

Multiple Resistance Traits Control *Plum pox virus* Infection in *Arabidopsis thaliana*

V. Decroocq,¹ O. Sicard,¹ J. M. Alamillo,² M. Lansac,¹ J. P. Eyquard,³ J. A. García,¹ T. Candresse,¹ O. Le Gall,¹ and F. Revers¹

¹UMR GDPP INRA-Université Victor Segalen Bordeaux 2, BP81, 33883 Villenave d'Ornon Cedex, France; ²CNB-CSIC, 28049 Madrid, Spain; ³U.R.E.F.V., INRA, BP81, 33883 Villenave d'Ornon Cedex, France

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Twelve *Arabidopsis* accessions were challenged with *Plum pox potyvirus* (PPV) isolates representative of the four PPV strains. Each accession supported local and systemic infection by at least some of the PPV isolates, but high variability was observed in the behavior of the five PPV isolates or the 12 *Arabidopsis* accessions. Resistance to local infection or long-distance movement occurred in about 40% of all the accession-isolate combinations analyzed. Except for Nd-1, all accessions showed resistance to local infection by PPV-SoC; in the Landsberg *erecta* (*Ler*) accession, this resistance was compromised by *sgt1* and *rar1* mutations, suggesting that it could be controlled by an *R* gene-mediated resistance pathway. While most of the susceptible accessions were symptomless, PPV induced severe symptoms on inflorescences in C24, *Ler*, and Bay-0 as early as 15 days after inoculation. Genetic analyses indicated that these interaction phenotypes are controlled by different genetic systems. The restriction of long-distance movement of PPV-EI Amar and of another member of genus *Potyvirus*, *Lettuce mosaic virus*, in Col-0 requires the *RTM* genes, indicating for the first time that the RTM system may provide a broad range, potyvirus-specific protection against systemic infection. The restriction to PPV-PS long-distance movement in Cvi-1 is controlled by a single recessive gene, designated *rpv1*, which was mapped to chromosome 1. The nuclear inclusion polymerase b-capsid protein region of the viral genome appears to be responsible for the ability of PPV-R to overcome *rpv1*-mediated resistance.

Additional keyword: Sharka.

Sharka disease is the major limiting factor in stone fruit tree production in Europe and North America. It is caused by *Plum pox potyvirus* (PPV) and is one of the most serious viral diseases in peach, apricot, and plum orchards. Losses from Sharka are due primarily to precocious fruit drop and decreases in fruit quality. The potential of PPV to severely affect the fruit tree industry has prompted the European Union to classify it as a quarantine pathogen (European Union council directive 2000/29/EEC, annex II), while the United States federal government has classified it as one of the top ten significant threats to U.S. agriculture (Public Health Security and Bioterrorism Act of

2002, PPV belongs to the family *Potyviridae*. Apart from the atypical EI Amar (EA) isolate (Wetzel et al. 1991a) and the sweet and sour cherry-infecting isolates (Crescenzi et al. 1997; Nemchinov and Hadidi 1996), most PPV isolates are classified into two major strains, M (from the isolate Marcus) and D (from the isolate Dideron) (Candresse et al. 1998). Recently, a new atypical strain, W3174, was discovered, but its origin is still unclear (James and Varga 2005).

Studies of the molecular mechanisms underlying the interactions of PPV with its woody hosts have been hampered by the difficulties inherent to the molecular genetic analysis of stone-fruit tree species, in particular the extended generation times and the length and space requirements of phenotypic tests. Four years of monitoring after inoculation are needed to assess the level of resistance or susceptibility of a given *Prunus* cultivar. Standardization of the resistance tests has similarly proved difficult because of delayed responses to inoculation, variability of the virus, physiological state of the host plant, and inoculation method.

In the past few years, the genetic and molecular advantages of model plant *Arabidopsis thaliana* were exploited for the identification of host factors contributing to virus infection. For example, two genes, *Tobamovirus multiplication 1* and *Tobamovirus multiplication 3* (*Tom1*, *Tom3*), were identified from a screen for *A. thaliana* mutants that show defective infection by *Tobacco mosaic virus* (TMV) (Yamanaka et al. 2000, 2002). Both *Tom1* and *Tom3* are putative transmembrane proteins that may serve as membrane anchors for the TMV replication complex. Similarly, movement-defective mutants and the corresponding RTM loci, which cooperate to restrict *Tobacco etch virus* (TEV) long-distance movement, were described (Mahajan et al. 1998; Whitham et al. 1999). *RTM1* encodes a jacalin-like protein, *RTM2* shows homology to the plant small heat-shock proteins with a predicted transmembrane domain (Chisholm et al. 2000; Whitham et al. 2000), and an additional RTM loci has been identified but not yet fully characterized. The mechanism whereby the three RTM loci cooperate to restrict long-distance movement is still unclear.

