The *Rx* Gene Confers Resistance to a Range of *Potexviruses* in Transgenic *Nicotiana* Plants

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Rx-mediated resistance was analyzed in *Rx*-expressing transgenic *Nicotiana* plants. The infection outcome of nine *Potato virus X* isolates mutated at amino acid positions 121 and 127 of the coat protein (CP) confirmed the key role of these amino acids but provided a more complex picture than previously reported. In particular, in *Rx*-expressing *Nicotiana* spp., eliciting activity modulated by amino acid 121 was conditioned by the nature of amino acid 127. These results suggest that the specificity of recognition might be modulated by host factors that are somehow subtly modified between *Rx*-expressing potato and *Rx*-expressing transgenic *Nicotiana* plants. Moreover, the CP of three *Potexviruses*, *Narcissus mosaic virus* (NMV), *White clover mosaic virus* (WCIMV), and *Cymbidium mosaic virus* (CymMV), are all recognized by the *Rx*-based machinery and able to trigger an *Rx*-dependant hypersensitive response. A smaller elicitor of 90 amino acids was identified in the CP of NMV and WCIMV, which contains the previously identified key positions 121 and 127. This elicitor is only weakly conserved (approximately 40% identity) among the CP of the various recognized viruses, suggesting that the *Rx* molecular machinery targets a conserved structural element of the *Potexvirus* CP rather than a conserved amino acid motif.

Additional keywords: extreme resistance, hypersensitive response.

Plant disease resistance can be mediated by a multilayered innate immune system which, in some aspects, shows similar features within animal immunity (Numberger et al. 2004). The first layer in this process is referred to as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Jones and Dangl 2006). PTI consists of recognition of conserved PAMPs followed by the induction of basal defenses. The second layer is referred to as effector-triggered immunity (ETI). ETI involves recognition by plant disease resistance (R) proteins of specific avirulence effectors (Avr) produced by the pathogen. This interaction is known as pathogen race-host plant cultivar-specific resistance and was first described genetically as the gene-for-gene interaction (Flor 1971). Any protein component of a virus (replicase, coat protein, or move-

ment protein) can function as the specific Avr determinant to elicit resistance mediated by a given R gene (Martin et al. 2003). It has been shown that a large subset of R genes, known as the nucleotide binding site leucine-rich repeat (NBS-LRR) resistance genes, can be implicated in this type of immunity (Jones and Dangl 2006). Despite the availability of several cloned R genes and their cognate Avr determinants, progress have been slow in the characterization of Avr determinants and in understanding how pathogen Avr proteins are recognized.

It is generally understood that R proteins directly or indirectly recognize an Avr elicitor (Dangl and Jones 2001) to trigger signal transduction cascades leading to defense and disease resistance. These responses are generally associated with a type of programmed cell death termed the hypersensitive response (HR) (Heath 2000; Shirasu and Schulze-Lefert 2000).

The genetically dominant *Rx* gene encodes a member of the NBS-LRR family of R proteins conferring race-specific resistance against *Potato virus X* (PVX, *Potexvirus*) (Cockeram 1970). This resistance can be described in terms of an elicitor-receptor model. Among the R proteins, the phenotype of resistance by *Rx* is unusual and is described as extreme resistance (ER) (Bendahmane et al. 1999; Kohm et al. 1993; Tozzini et al. 1991). After inoculation of *Rx*-expressing plants with an avirulent PVX isolate (such as PVX_{CP4}), resistance is induced very early and involves the arrest of PVX accumulation without development of HR. As a consequence, the detection of PVX is almost impossible in inoculated leaves (Bendahmane et al. 1999, 2000; Hajimorad and Hill 2001). Moreover, experiments in potato protoplasts indicated that *Rx* can be active at a single cell level and that a nonspecific response restricts virus accumulation (Kohm et al. 1993). However, in transient expression experiments, *Rx* is able to trigger an HR reaction showing that, under these specific conditions, the nonspecific antiviral response is epistatic over the HR.

The NBS-LRR proteins form the largest class of plant resistance proteins and contain a coiled-coil (CC) domain at the N-terminus, an NBS domain, and an LRR domain at the C-terminus (Ellis et al. 2000; Jones and Jones 1997; Young 2000). The C-terminus of the NBS is referred to as the ARC domain because of its presence in Apaf-1, R proteins, and CED-4 (Aravind et al. 2004; Leipe et al. 2004; Van der Biezen and Jones 1998). The NB-ARC central domain of these proteins has an N-terminal subdomain with a consensus kinase P-loop domain. This domain has been shown, in vitro, to bind and hydrolyze ATP in the case of *I-2* and *Mi-1* tomato genes. However, it is assumed that the NBS domain acts as a nucleotide-dependent molecular switch which can regulate the conformation and, subsequently, the signaling potential of the proteins of this family (Takken et

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al. 2006; Tameling et al. 2002). The LRR domain, specifically the C-terminal region, is implicated in the recognition of non-self molecules which, in plants, can be direct or indirect (De Young and Innes 2006). Genetic studies have shown that the LRR domain can recognize specific elicitors in both *Arabidopsis thaliana* (Bittner-Eddy et al. 2000; Botella et al. 1998; McDowell et al. 1998; Noel et al. 1999) and lettuce (Meyers et al. 1998).

Recently, a model for R protein activation was proposed based on the nucleotide status and intramolecular interactions within the R protein (Moffett et al. 2002; Tameling et al. 2002, 2006). In this model, two different states of the R protein (inactivated and activated) are differentiated by intramolecular interactions between protein domains as well as ADP/ATP binding status. When the NB-ARC domain interacts with both CC and LRR domains in the presence of ADP, the R protein is inactivated. Recognition of an elicitor initiates a series of conformational changes involving, first, the dissociation of the LRR from the CC-NB-ARC part of the protein with the concomitant release of ADP and, second, dissociation of the interactions between the CC and NB-ARC domain and the binding of ATP. Consequently, the R protein is activated and can initiate cellular signaling leading to disease resistance (Moffett et al. 2002; Tameling et al. 2006).

Transient expression and reverse genetic experiments have shown that the coat protein (CP) of PVX is the sole elicitor recognized by the product of the *Rx* gene (Bendahmane et al. 1995; Goulden et al. 1993; Kavanagh et al. 1992; Kohm et al. 1993).

