNA were selected by colony hybridization by using insert DNA from BAC9 as a probe. The sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer) and M13 universal forward and reverse primers. The sequencing reactions were resolved on an ABI377 automated sequencer (Applied Biosystems, La Jolla, CA). Sequence contigs were assembled using UNIX versions of the Staden programs package (R. Staden, Medical Research Council, Cambridge, UK). Homology searches were done using the BLAST program (Altschul et al., 1990). The genomic DNA sequence has EMBL accession number AJ011801.

**Protoplasts Assay**

Infectious viral RNA were electroporated into potato protoplasts, and RNA gel blot analysis was used to measure the accumulation of viral RNA, as described previously (Köhlm et al., 1993). The probe used to detect PVX RNA was antisense RNA probe transcribed from the T7 promoter of pHB-RP plasmid linearized with Apal and was described previously (Köhlm et al., 1993).

**Graft Inoculation**

Graft inoculations involved the grafting of healthy scions to infected stocks. First, seedlings of *N. benthamiana* were sap inoculated with PVX strains. Later, when the plants exhibited symptoms on the systemic leaves, the apex of a healthy plant to be tested was grafted onto the infected stock. A diagonal cut was made in the stem of the third internode to insert the infected scion. The 2- to 6-cm scions were given a V shape at the end that would be inserted into the cut on the stock plant to ensure good contact between vascular systems. The graft was secured with Parafilm M (Sigma), and the whole plant was covered with a thin transparent plastic bag to avoid dehydration of the scion. The bag was removed 7 days after grafting. The presence of virus in the stock and the scion was determined 4 weeks after grafting by using RNA gel blot analysis. All graft inoculation experiments were repeated at least twice.

**DNA Rearrangement**

The FspI-PvuII fragment of BAC9 (19,285 bp) was cloned into the pSLJ 7292 binary vector (Jones et al., 1992) digested with Ecl316II to create pSLJ9. This clone was introduced into Agrobacterium strain LBA4404. Transformation of *N. benthamiana* and *N. tabacum* was conducted by Agrobacterium-mediated leaf disc transformation (Horsch et al., 1985). Transformation of potato cultivar Mars Bard was performed as described previously (Gilbert et al., 1998). The transformation of *N. benthamiana* with the N disease resistance (R) gene was conducted by using Agrobacterium strain AGL1, which carries the binary vector pTG34 and was kindly provided by B. Baker (University of California, Berkeley; Whitham et al., 1994).

**Rapid Amplification of cDNA Ends**

The 5’ and 3’ ends of the Rx cDNA were determined by rapid amplification of cDNA ends (RACE) by using the MARATHON cDNA amplification kit (Clontech Laboratories, Palo Alto, CA). To obtain specific RACE products, two consecutive rounds of amplification (35 cycles each) were necessary. Conditions for the first round of polymerase chain reaction (PCR) were as recommended by the manufacturer. PCR samples of the first amplification round were diluted 1:10 with distilled water, and a 1-μL aliquot was used as a template for the second round of PCR (15 sec at 94°C, 15 sec at 65°C, and 1 min at 72°C). Two sets of oligonucleotides were used in combination with the adapter primers (AP1 and AP2) of the kit: K3 (5′-ACTGGAGTCTTTATACTATCATGG-3′) and K4 (5′-GAAGAATTTCTCTGGATGGAAC-3′) for the 3’ end of Rx and oligonucleotides K13 (5′-GCTTACTGGTTTCTACCTCAG-3′) and K15 (5′-GCAGGTTTCTCCAGAATTAGCTTCTCA-3′) for the 5’ end of Rx. RACE products were

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subcloned into the pGEM-T plasmid (Promega), and sequences of 10 independent 3' and 5' end clones were determined as given above. Sequences of 5' and 3' RACE PCR products overlapped with the sequence of the PCR product corresponding to the central region of the Rx cDNA amplified using primers asK4 (5'-GAGACAATCTCTCCTGTAAC-3') and asK15 (5'-GGCAATCTCTAAGAATACCTCTGC-3').

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REFERENCES


**NOTE ADDED IN PROOF**