Through studies of the interaction of eukaryotic translation initiation factors with viral proteins and genetic and reverse-genetic analysis, eIF4E and eIF(iso)4E were linked to the potyvirus infectious cycle in various plant species (Duprat et al. 2002; Gao et al. 2004; Kang et al. 2005; Lellis et al. 2002; Nicaise et al. 2003; Ruffel et al. 2002; Stein et al. 2005). Our own analyses demonstrated a cosegregation of the *Prunus eIF(iso)4E* ortholog with a major quantitative trait locus (QTL) of resistance to sharka in peach and apricot (Decroocq et al. 2005). To identify new host factors involved in plant-potyvirus interactions, in particular with a potyvirus infecting perennial

Corresponding author: Véronique Decroocq;
E-mail: decroocq@bordeaux.inra.fr

*The e-Xtra logo stands for “electronic extra” and indicates the HTML abstract available on-line contains supplemental material not included in the print edition. A table lists the twelve *Arabidopsis* accessions and the five PPV isolates used in this study and Figure 1 appears in color online.

host plants, *A. thaliana* accessions were evaluated for their susceptibility to a range of PPV isolates representing a large part of the pathogen diversity.

Using this approach, we showed considerable variation in the susceptibility to PPV infection of *A. thaliana* accessions. Phenotypes ranging from complete invasion of the plant (accompanied or not by symptom-induction) to the inability of the virus to mount a productive replication in the initially inoculated leaves were observed and the genetic bases of some of these interaction phenotypes were analyzed.

RESULTS

Twelve *Arabidopsis* accessions of diverse geographical origin were inoculated with five isolates of PPV. Responses of *Arabidopsis* to PPV were classified as susceptible (referred to as S in Table 1) when the virus was detected in the uninoculated inflorescence tissues by enzyme-linked immunosorbent assay (ELISA) or by reverse transcriptase-polymerase chain reaction (RT-PCR). While in most cases, the infected plants remained asymptomatic, there were several accession-isolate combinations in which symptoms were observed, such as reduction of plant growth, chlorotic stems, cauline, and rosette leaves, severe inflorescence stunting, and distortion accompanied or not with curling of the cauline leaves (Fig. 1). In Table 1, the severity of the observed symptoms is indicated by the number of + signs. Plants in which both ELISA and RT-PCR assays were negative for PPV detection in inflorescence tissues were noted as resistant. Based on the results of PPV detection by ELISA or RT-PCR in the inoculated leaves, resistance phenotypes were further separated in resistance to local infection (Ri, no virus detected in inoculated leaves) and resistance to long-distance movement (Rsys, virus detected in inoculated leaves but not on distal uninoculated tissues). In preliminary experiments, the plants classified as Ri were assayed for PPV accumulation at 30 days postinoculation (dpi), but no accumulation was detected, and therefore, sampling at such late stages of *Arabidopsis* development was not routinely continued and plants were sampled at 9 and 21 dpi.

PPV is able to systemically infect *Arabidopsis thaliana*.

Although the response of *Arabidopsis* accessions to the five PPV isolates varied, all 12 accessions studied were infected by at least one PPV isolate, with or without development of visible symptoms (Table 1). However, none of the PPV isolates tested

was able to infect the *AteIF(iso)4E-1* transposon-disrupted mutant line (Duprat et al. 2002), suggesting that eIF(iso)4E plays an essential role during PPV infection in *A. thaliana*, as already shown for other potyviruses (Duprat et al. 2002; Lellis et al. 2002). Remarkably, the PPV-SoC isolate was able to systemically infect all tested accessions, while infection of uninoculated tissues was never observed for the PPV-SoC isolate (Table 1). In fact, only the Nd-1 accession was able to support local replication of PPV-NAT isolate, while the virus was never detected in the inoculated leaves of any of the other accessions tested. Although Ri is often associated with a hypersensitive response characterized by localized cell death at the infection site, a local lesion response was not macroscopically observed in any of the experiments reported here (data not shown).

In a number of accession-isolate combinations, PPV was only detected (by ELISA or by RT-PCR) in the inoculated leaves, indicating that the virus was unable to spread in the uninoculated tissues. Such a phenotype was observed in most of the accessions inoculated with the PPV-EA isolate. Absence of any long-distance movement was also observed when Cvi-1 was inoculated with PPV-PS and when Shahdara and Kas-1 were infected with PPV-R (Table 1). In contrast, the C24 accession was highly susceptible to PPV infection and developed severe growth defects when infected with M, D, and EA PPV strains. Severe symptoms were also observed when the Landsberg *erecta* (*Ler*) accession was infected with the PPV-R isolate, and slightly less severe ones were observed when Bay-0 was infected either with the PPV-NAT or PS isolates (Table 1). Two to three weeks after inoculation, chlorosis was observed on distal uninoculated tissues, such as cauline leaves and inflorescences. During later stages of infection, the plants exhibited severe growth stunting and occasionally lethal necrosis (Fig. 1).