According to the general R model, recognition and response stage are separated in the PVX-*Rx* system (Bendahmane et al. 1999; Staskawicz et al. 1995). The recognition stage involves (either direct or indirect) interaction between the product of the *Rx* gene and the PVX CP (Bendahmane et al. 1995; Kohm et al. 1993). This stage involves a high degree of specificity, in contrast to the response phase, which is not virus specific (Bendahmane et al. 1999). *Rx*-mediated resistance is highly effective not only in the degree of resistance afforded but also in the specificity of the resistance. Indeed, to date, only a single isolate described, PVX_{HB}, is able to overcome the *Rx*-mediated resistance (Moreira et al. 1980; Querci et al. 1993). Mutational analysis of CP has revealed a major role of the conserved threonine at position 121 of the CP in avirulence toward *Rx*. Substitution of a lysine (T121K) as observed in PVX_{HB} completely abolishes recognition by potato genotypes carrying *Rx* (Goulden et al. 1993). However, PVX isolates containing this T121K mutation alone are severely affected not only in their infectivity but also in their ability to systemically invade susceptible hosts (Goulden et al. 1993). A second mutation, R127K, also observed in PVX_{HB}, contributes to the restoration of the fitness of T121K variants (Goulden et al. 1993).

The recognition stage can involve direct contacts between the elicitor and the R protein, as with the *N* gene and the *Tobacco mosaic virus* p50 helicase (Ueda et al. 2006) or indirect interactions involving other host proteins, as observed with bacterial resistance genes (Belkadir et al. 2004; Mackey et al. 2003; Van der Biezen and Jones 1998). Recognition via indirect interactions can be explained by way of the guard hypothesis model (Jones and Dangl 2006; Van der Biezen and Jones 1998).

The guard hypothesis model has been proposed for the recognition stage in the PVX-*Rx* system (Farnham and Baulcombe 2006). Recently, a protein, RanGAP2, has been shown to interact with the CC domain of *Rx* protein (Tameling and Baulcombe 2007). RanGAP2 has also been shown to associate with close homologs of *Rx* such as *Rx2* and *Gpa2* (Sacco et al. 2007). Based on the guard model, it can be hypothesized that

Rx is part of a protein complex that includes RanGAP2 as a virulence target of the PVX CP. However, evidence of a direct physical interaction between RanGAP2 and PVX CP is lacking.

In an effort to understand the detailed mechanisms of *Rx*-mediated resistance, interactions between the CP elicitor and the *Rx* protein were investigated using two different approaches.

In the first approach, a series of mutations at the previously identified essential T121 and K127 residues were generated. Analysis of the resultant *Rx*-mediated resistance was performed in *Rx*-expressing transgenic *Nicotiana* plants. The results show that the biochemical nature of the amino acids at these key positions is able to modulate the specificity of CP recognition. It seems also that various host factors are able to modulate this recognition stage.

In the second approach, the ability of *Rx*-mediated resistance to be triggered by the CP of other filamentous viruses was investigated. The results show that, in addition to PVX, the *Rx*-based sensing mechanism can recognize other *Potexviruses*, although with different degrees of resistance against these viruses.

RESULTS

Mutations of the PVX CP influence PVX interaction in *Rx*-expressing plants.

To characterize the interaction between the PVX CP and the *Rx* resistance gene product, the combination of the two key amino acids at positions 121 and 127 of the PVX CP was modified. The avirulent strain PVX_{CP4} and the virulent strain PVX_{HB} differ at 79 nucleotide positions, but only 7 differences affect the primary structure of the CP (Goulden and Baulcombe 1993). PVX_{CP4} and PVX_{HB} have a threonine or a lysine in position 121 and a lysine or an arginine in position 127, respectively. Mutations introduced by polymerase chain reaction (PCR) into the PVX_{CP4} cDNA changed the codon at position 121 from a threonine to five other amino acids and the codon at position 127 from a lysine to an arginine. Wild-type (WT) or transgenic *Rx*-expressing *Nicotiana benthamiana* (Bendahmane et al. 1999) were mechanically inoculated with the following transcripts: PVX_{CP4}, PVX_{CP4}-RK, PVX_{CP4}-VK, PVX_{CP4}-SK, PVX_{CP4}-PK, PVX_{CP4}-KK, PVX_{CP4}-RR, PVX_{CP4}-VR, PVX_{CP4}-SR, PVX_{CP4}-PR, and PVX_{HB}. Symptoms were observed 15 days after inoculation. As shown by reverse-transcription (RT)-PCR and Western blot analysis, the accumulation of the different PVX strains was comparable in WT *N. benthamiana* leaves (Fig. 1; Supplementary Fig. 1).

Fig. 1. Effects of mutations of the *Potato virus X* (PVX) coat protein (CP) on the infection phenotype in transgenic *Rx*-expressing *Nicotiana benthamiana*. Symptoms were observed 15 days postinoculation. **1**, Inoculated leaf of *Rx*-expressing *N. benthamiana*. **2**, Systemic leaf of *Rx*-expressing *N. benthamiana*. The two letters indicate the nature of residues 121 and 127 in the PVX CP. The mutant constructs were classified based on the *Rx*-mediated response observed from a to e. **a**, PVX_{CP4}; **b**, PVX_{CP4}-SK; **c**, PVX_{CP4}-VK; **d**, PVX_{CP4}-RR; **e**, PVX_{HB}.

As expected, the two parental isolates PVX_{CP4} and PVX_{HB} were able to accumulate and spread in WT plants. In the *Rx*-expressing plants, PVX_{CP4} induced extreme resistance such that no symptoms were observed on inoculated leaves (Fig. 1-1a). In addition, no viral accumulation could be detected in either inoculated or noninoculated leaves (data not shown). In parallel, PVX_{HB} induced systemic necrosis in the *Rx*-expressing plants (Fig. 1-1e), and virus could be detected in both inoculated and noninoculated leaves (data not shown). These results show that PVX_{HB} replication as well as cell-to-cell and long-distance movements are permitted in the *Rx*-expressing plants (Fig. 1-1e), therefore suggesting that the resistance-breaking status of this strain, observed in potato, is not as complete as in *Nicotiana* spp.