These results show that a high degree of variation exists in the outcome of *Arabidopsis*-PPV interactions and that, in a significant number of cases (40%, 24 out of 60 accession-isolate combinations), some form of resistance against viral infection was observed. The genetic bases of some of the phenotypes observed were then analyzed using either forward or reverse genetics strategies.

Genetic basis of symptom development in the *Ler* accession infected with PPV-R isolate.

Limited information on the genetic bases underlying symptom development in virus-plant pathosystems is available so

Table 1. Phenotypic variation of *Arabidopsis*–*Plum pox potyvirus* (PPV) interactions

<i>Arabidopsis</i> accessions	PPV isolates ^a				
	NAT (D)	R (D)	PS (M)	SoC	EA
C24	S+++	S+++	S+++	Ri	S+++
Landsberg <i>erecta</i> (<i>Ler</i>)	S	S+++	S	Ri	S
BayO	S++	S	S++	Ri	Rsys
Col-0, Col-3, Col-5	S	S+	S	Ri	Rsys
Ws, Ws-2	S	S	S	Ri	Rsys
Cvi-1	S	S	Rsys	Ri	S
Kas-1, Shahdara	S	Rsys	S	Ri	Rsys
Nd-1	S	S	S	Rsys	Rsys
<i>Arabidopsis</i> mutants in Col-0 background ^b					
<i>AteIF(iso)4E-1</i>	Ri	Ri	Ri	Ri	Ri
<i>Arabidopsis</i> mutants in <i>Ler</i> background ^c					
<i>sgt1b, rar-10</i>	S	nd	S	Rsys	S
<i>sgt1b / rar-10</i>	S	nd	S	Rsys	S

^a S = susceptible but symptomless; S+ = mild symptoms appeared occasionally (1 or a few plants over 12 infected) and did not affect plant growth; S++ = an intermediate situation between the two extremes; S+++ = all plants infected developed severe growth defects leading, in most cases, to death at 3 to 4 weeks postinoculation; Ri = resistance to PPV inoculation; Rsys = resistance to PPV long-distance movement; nd = not determined.

^b *AteIF(iso)4E-1* was obtained by insertion of a transposon within the *eIF(iso)4E* gene (Duprat et al. 2002).

^c Provided by J. Parker (Max Planck Institute, Cologne, Germany) and coworkers.

far. Whereas severe symptoms have been observed in *Ler* in response to infection by PPV-R isolate, only very mild symptoms were observed in the Col-0 accession (Table 2 and Fig. 1). To determine the number of loci controlling severe symptom development in the *Ler* accession infected by the PPV-R isolate, we used a recombinant F2 population between *Ler* and Col-0. Parental and F2 plants were scored for development of symptoms and virus accumulation, as determined by ELISA. The results showed that the ratio of symptomatic versus asymptomatic plants did not fit with simple genetic models, suggesting that several loci are involved in the control of this trait. A total of 62 individuals out of 115 developed symptoms, which

is consistent with a 9:7 segregation ratio, indicating that this trait is controlled by at least two loci ($P = 0.6134$ to 0.5311 ; Table 2). Some F2 individuals developed more severe symptoms than the *Ler* parental line; these plants showed severe stunting and died within 21 dpi. This observation suggests that the Col-0 parent might contribute with one or more supplementary genetic factors enhancing or accelerating the severity of the symptoms.

Analysis of local resistance to inoculation by the PPV-SoC isolate.

The Ri observed during the interaction between PPV-SoC and most ecotypes tested, failed to detect any virus in the inoculated leaves, suggesting either a link with either *R* gene-mediated resistance pathways or a nonhost passive resistance. The first possibility was evaluated in the *Ler* accession by analyzing the behavior of available mutant lines affecting two genes identified as essential in *R* gene-triggered disease resistance, *RAR1* and *SGT1* (Austin et al. 2002; Azevedo et al. 2002; Tornero et al. 2002). Table 1 shows that, contrary to the wild-type *Ler* accession, *sgt1b* and *rar1* mutants and the *sgt/rar1* double mutant supported the local accumulation of the PPV-SoC isolate. However, these mutants still failed to support PPV-SoC systemic infection, indicating the existence in these plants of two levels of resistance, one restricting local PPV-SoC infection, compromised by the *rar1* or *sgt1b* mutations, and the other, independent of these factors, acting to block the long-distance spread of the virus.

Analysis of the resistance to PPV-EA long-distance movement in Col-0.

Segregation data obtained for resistance to PPV-EA infection in the Col-0 × *Ler* F2 population was consistent with a single dominant gene model (79:17 resistant/susceptible, $P = 0.098$, for a segregation ratio of 3:1). In *A. thaliana*, three dominant loci have been shown to confer resistance to the long-distance movement of another potyvirus, TEV, and were designated RTM for restricted TEV movement (Mahajan et al. 1998). Mapping of the resistance trait associated with restriction of PPV-EA systemic spread in the recombinant inbred lines (RIL) of Lister and Dean (1993) allowed the identification of a single region, in the upper part of chromosome 1 in the vicinity of the *RTM1* gene (data not shown) (Mahajan et al. 1998). To analyze the possible involvement of the RTM in the resistance to PPV-EA, chemically induced *rtm* mutants (Mahajan et al. 1998; Whitham et al. 1999) were challenged with the PPV-EA isolate (Table 3). In all three mutants, PPV-EA was able to infect uninoculated tissues and was readily detected in cauline leaves and inflorescence tissues. In order to determine whether the *RTM* loci are also required for restriction of the long-distance movement of other potyviruses, the *rtm* mutants were inoculated with the AF199 isolate of *Lettuce mosaic virus* (LMV), which was previously reported to be unable to systemically invade the Col-0 accession (Revers et al. 2003). Again, restric-

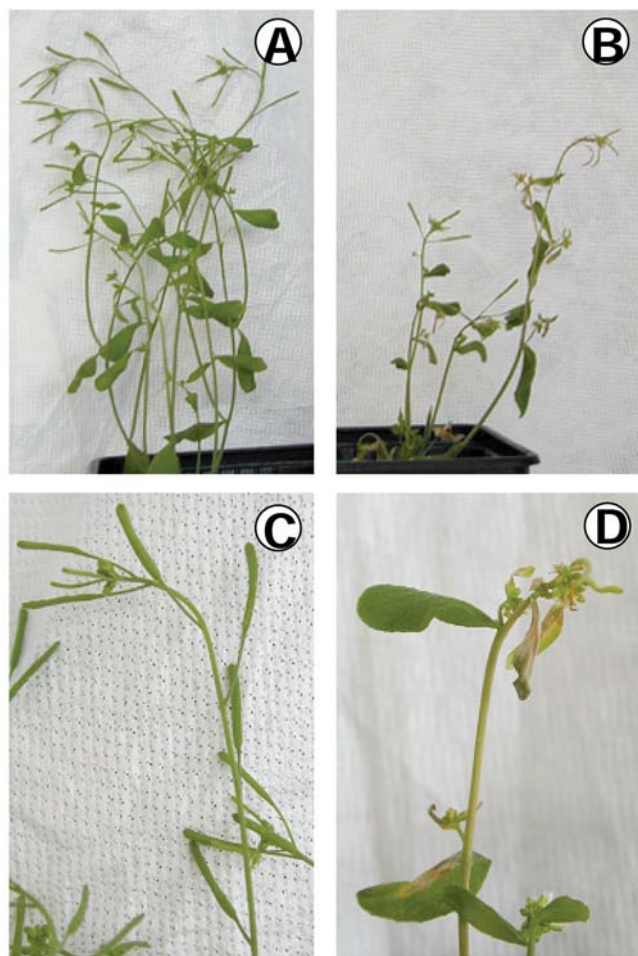


Fig. 1. Symptoms exhibited by *Arabidopsis thaliana* accession *Ler* when infected with *Plum pox potyvirus* (PPV)-R. **A**, Healthy plant 3 weeks after inoculation with PPV-R. **B**, Infected *Landsberg erecta* (*Ler*) plant with severe symptoms and stunting, 3 weeks after inoculation. **C**, Close-up of **A** showing healthy inflorescences. **D**, Close-up of *Ler* inflorescences infected with the PPV-R isolate and showing leaf curling and chlorosis and severe inflorescence distortion.

Table 2. Statistical inheritance analysis in selected *Arabidopsis-Plum pox potyvirus* (PPV) interactions

<i>Arabidopsis</i> F2 populations	PPV isolate	Number tested	Segregation	Genetic determinism	χ^2 (P value) ^a
Symptom intensity (ratio of symptomatic over asymptomatic plants)					
Col-0 × <i>Ler</i> ^b	R	115	62:53	9R:7S	0.6134
		118	63:55	9R:7S	0.5311
Virus accumulation (ratio of resistant over susceptible plants)					
Col-0 × <i>Ler</i>	EA	96	79:17	3R:1S	0.0989
<i>Cvi</i> × <i>Ler</i>	PS	96	17:79	1S:3R	0.0989

^a P values calculated for a χ^2 of 1 degree of freedom resulted from a chi-square test of fit of the data to two dominant genes (9R:7S), a single dominant gene (3R:1S), and a single recessive gene models (1R:3S). When $P > 5\%$, the hypothesis that the observed ratio of segregation is consistent with the expected ratio cannot be rejected.