Following inoculation with PVX CP mutants, three types of interaction phenotypes were observed. The first type of interaction, represented by seven mutants (PVX_{CP4-SK}, PVX_{CP4-VK}, PVX_{CP4-RK}, PVX_{CP4-KK}, PVX_{CP4-PK}, PVX_{CP4-VR}, and PVX_{CP4-SR}), allows accumulation and spreading of the virus in WT plants. However, in *Rx*-expressing plants, no symptoms were observed (Fig. 1b) and virus did not detectably accumulate (data not shown). The second type of interaction is represented by the mutant PVX_{CP4-RR}, which also was able to accumulate and spread normally in WT plants. It induced necrotic local lesions on inoculated leaves of *Rx*-expressing transgenic plants (Fig. 1-1c) but was unable to accumulate in the noninoculated leaves of these plants (Fig. 1-2c). The final type of interaction is represented by the mutant PVX_{CP4-PR}. Again, accumulation and spreading of the virus occurred in WT plants; however, in *Rx*-expressing *N. benthamiana* plants, PVX_{CP4-PR}-induced necrotic local lesions on inoculated leaves were observed (Fig. 1-1d), as well as necrotic lesions on noninoculated leaves (Fig. 1-2d). These results demonstrate that the virus was able to spread from the inoculated leaves to the entire plant.

Sequencing of the entire CP open reading frame of progeny viruses following infection confirmed the stability of introduced mutations (data not shown).

Three other *Potex*viruses are recognized by *Rx*-mediated resistance.

To test whether the resistance gene *Rx* could also mediate the recognition of other viruses more or less distantly related to PVX, different viruses from the *Flexiviridae* family, *Narcissus mosaic virus* (NMV), *White clover mosaic virus* (WCIMV), and *Cymbidium mosaic virus* (CymMV) in the genus *Potexvirus* and *Helenium virus S* (HVS) in the genus *Carlavirus*, were selected and used to inoculate WT or *Rx*-expressing *N. benthamiana* plants. The different viruses were selected based on their ability to systemically infect and induce symptoms in WT *N. bentha-*

miana control plants (Fig. 2; Supplementary Fig. 2). Phenotypes were observed 21 days after inoculation.

Four different phenotypes were observed in *Rx*-expressing *N. benthamiana* plants. Inoculation with HVS induced mosaic symptoms. Inoculation with PVX_{HB} resulted in systemic necrosis. No symptoms were observed following inoculation with NMV, WCIMV (Fig. 2A and B), or PVX_{CP4} representing a third phenotype. Transcript levels of NMV and WCIMV were determined by RT-PCR in both inoculated and systemic leaves of the WT plants. However, no CP coding region could be detected in leaves of *Rx*-expressing plants (Fig. 3), confirming absence of accumulation and movement of WCIMV and NMV viruses in these plants. The fourth phenotype was observed in *Rx*-expressing plants inoculated with CymMV, where a pronounced mottling and stunted phenotype was seen (Fig. 2C). Accumulation and spread of CymMV in the WT and *Rx*-expressing plants was confirmed by RT-PCR analysis of CP expression (Fig. 3).

These results show that, for the three *Potex*viruses tested (NMV, WCIMV, and CymMV), differences in infection phenotypes were observed between WT and *Rx*-expressing plants (Fig. 2). In contrast, these differences were not observed with the *Carlavirus*, HVS, suggesting that these three *Potex*viruses, and not the *Carlavirus*, are specifically affected by *Rx*-mediated resistance mechanisms. To evaluate this hypothesis, we then assayed the ability of the CP of these viruses to specifically induce an HR when transiently expressed in leaves of *Rx*-expressing plants.

The CP of NMV, WCIMV, and CymMV elicit an *Rx*-mediated HR.

The CP of two parental PVX isolates, the three other *Potex*viruses (NMV, WCIMV, and CymMV), and the *Carlavirus* (HVS) were cloned in an *Agrobacterium* binary vector and transiently expressed, by agroinfiltration, in WT and *Rx*-expressing *N. tabacum* leaves which are more resistant to multiple agroinfiltrations.

In contrast, on WT plants, no HR was induced following infiltration (Fig. 4). In *Rx*-expressing plants, an HR was observed

Fig. 2. Symptoms induced after inoculation with various *Potex*viruses on *Nicotiana benthamiana* plants. Wild-type (wt; left) and *Rx*-expressing (right) *N. benthamiana* plants were inoculated with **A**, *White clover mosaic virus*; **B**, *Narcissus mosaic virus*, and **C**, *Cymbidium mosaic virus*. Magnification of WT and *Rx*-expressing *N. benthamiana* leaves showing specific symptoms are also presented. Pictures were taken 21 days after inoculation.

Fig. 3. Accumulation of the various *Potex*viruses in *Nicotiana benthamiana* as revealed by reverse-transcription polymerase chain reaction analysis (RT-PCR). The amplified products corresponding to the sequence of complete open reading frames (ORFs) of the coat protein of *Narcissus mosaic virus* (NMV), *White clover mosaic virus* (WCIMV), *Cymbidium mosaic virus* (CymMV), and to actin ORF are indicated. Total RNA extracted from tissue of inoculated leaves (IL) and systemic leaves (SL) 21 days after inoculation of wild-type (wt) or *Rx*-expressing tobacco was used for RT-PCR analysis.

Fig. 4. Hypersensitive reaction observed in wild type (wt) and *Rx*-expressing (*Rx*) *Nicotiana tabacum* leaves following agroinfiltration with *Agrobacterium* harboring constructs allowing the transient expression of the complete *White clover mosaic virus* (WCIMV) coat protein (CP). Abbreviations indicate *Agrobacterium* culture carrying: CP4 = complete CP open reading frame (ORF) of *Potato virus X* (PVX)_{CP4}, CP = construct with complete CP ORF of WCIMV, HB = complete CP ORF of PVX_{HB}, GFP = complete ORF of green fluorescent protein, and pBIN61 = empty pBIN 61 vector. Pictures were taken 6 days after infiltration.

following expression of PVX_{CP4} CP but not of PVX_{HB} CP (Fig. 4). Infiltration of *agrobacteria* containing the empty vector did not induce an HR (Fig. 4).

A necrotic reaction similar to the one induced by the CP of PVX_{CP4} was observed 6 days after infiltration by the WCIMV CP (Fig. 4), NMV CP, and CymMV CP. No reaction was observed following transient expression of HVS CP (Supplementary Fig. 3). These results show that the sensing system mediated by the *Rx* gene is able to recognize the CP of these three other *Potexviruses* and to trigger an HR.

Identification of a smaller elicitor of *Rx*-mediated responses.

The domain of PVX able to elicit the *Rx*-mediated response was previously shown to be limited to amino acids 30 and 139 of the PVX CP (Bendahmane et al. 1995; Moffet et al. 2002). Therefore, the possibility that a similar domain of three other *Potexvirus* CP could trigger *Rx*-mediated response was investigated.