^b The Col-0 × *Landsberg erecta* (*Ler*) F2 population was tested twice for symptom appearance.

tion to long-distance movement was found to be abolished in all three *rtm* mutants (Table 3). As further confirmation of the involvement of the RTM system, a T-DNA insertion knockout (KO) line for *RTM2* in the Col-0 background was obtained from the SALK collection, and homozygous mutant plants were selected by PCR on genomic and complementary DNAs. The *rtm2* KO mutant showed no obvious morphological defects, and the chemically induced *rtm2* mutant (Whitham et al. 1999) allowed long-distance movement of PPV-EA and LMV-AF199 (Table 3). The results indicated that the resistance of the Col-0 accession to the long-distance movement of several potyvirus isolates, including PPV-EA and LMV-AF199, is conditioned by the *RTM* genes. In contrast, the Col-0 acces-

sion was found to be fully susceptible to the PPV M and D strains (Table 1), indicating that some PPV isolates are able to overcome RTM resistance or are RTM independent.

Analysis of the resistance to PPV-PS long-distance movement in Cvi-1.

The resistance that confines PPV-PS infection to inoculated leaves in the Cvi-1 accession was analyzed in further detail. PPV-PS replication does not appear to be inhibited, since the virus was detected by RT-PCR or by ELISA at 9 dpi in inoculated rosette leaves (data not shown). The presence of infectious PPV particles was confirmed by successful back-inoculation from Cvi-1-inoculated rosette leaves to *Nicotiana benthamiana* plants, which developed typical vein clearing and chlorosis PPV symptoms (data not shown). Absence of virus in the upper uninoculated tissues indicated that the PPV-PS long-distance movement was disturbed.

Replication, local movement within the inoculated leaf, and long-distance movement to distal tissues were assessed by inoculation of the Cvi-1 accession with a green fluorescent protein (GFP)-tagged PPV-PS recombinant isolate. Inoculation of

Table 3. Comparison of susceptibility of *rtm* Col-0 mutants to infection by three different potyviruses

Arabidopsis accessions	Potyvirus isolate ^a		
	TEV-GUS ^b	PPV-EA	LMV-AF199
Col-0	Rsys	Rsys	Rsys
<i>rtm1-1</i> , <i>rtm1-2</i> ^b	S	S	S
<i>rtm2-1</i> ^b	S	S	S
<i>rtm3-1</i> ^b	nd	S	S
<i>rtm2</i> ^c	nd	S	S

^a TEV = Tobacco etch virus, PPV = Plum pox potyvirus, LMV = Lettuce mosaic virus; nd = not determined.

^b Ethylmethylsulfonate mutants and phenotypes when infected with TEV (Whitham et al. 2000).

^c T-DNA insertion line (SALK_010448).

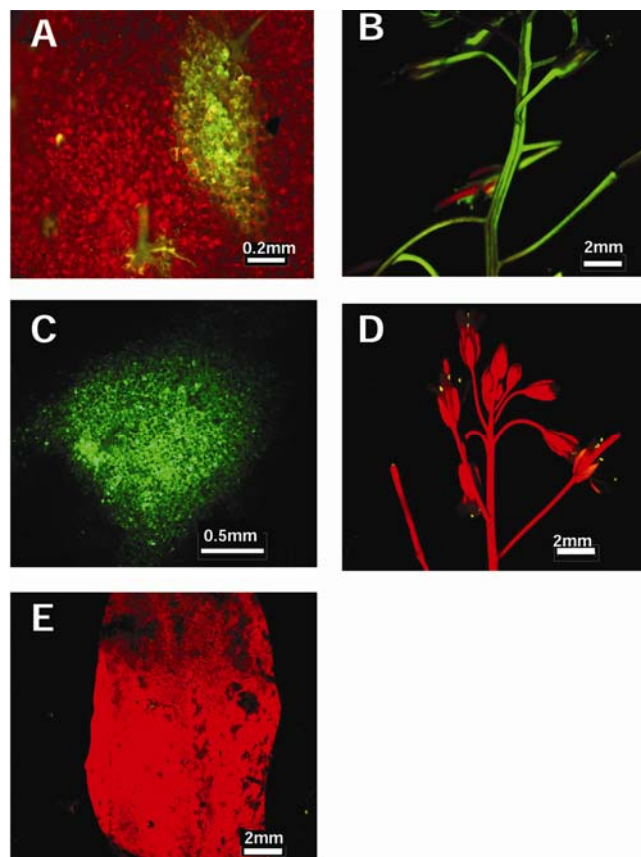


Fig. 2. Green fluorescent protein (GFP)-tagged *Plum pox potyvirus* (PPV)-PS movement is restricted to inoculated leaves of the Cvi-1 accession. Photographs taken under UV light of A and B, PPV-R GFP and C, and D, PPV-PS GFP in Cvi-1-inoculated (A and C) and -uninoculated (B and D) tissues. E, Control consisting of *AtelF(iso)4E-1* mutant plants inoculated with PPV-PS GFP. The photographs were taken at 8 (A, C, and E) and 21 (B and D) days postinoculation.