The region comparable to positions 30 to 139 of PVX CP was determined for the three other viruses (Figs. 5, fragment 7, and 6A; Supplementary Fig. 4) by sequence alignment. Three shorter constructs were also generated for the three *Potexvirus* CP (Fig. 6A, fragments 2, 5; and 6) which were truncated at their N-terminus by 20 amino acids or their C-terminus by 12 amino acids. All truncated versions were cloned into the *Agrobacterium* binary vector and transient expression was performed in WT and *Rx*-expressing *N. tabacum* leaves. As expected, no necrotic reaction was observed with any of the constructs in infiltrated leaves of the WT plants (Fig. 6). Construct 1 of the WCIMV CP (corresponding to positions 30 to 139 of the PVX

	1	MPPKVAPESS	DAVSSQEOPQ	RPPPATPPVP	TPPPGRREEV	GDRAEDPILQ	50
HVS							
NMV							
CymMV							
PVX-HB							
PVX-CP4							
WCIMV							
							
HVS		RLESLTALLR	SERSAVRVTN	ASFETGRPAL	QPTADMGRDV	TNMYNRPSTD	100
NMV							
CymMV							
PVX-HB							
PVX-CP4							
WCIMV							
							
HVS		SLWAVKPKPI	SNNMATSDEM	VKI.KVALEG	LGVPTHEITG	IYQMCIFYCA	150
NMV		DLKKIKYESA	TTAVATPAEI	QLGLDLF.KK	LGLDANSVAP	AMWDLARAYA	
CymMV		DLAALKYSPV	TSSIAFPBEI	KAITQLWVNN	LGLPADTVGT	AAIDLARAYA	
PVX-HB		FFSTAKVVA	SNVATNEDL	TRIKIWKD	MKIPSDTMAQ	AAWDLVRHCA	
PVX-CP4		FFSTAKVVA	SNVATNEDL	AKIQEIWKD	MKIPSDTMAQ	AAWDLVRHCA	
WCIMV		DIRILKTTSS	TVSVASPAEI	BAITKTWAEI	FKIPNDVLPL	ACWDLARAF	
							
HVS		STSSSSYQDP	KGVFEWPGGA	IMVDDVMGKV	QEIAGIRVVC	RLYAPVTWNY	200
NMV		DVOASRSVAVL	SGTTSPNPAP	TRQALARQFY	VINITPROFC	MYFAKVVVNL	
CymMV		DVGAKSATL	LGFCEPKPDV	RRALAAQIF	VANVTPROFC	AYYAKVVVNL	
PVX-HB		DVGSSAQTEM	IGTGYPYNGV	SRARLAAAIK	EV.CKLRQFC	KRYAPVVVNN	
PVX-CP4		DVGSSAQTEM	IGTGYPYNGV	SRARLAAAIK	EV.CTLRQFC	KRYAPVVVNN	
WCIMV		DVGASSKSEL	TGDSAAALAGV	SRKQLAAAIK	.IHCITRQFC	MYFANIVVNI	
							
HVS		MHIHDSHPSD	WASMGFAPNV	KYAAEDCEDY	VENPAAVOEL	GGVIRPRTRD	250
NMV		LLDSNVBPAG	WAKQGLPDDC	KFACPFDFEG	VLSPAALDEA	DGLIRBPESQ	
CymMV		MLATNDHPAN	WAKAGFOEDT	RFAAFDFEFA	VDSTAALDEA	EQQ.RRBTDR	
PVX-HB		MLTINSHBAN	WQACGFKPEH	KFAAFDFEFG	VTNPAAITPK	EGLIRBPFEA	
PVX-CP4		MLTINSHBAN	WQACGFKPEH	KFAAFDFEFG	VTNPAAITPK	EGLIRBPFEA	
WCIMV		MLTKTHBAS	WSKLGKYES	KFACPFDFEG	VNHPAALMPA	DGLIRGSDV	
							
HVS		EYVAYNAYKL	IVLNKAN.NN	DTYGNFSAQI	TGGRMGPTIE	HNFNANNKKQ	301
NMV		EIQAHSTAKY	GALARQRLQN	GNFVSTLAEV	THGRVGVGNA	MYAIEAPPPL	
CymMV		ERAAHSIGKY	GALARQRIQN	GNLITNTIAEV	TKGHLGSTNA	LYALPAPPRI	
PVX-HB		EMNAQTATF	VKITKARAQS	NDFASLDAAV	TRGRITGTTA	AEAVISLPPF	
PVX-CP4		EMNAQTAAAF	VKITKARAQS	NDFASLDAAV	TRGRITGTTV	AEAVVSLPPF	
WCIMV		EILAHQTAKQ	VALHRDAKRR	GTNVVNSV..			

Fig. 5. Multiple alignment of the amino acid sequences of the coat protein (CP) of *Narcissus mosaic virus* (NMV), *White clover mosaic virus* (WCIMV), *Cymbidium mosaic virus* (CymMV), and *Helenium virus S* (HVS) with those of the CP4 and HB strains of *Potato virus X* (PVX). Published sequences for the two PVX isolates and sequences determined in this work for other viruses were aligned using MultAlin software. The amino acids conserved in all sequences (even in HVS) are highlighted in dark gray; amino acids conserved only in *Potexviruses* are in gray. The arrowheads indicate key amino acids 121 and 127. The minimal elicitor of PVX for *Rx*-mediated-resistance, described by Bendahmane and associates (1995), is boxed.

CP) was able to elicit *Rx*-mediated HR whereas no necrosis could be observed on the infiltrated WT leaves (Fig. 6B). The same HR was observed after infiltration of construct 6 (corresponding to positions 50 to 139 of the PVX CP) (Fig. 6B). After transient expression of constructs 2 or 5 (corresponding to positions 50 to 127 and 30 to 127 of the PVX CP, respectively), no necrotic reaction was observed (Fig. 6B). As shown in the RT-PCR results (Fig. 6C), the constructs containing the coding sequence of the different fragments of WCIMV CP agroinfiltrated in WT and *Rx*-expressing *N. tabacum* led to the synthesis of the corresponding RNAs in the same quantity. These results suggest that the lack of symptoms on *Rx*-expressing leaves, observed for the fragments 2 and 5 of the WCIMV CP (Fig. 6B), is not due to the absence of the corresponding

RNA but to the inability of these CP fragments to elicit the *Rx*-mediated resistance.

Similar results were observed with the corresponding constructs derived from the CP of NMV (Supplementary Fig. 5); however, no necrotic reaction was observed following infiltration of constructs derived from the CP of CymMV. As expected, construct 1 derived from the HVS CP failed to elicit an HR.

These results demonstrate that, for two *Potexviruses* (NMV and WCIMV), the N- and C-terminal parts of CP are not required for elicitation of the *Rx*-mediated response. Therefore, the size of the minimal region of the CP essential for elicitation was reduced to a domain of 90 amino acids corresponding to positions 50 to 139 of the PVX CP.

Quantification of elicitor activity induced by the various *Potexvirus* CP.

In an effort to characterize the CP-*Rx* interaction, we quantified and compared the *Rx*-mediated HR in response to the recognized three *Potexvirus* CP and the CP of PVX_{CP4} and PVX_{HB} (controls). All CP were transiently expressed in WT or *Rx*-expressing *N. benthamiana* leaves and the cell death response was quantified by measurement of electrolyte leakage at 24, 48, and 72 h after infiltration (Fig. 7).

Expression of the PVX_{HB} CP, which is unable to elicit an HR, failed to induce an increase in conductivity after 24 h but induced a weak electrolyte leakage after 48 and 72 h (Fig. 7). Similar results have been reported in *N. benthamiana* (Farnham and Baulcombe 2006), where a weak response was observed in WT plants after transient expression of PVX_{HB} CP. Moreover, the expression of the PVX_{CP4} CP induced a strong and lasting increase of conductivity in *Rx*-expressing plants, reflecting an *Rx*-mediated HR (Fig. 7A). In contrast, expression of HVS CP failed to induce a significant increase in conductivity in *Rx*-expressing plants (Fig. 7A). Expression of WCIMV, NMV, and CymMV CP in *Rx*-expressing *N. benthamiana* resulted in electrolyte leakage as with PVX_{CP4} (Fig. 7B). However, the magnitude of the reaction with WCIMV seemed to be higher after 24 and 48 h than with the other CP, then decreased after 72 h. The differences observed with CymMV compared with PVX-CP4 were not significant. The absence of any significant electrolyte leakage following infiltration of all CP in WT *N. benthamiana* confirmed that the observed phenotype is *Rx* mediated (Fig. 6B).

Fig. 6. Identification of a smaller elicitor of the *Rx*-mediated response. **A**, Schematic representation of fragments of coat protein (CP). Fragments 1 to 6 of CP of different viruses are tested for their ability to elicit an *Rx*-mediated response. The amino acids encompassed by the different fragments and the overall length are indicated for *Potato virus X* (PVX) and *White clover mosaic virus* (WCIMV). **B**, Hypersensitive reaction observed in wild-type (wt) and *Rx*-expressing (*Rx*) *Nicotiana tabacum* leaves following agroinfiltration with *Agrobacterium* harboring constructs allowing the transient expression of the partial WCIMV CP. Abbreviations indicate *Agrobacterium* culture carrying: F1 = construct with coding sequence for fragment 1 of WCIMV CP, F2 = construct with coding sequence for fragment 2 of WCIMV CP, F5 = construct with coding sequence for fragment 5 of WCIMV CP, and F6 = construct with coding sequence for fragment 6 of WCIMV CP. Pictures were taken 6 days after infiltration. **C**, Accumulation of the various WCIMV CP fragments as revealed by reverse-transcription polymerase chain reaction (RT-PCR). The amplified products corresponding to the sequence of complete ORF of coat protein of PVX-TK, PVX-KR, WCIMV, and fragments 1, 2, 5, and 6 of WCIMV are indicated. Total RNAs extracted from tissue of wt *N. tabacum* leaves harvested 6 days after agroinfiltration were used for RT-PCR analysis. For WCIMV CP fragments, the same oligonucleotides have been used, resulting in the same size product (273 bp). For the complete CP, the expected size is 702 bp. The expression of actin has been used as control (bottom panel).

DISCUSSION

The PVX CP is known to be the avirulence determinant triggering *Rx*-mediated defense response in potato (Kavanagh et

al. 1992; Kohm et al. 1993). Mutagenesis of codons 121 or 127 impairs the ability of the virus to accumulate and spread in the infected plant (i.e., viral fitness) (Goulden et al. 1993) but also affects the ability of the CP to be recognized by the *Rx*-sensing

Fig. 7. Conductivity of the intercellular fluid extracted from leaves of wild-type or *Rx*-expressing *Nicotiana benthamiana* plants following infiltration with *Agrobacteria* carrying constructs allowing the expression of the coat protein (CP) of various potexviruses. Conductivity ($\mu\text{S cm}^{-1}$) measurements were derived from samples processed at the indicated time (h) after infiltration. Higher conductivity levels are indicative of increased membrane leakage and imminent cell death. **A**, Expression of the CP of parental viruses and *Helenium virus S* (HVS) are tested on wild-type (wt) and *Rx*-expressing plants. **B**, Expression of the CP of other viruses on wt and *Rx*-expressing plants compared with results obtained with parental viruses on *Rx*-expressing plants. Results are calculated as the mean $\pm$ SD. PVX = *Potato virus X*, NMV = *Narcissus mosaic virus*, WCIMV = *White clover mosaic virus*, and CymMV = *Cymbidium mosaic virus*.

machinery. According to previous studies (Goulden et al. 1993), the resistance is induced in potato only when a threonine residue is present at position 121 of the CP. Interestingly, this threonine residue is conserved in the CP of all known isolates of PVX, with the exception of PVX_{HB} (Huisman et al. 1988; Kavanagh et al. 1992; Orman et al. 1990; Skryabin et al. 1988). In addition, the lysine at position 127 may act as a secondary feature of the avirulence determinant in *Rx*-expressing potato (Goulden et al. 1993).

In our experiments using *N. benthamiana* WT plants, all mutations affecting these amino acids showed a strong leaf mosaic phenotype, reflecting the efficient accumulation and spreading of the virus as revealed by RT-PCR and Western blot analysis. This is somewhat surprising in view of the debilitating effects of the T121K mutation reported previously (Goulden et al. 1993). This discrepancy may be explained by the previous experiments being performed in potato whereas the experiments reported here were performed in *N. benthamiana*. There are numerous reports describing similar situations in which modified viruses were able to mount a systemic infection in some hosts but not in others, with *N. benthamiana* frequently appearing as a more permissive host (Fujita et al. 2000; Lee et al. 2002; Spetz and Valkonen 2004; Wang et al. 1998). It also appears that the negative effects of the T121K mutation on viral systemic movement reported previously (Goulden et al. 1993) are in fact host specific and observed in potato but not in the more permissive *N. benthamiana*.