LG1

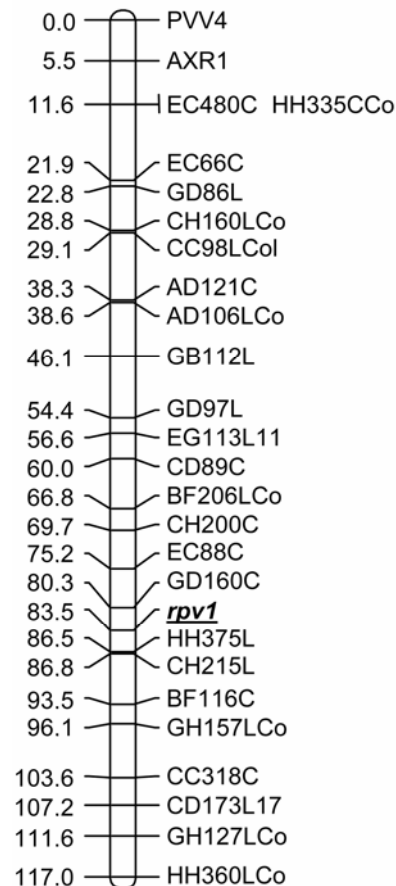


Fig. 3. Map position of *rpv1* on chromosome 1 of *Arabidopsis thaliana* Cvi-1 accession. The *rpv1* (restricted *Plum pox virus*) locus was mapped as a single gene by MAPMAKER analysis and was positioned between GD160C and HH375L on linkage group 1 (LG1). The figure was drawn with MapChart and indicates the genetic distance on the left side (in cM), the markers on the right side, and the target locus *rpv1* in bold and underlined.

Cvi-1 with a GFP-tagged PPV-R recombinant or inoculation of the *AteIF(iso)4E-1* mutant plants served as controls. In the Cvi-1 accession, the GFP-tagged PPV-R isolate replicated within the inoculated leaves (Fig. 2A) and spread systemically into the uninoculated tissues and inflorescences (Fig. 2B). In contrast, the GFP-tagged PPV-PS isolate accumulated only in the Cvi-1 inoculated leaves (Fig. 2C) and GFP fluorescence was never detected in the uninoculated inflorescence tissues (Fig. 2D). In control inoculated leaves of the *AteIF(iso)4E-1* mutant, no GFP fluorescence was observed, irrespective of the recombinant PPV isolate used (Fig. 2E).

Genetic analysis of the F2 progeny derived from a cross made between Cvi-1 and *Ler* (Table 2) showed that restriction of PPV-PS infection in Cvi-1 segregated as a single recessive gene (segregation ratio of 1:3; $P = 0.098$). This Cvi-1 locus was designated as *rpv1* for “restricted plum pox virus.” Since the *rpv1* resistance is recessive, while that conferred by the RTM genes is dominant, restriction of PPV-PS long-distance movement in Cvi-1 should correspond to a mechanism distinct from the RTM-mediated pathway.

A mapping population of 162 RIL developed from a cross between the Cvi-1 and *Ler* accessions (Alonso-Blanco et al. 1998) was used to identify the genomic region encompassing *rpv1*. A single region for the position of *rpv1* in the *A. thaliana* genome was identified with the different statistical procedures

assayed. It mapped as a single gene by MAPMAKER analysis or as a single QTL by interval mapping and composite interval mapping (CIM) using MultiQTL analysis. The *rpv1* locus was located on chromosome 1 between the GD160C and HH375L markers (Fig. 3) and was recurrent over two different phenotyping scoring data sets (log of the likelihood ratio [LOD] score of 12 by MAPMAKER analysis). One marker in this region, HH375L, had the strongest correlation seen in the QTL analysis (LOD score of 4.58 by CIM). Interestingly, while the susceptible parental line *Ler* was asymptomatic when inoculated with the PPV-PS isolate, the ability to develop symptoms in response to PPV segregated as a single gene in the Cvi-1 × *Ler* progeny (F2 population and RIL). This factor of symptomatology should be provided by the Cvi-1 accession but was masked in the parental line by the resistance conferred by the *rpv1* homozygous locus.