All PVX strains containing a lysine at position 127 failed to accumulate or induce symptoms in *Rx*-expressing *N. benthamiana* (Fig. 1). This result is contrasted by previous experiments which show that, among PVX strains harboring a lysine at position 127, PVX_{CP4-KK} and PVX_{CP4-RK} were the only ones able to accumulate in *Rx*-expressing potato protoplasts (Bendahmane et al. 1995), and that PVX_{CP4-KK} systemically invades *Rx*-expressing potato plants (Goulden et al. 1993). It is difficult to reconcile these previous observations with our results, other than postulating that, particularly in the case of PVX_{CP4-KK}, the specificity of the *Rx*-based recognition system is subtly modified between the original *Rx*-expressing potato and the *Rx*-expressing *N. benthamiana* used in these experiments. As a consequence, it seems that the genetic background in which the resistance gene is expressed could influence the specificity of recognition. Alternatively, it has been shown, for different resistance genes (*I* gene and *Tm* gene), that virus accumulation observed in protoplasts does not necessarily correlate with results observed at a whole-plant level (Cadle-Davidson and Jahn 2005; Collmer et al. 2000; Fraser and Loughlin 1980; Motoyoshi and Oshima 1977). Taking into consideration the guard hypothesis (Dangl and Jones 2001) of the mode of action of *R* proteins, another explanation for the discrepancies could be that, in *Rx*-expressing *N. benthamiana*, interaction of the *R* gene product with *Nicotiana* orthologs of the potato cellular interactors could somehow modulate the specificity of the *Rx* recognition complex.

The situation is different for the mutants, which possess an arginine at position 127. Very different infection outcomes were observed, ranging from extreme resistance with PVX_{CP4-SR} and PVX_{CP4-VR} to systemic necrosis with PVX_{HB} and two intermediate phenotypes, local lesions without systemic infection for PVX_{CP4-RR}, and an HR with systemic infection for PVX_{CP4-PR} (Fig. 1). These different infection phenotypes probably reflect differences in recognition efficiency of the CP modulated by the amino acid present at position 121. Similar variations in elicitor activity are reported in *Rx*-expressing potato protoplasts using PVX_{CP4}-derived mutants (Bendahmane et al. 1995). Despite the fact that no HR was observed upon transient expression of the PVX_{HB} CP in *Rx*-expressing leaves (Fig. 4), both the *Rx*-dependent systemic infection phenotype of the virus and the weak but significant *Rx*-dependent increase in conductivity observed upon PVX_{HB} CP expression (Fig. 7B) indicate that PVX_{HB} is not fully avirulent but has a low level of interaction with the *Rx*-mediated sensing system in the *Rx*-expressing *N. benthamiana*. Weak or absent *Rx*-mediated cell death following transient expression of CP PVX_{HB} in *N. benthamiana* has previously been observed (Farnham and Baulcombe 2006).

Taken together, the results reported here reinforce the key role of the amino acids at positions 121 and 127 in the recognition of PVX CP by the product of the *Rx* gene. In contrast to previous results claiming that T121 has a central role in the recognition process (Goulden et al. 1993), our results suggest an important role for amino acid 127 as well, because all mutants with a lysine at this position were efficiently recognized, regardless of the nature of the amino acid at position 121 (Fig. 1).

Moreover, our results suggest that the recognition stage may be slightly different depending on the genetic background, revealing a more complex structure–function relationship pattern than was previously described (Bendahmane et al. 1995; Goulden et al. 1993). Although it has been shown that *R* genes function differently when they are transferred to different genetic backgrounds, the situation seems to be different for *Rx*, because its expression allows a greater recognition spectrum in *Nicotiana* spp. compared with potato. Furthermore, our results suggest that the fine specificity of recognition between the CP and the *Rx* gene product may also be modulated by host factors differing between *Rx*-expressing potato and *Nicotiana* spp.

The second set of experiments presented in this article aimed to determine whether the *Rx* gene product is able to recognize CP other than the PVX, and to see whether *Rx*-mediated resistance could control the multiplication of other viruses. This appears to be the case for at least the three *Potexviruses* evaluated (Fig. 2), in particular for NMV and WCIMV, in which systemic invasion was blocked in an *Rx*-dependent manner (Figs. 2 and 3). In the case of CymMV, elicitor activity of the CP was demonstrated by both *Rx*-dependent HR observed in CP transient expression experiments and *Rx*-dependent in-

Table 1. Presence (+) or absence (–) of a hypersensitive reaction following transient expression through agroinfiltration of the coding sequence of the coat proteins (CPs) or of fragments derived from the CP of different viruses in the leaves of *Rx*-expressing *Nicotiana tabacum*

Cultures ^b	Virus ^a					
	PVX _{CP4}	PVX _{HB}	WCIMV	NMV	CymMV	HVS
CP	+	–	+	+	+	–
F1	ND	ND	+	+	–	–
F2	ND	ND	–	–	–	ND
F5	ND	ND	–	–	–	ND
F6	ND	ND	+	+	–	ND

^a PVX_{CP4} and PVX_{HB} = isolates of *Potato virus X*, WCIMV = *White clover mosaic virus*, NMV = *Narcissus mosaic virus*, CymMV = *Cymbidium mosaic virus*, HVS = *Helenium virus S*, and ND = not determined.

^b CP = *Agrobacterium* culture expressing the relevant complete CP; F1 to F6 = *Agrobacterium* cultures expressing fragment 1 to 6 of the relevant CP.

creased symptomatology in the whole-plant assay. However, during these experiments, the fragments of CymMV CP failed to elicit an HR and only the complete CymMV CP was able to elicit an *Rx*-dependant HR. The CP of this virus may be a weak elicitor, which could also explain phenotypes observed on the whole plant following inoculation. On the other hand, the CP of the *Carlavirus* HVS, which is more divergent (Fig. 5), is not able to elicit *Rx*-mediated responses. An alignment of CP sequences indicates that the PVX CP shows 33, 39, and 35% identity with the CP from NMV, WCIMV, and CymMV, respectively, and only 25% with the CP of HVS (Fig. 5). However, these sequence comparisons do not show an obvious conserved domain of the CP that could represent the *Rx*-recognition motif. Indeed, only 40 amino acids are identical between the four *Potexvirus* CP and these are not particularly clustered. In addition, 26 of them are also present in the CP sequence of HVS (Fig. 5). An interesting observation, however, is that the four *Potexvirus* CP have a threonine at position 121 but the HVS CP has a glycine in this position. The other amino acid, important for PVX CP recognition, at position 127, is not conserved between the different *Potexviruses*, CymMV having an alanine and NMV and WCIMV a methionine in this position. This threonine/methionine combination is also present in the CP of different PVX avirulent strains, including UK3 and DX (Santa Cruz and Baulcombe 1995). Moreover, among the CP sequences of the *Flexiviridae* family, particularly *Potexvirus* and *Carlavirus*, a range of combinations for the two amino acids at position 121 and 127 can be found.