Five recombinant viruses constructed between infectious pGPPV-PS and pGPPV-R full-length clones (Sáenz et al. 2000) were used to locate the genetic determinant responsible for breaking the *rpv1* resistance by PPV-R. As shown in Figure 4, the R/P₂₂₁₂₋₃₆₂₈ and R/P₂₂₁₂₋₇₆₇₇ viruses, carrying the 5' and 3' ends from PPV-R, were able to systemically spread on Cvi-1 plants, whereas the complementary recombinant viruses, P/R₂₂₁₂₋₃₆₂₈ and P/R₂₂₁₂₋₇₆₇₇, in which the 5' and 3' sequences come from PPV-PS, did not move outside of the inoculated

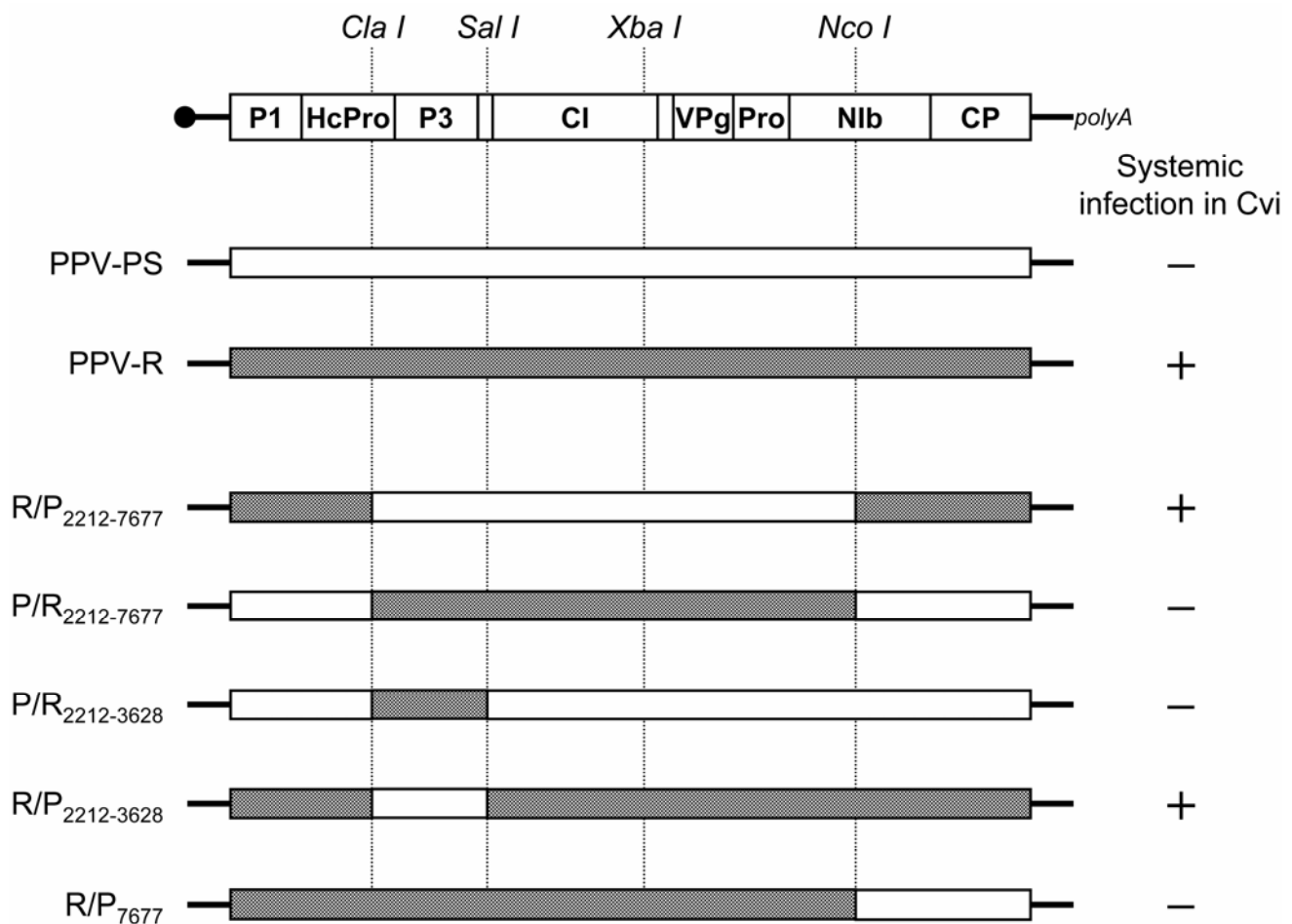


Fig. 4. Schematic representation of the genome of *Plum pox potyvirus* (PPV) and of the R and PS PPV recombinant viruses tested on the Cvi-1 accession. PPV-PS and PPV-R sequences are shown as open and filled boxes, respectively. Restriction sites used in the cloning are indicated. A genetic map of PPV, indicating the positions of the encoded proteins, is shown at the top of the figure. Infectious clones and recombinants are fully described (Sáenz et al. 2000). The names of the virus proteins are indicated in the genome map at the top of the figure. HcPro is the aphid transmission helper component-proteinase with a gene silencing suppression ability; CI is the cylindrical inclusion protein with a role in cell-to-cell movement and an RNA helicase activity; VPg is linked to the 5' end of the genome (circle); Pro is the major proteinase responsible for polyprotein processing; Nib is the RNA-dependent RNA polymerase; CP is the capsid protein (Urcuqui-Inchima et al. 2001).