Transient expression of CP fragments from the different viruses (Fig. 6B) confirms, as previously shown for PVX CP, that the 5' and 3' extremities of the CP are not required for the elicitation of the *Rx*-mediated resistance. Only 110 amino acids of the PVX CP were known to be necessary to induce *Rx*-mediated resistance and to suppress PVX RNA accumulation (Bendahmane et al. 1995; Moffet et al. 2002). Our results suggest that fragments of NMV and WCIMV CP corresponding to the same 110 amino acids are able to induce the *Rx*-mediated resistance, as indicated by both the induction of the *Rx*-dependent HR (Fig. 4) and the *Rx*-dependent increase in conductivity (Fig. 7). Furthermore, the minimal region of these different CP was reduced by the demonstration that the first 20 amino acids of this fragment are not required for elicitor activity (Fig. 6B). This smaller elicitor of 90 amino acids corresponds to amino acids 50 to 139 of PVX CP containing the key residues 121 and 127. In this region, the CP of the two *Potexviruses* (NMV and WCIMV) and of PVX show an average identity of only 40%, indicating a high level of flexibility in the recognition pattern of the *Rx*-based sensing machinery. The deletion of the 12 carboxy-terminal amino acids of this minimal elicitor fragment, including the key position 127 (constructs 2 and 5, corresponding to position 50 to 127 and 30 to 127, respectively, of the PVX CP), abolishes the eliciting activity (Fig. 6B), indicating that the deleted region (from 128 to 139) is required for the elicitor activity. The expression of the short deleted fragment in *Rx*-expressing plants could indicate whether this region of 12 amino acids is sufficient to induce an HR; however, this seems unlikely because this fragment does not contain the key residue 121.

The results reported here show that the product of the *Rx* gene can recognize the CP of several viruses belonging to the same genus (Table 1), even if these proteins show only a distant relationship in the required region for elicitation. The most likely hypothesis to explain these observations is that the *Rx* molecular sensor targets a conserved structural element of these CP rather than a linear amino acid sequence. The possibility of recognition of a three-dimensional structure has already been raised for the PVX-*Rx* pathosystem (Chapman et

al. 1992; Goulden et al. 1993). Secondary structure predictions of the PVX CP (Baratova et al. 1992a and b) have shown that the key amino acids 121 and 127 are likely to be buried inside the CP and that their mutation could induce conformational changes of the CP (Querci et al. 1993). A new organization of the PVX CP has recently been proposed by Nemykh and associates (2008) in which a majority of the secondary elements coincide with the model predicted by Baratova and associates (1992a and b). In this new model, the fraction of predicted α helices (55%) is even larger than before. However, it seems that the three-dimensional arrangement of α helices and β turns could be quite different from the model predicted before. An experimental analysis of the three-dimensional structure of NMV, WCIMV, CymMV, and PVX CP will provide further information on the nature of a putative conserved structure.

Such a conserved element could be assimilated to a PAMP for plant viruses as has been described for microbes (Brunner et al. 2002; Parker 2003). Penetration of the cell wall exposes the host plasma membrane to microbes, where they encounter extracellular surface receptors that recognize PAMPs. Our most complete understanding of the plant response to PAMPs relates to perception of flagellin, the protein subunit of flagella (Gomez-Gomez and Boller 2000). Flagellin is recognized as a PAMP by many plant species. In *Arabidopsis*, a 22-amino-acid peptide is sufficient for host receptor activation by FLS2, which is homologous to TLR5 in animal cells (Felix et al. 1999; Gomez-Gomez and Boller 2000; Underhill and Ozinsky 2002). Identification of this potent peptide elicitor facilitated detailed analysis of responses to flagellin using whole-plant and protoplast systems. It may be that multiple PAMP signaling pathways converge and activate defenses via overlapping mitogen-activated protein kinase cascades and transcription factors (Asai et al. 2002). The identification of a minimal elicitor pattern in the CP of *Potexviruses* could facilitate the determination of the three-dimensional structure recognized by the *Rx* machinery.

The guard model raises the hypothesis that host proteins will be able to recognize different elicitors and the change in functional properties of the elicitor (Dangl and Jones 2001). Our results support this hypothesis, suggesting that host proteins are able to detect structural or functional differences between different strains of PVX CP as well as CP of different *Potexviruses*. Recent identification of the GTPase, RanGAP2, as a potential "guardee" of *Rx* or *Rx* homologs (Sacco et al. 2007; Tameling and Baulcombe 2007) is an important stage in the understanding of the *Rx*-mediated resistance mechanism, although a direct physical interaction between RanGAP2 and PVX CP has not yet been shown. Further studies are now needed to identify the different partners and the conserved structure of CP implicated in *Rx*-mediated resistance in different genetic backgrounds.

MATERIALS AND METHODS

Plant material.

Untransformed control plants of *N. benthamiana*, *N. tabacum* cv. Samsun, and transgenic lines expressing the *Rx* gene under its own promoter were used in this study. The *Rx*-expressing plants were obtained by *Agrobacterium*-mediated transformation using the pSLJ9 recombinant plasmid (Bendahmane et al. 1999). Plants were grown in glasshouses under controlled temperature (23 and 19°C, day and night, respectively) and light (16-h day length).

Mutagenesis of the PVX CP.

Oligonucleotide-directed mutagenesis (Ho et al. 1989; Horton et al. 1989) was used to introduce specific mutations into the CP gene of full-length infectious cDNAs clones of CP of PVX_{CP4}.

The presence of the mutation was confirmed by sequence analysis. The transcripts of these mutant constructs were inoculated first to WT *N. benthamiana* and subsequently to WT and *Rx*-expressing transgenic plants.

The mutations introduced in the CP of PVX_{CP4} changed the codon at position 121 from ACA(T) to GTG(V), AGC(S), CCG(P), CGG(R), or AAA(K) and the codon at position 127 from AAA(K) to CGG(R).

Virus isolates and inoculation assays.

All the viruses used in this study—CymMV, HVS, NMV, PVX strain CP4 (accession number AF172259), PVX strain HB (accession number X72214), and WCIMV—were maintained in WT *N. benthamiana*. To carry out the *Rx*-resistance assays, each virus was mechanically inoculated into WT and *Rx*-expressing plants. Inoculations were repeated five times on two WT and two *Rx*-expressing plants for each virus. Plants were inoculated at the two- to four-leaves stage, with Carborundum (400 mesh) used as abrasive and sap prepared by grinding with sand one leaf of an infected *N. benthamiana* plant in 3 ml of 10 mM phosphate buffer (pH 7), and centrifuging the resulting homogenate for 2 min at 800 × *g* before use. The inoculated plants were scored for symptoms of viral infection 21 days after inoculation. The presence of virus was verified by RT-PCR using primers designed to amplify the viral CP (Supplementary Table 1).

RNA isolation.

Total RNA was extracted from inoculated leaves as previously described (Devic et al. 1989). Specifically, 2 g of leaf tissue was ground in liquid nitrogen using a mortar and pestle. The powdered material was dispersed in 4 ml of homogenization buffer (0.1 M NaCl, 2% sodium dodecyl sulfate [SDS], 10 mM EDTA, and 50 mM Tris-HCl, pH 9.0), mixed with 4 ml of phenol/chloroform (1:1), and centrifuged at 3,000 × *g* for 20 min. The supernatant was further extracted once with phenol/chloroform. The nucleic acids were precipitated with 5 ml of 100% ethanol, washed with 70% ethanol, dried, and resuspended in 150 µl of diethylpyrocarbonate (DEPC) water.

DNase treatment.

Total RNA (20 µg) was treated with DNase I (Promega, Madison, WI, U.S.A.). The reaction mix of 25 µl (7.5 U of DNase I and 25 U of RNaseOUT) was incubated at 37°C for 10 min and completed with 75 µl of H₂O. The mix was further extracted once with phenol/chloroform. The nucleic acids were precipitated with 300 µl of 100% ethanol and 15 µl of NaAc (3 M, pH 5.5), washed with 70% ethanol, dried, and resuspended in 30 µl of DEPC water.

Reverse transcription of the viral CP.

Total RNA (2 µg) was used for first-strand cDNA synthesis, which was primed with 0.02 picomoles of oligonucleotide (dT)₂₀ (Invitrogen, Carlsbad, CA, U.S.A.). This first reaction mix of 11.5 µl was heated at 70°C for 10 min and then cooled on ice for 5 min. The reaction mix was completed at 20 µl (4 µl of 5× superscript buffer [Invitrogen], 2 µl of 100 mM dithiothreitol [DTT], 1 µl of 5 mM dNTP, 40 units of RNaseOUT [Invitrogen]) and incubated at 37°C for 5 min, after which 100 units of SuperscriptIII (Invitrogen) was added. The reaction was incubated at 37°C for 2 h. The first-strand cDNA was converted into double-strand DNA by PCR. These RT-PCR were performed with oligonucleotides using 25 cycles of amplification.

In the same manner, the viral CP were reverse transcribed from infected or agroinfiltrated tissues. Sequencing before and after propagation in plants was used to test the stability of the viral genome sequence.

Agrobacterium tumefaciens-mediated transient expression of viral CP.

The CP coding sequences from the different viruses were inserted between the 35S promoter and terminator in the binary vector pBin61 (Bendahmane et al. 1999). These constructs were transformed into *Agrobacterium* strain C58C1 carrying the virulence helper plasmid pCH32 (Hamilton et al. 1996). pCH32 expresses VirG and VirE and is used to enhance T-DNA transfer. The binary constructs were transiently expressed in *N. tabacum* leaves as previously described (Bendahmane et al. 2000).

Western blot analysis.

Inoculated leaves (500 µg) were ground in liquid nitrogen and 500 µl of 2× Laemmli solution (10% glycerol, 3% SDS, 0.2 M DTT, 0.004% bromophenol blue, 0.12 M Tris HCl, pH 6.8, in 0.09% SDS) was added. The samples were boiled at 100°C for 10 min and centrifuged for 10 min at 10,000 × *g*. The supernatants were stored at −20°C.

Each sample (20 µl) was loaded and separated on a 12% acrylamide gel and blotted on polyvinylidene difluoride (PVDF) membrane (immunoblot PVDF membrane; Biorad, Hercules, CA, U.S.A.) for an hour in transfer buffer (25 mM Tris, pH 8.3; 192 mM glycine; 20% ethanol). The membrane was washed for 5 min in TTBS (0.1 M Tris; 0.15 M NaCl, pH 7.5; and 0.1% Triton X100) and blocked for 1 h in TTBS + 10% nonfat milk. The membrane was then washed for 5 min in TTBS and incubated with the primary antibody (immunoglobulin G [IgG] polyclonal antibody against PVX; DSMZ, Braunschweig, Germany) at 1/500 dilution overnight at 4°C. The membrane was then washed three times for 5 min in TTBS and incubated through the day with secondary antibody (goat anti rabbit IgG linked to alkaline phosphatase; DSMZ) at 1/1,000 dilution. After four washes of 10 min in TTBS, the membrane was developed using the AP kit (AP conjugate substrate kit; Biorad).

Molecular markers used were low prestained markers (Precision plus Protein all blue standard, Biorad; Page ruler protein ladder, Fermentas, Vilnius, Lithuania).

Electrolyte leakage assays.

These measurements were performed as previously described (Farnham and Baulcombe 2006). Four 7-mm-diameter leaf disks from the same *Agrobacterium*-infiltrated leaf were collected at 24, 48, or 72 h after infiltration and placed in a 1.5-ml tube before adding 1 ml of analytical grade H₂O. Samples were incubated for 10 min at 37°C at a pressure of 1 Pascal. Leaf disks were then incubated at room temperature for 30 min, removed from the tubes, and stored at −20°C. Conductivity was measured using a Horiba B-173 conductivity meter. Electrolyte solutions were thawed at room temperature and 0.8 ml of the solution was added to 3.2 ml of analytical grade H₂O in a 15-ml tube. For each data point, five independent replicates were analyzed. Results are shown as the mean ± standard deviation.

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