leaves, indicating that the ability of PPV-R to break the *rpv1* resistance should either be located in the first 2,212 nt ([5' noncoding region [NCR], P1, and part of the HcPro coding sequences) or in the 3' terminal region of the genome (from nt 7,677 to the end, corresponding to the end of the nuclear inclusion polymerase b (Nib) and capsid protein [CP] coding sequences, plus the 3' NCR) or that both of these regions are involved. R/P₇₆₇₇ revealed that the 3' terminal region from PPV-R is necessary to overcome the *rpv1* resistance. However, we cannot yet rule out the possibility of a role of the 5' part of the PPV-R genome.

DISCUSSION

The results presented here demonstrate that *A. thaliana* is a suitable host to search for genetic factors involved in controlling susceptibility or resistance to PPV infection. PPV isolates exhibit very different behavior when infecting various *A. thaliana* accessions, providing a wide panel of infection phenotypes ranging from complete local resistance to full susceptibility, with systemic infection accompanied or not by severe symptoms. *A. thaliana* is a small herbaceous plant with a short life cycle that has become an excellent model for genetic studies. It is notably different from the usual PPV host plant *Prunus* spp., but the fact that several PPV strains and isolates infect *Arabidopsis* plants with different efficiency provides an excellent system to study PPV-plant interactions.

The infection of *A. thaliana* by many viruses has been previously reported. Many other viruses systemically infect *Arabidopsis* accessions but show mild or no symptoms. A few of them induce severe symptoms in infected organs, such as *Turnip crinkle virus* (TCV) (Simon et al. 1992) and *Turnip mosaic virus* (TuMV) (Martin-Martin et al. 1999), but they generally display low variation in the severity of these symptoms. Consequently, limited information is currently available on plant genes that may condition symptom development in a susceptible host. In contrast, the results reported here highlight the existence of wide, isolate- or accession-specific variations in symptom severity in the PPV-*Arabidopsis* pathosystem. The development of PPV-PS-induced symptoms in a Cvi-1 background appears to be controlled by a single locus, while symptom severity in *Ler* infected with PPV-R is conditioned by at least two genes. The genetic determinants of symptom development in *Ler* infected with PPV-R and Bay-0 infected with the PPV-NAT or PS isolates are currently under study, using the Lister and Dean and the Bay-0 × Shahdara RIL progenies, respectively (Loudet et al. 2002).

Variations in symptom severity may correspond to different processes. In particular, they may reflect variations in the timing or intensity of virus accumulation or, alternatively, may result from differential host behavior under comparable virus accumulation conditions (tolerance). Preliminary results tend to indicate that in the Cvi-1 × *Ler* progeny, symptomatic and asymptomatic plants accumulate PPV-PS to essentially similar levels. Some single loci controlling symptom development in *A. thaliana* have been identified by genetic screens of *Arabidopsis* mutants or accessions (Fujisaki et al. 2004; Lee et al. 1996; Park et al. 2002; Sheng et al. 1998) and, similar to the Cvi-1 × *Ler* situation reported here, virus distribution and accumulation were similar in symptomatic and asymptomatic plants; none of these genes has been cloned so far. In contrast, Dardick and associates (2000) showed that, although the Shahdara accession supports rapid accumulation of TMV and develops distinctive disease symptoms, full sensitivity is controlled by at least two genes. One confers rapid virus accumulation, while the other determines the severe symptom phenotype. This second situation could be parallel to the situation

observed in *Ler* inoculated with PPV-R and in which preliminary results indicate that there could be at least a partial correlation between virus accumulation and symptom severity.

Two *Ler* mutants debilitated for *R* gene-mediated resistance, *rar1* and *sgt1b*, were susceptible to PPV-SoC, suggesting that the resistance to inoculation of *Ler* to this isolate is possibly controlled by a dominant *R* gene. Dominant genes controlling resistance to other viruses in *A. thaliana* have been described: *HRT* for TCV (Cooley et al. 2000), *RCY1* for *Cucumber mosaic virus* (Takahashi et al. 2001), and *LLMI* for LMV (Revers et al. 2003). Further analysis of other accessions showing Ri to PPV and other mutants affected in the *R* pathway will be required to evaluate the generality of the relationship between an Ri phenotype and one or more *R* genes. Indeed, many components of the *R*-gene signaling pathways are also involved in nonhost resistance independently of *R* genes.

The RTM mechanism restricting virus long-distance movement was initially described as being specific to TEV (Chisholm et al. 2000; Mahajan et al. 1998; Whitham et al. 1999, 2000). This conclusion was based on the observation that *Arabidopsis* accessions RTM-restrictive for TEV, such as Col-0 or Ws-2, were fully susceptible to isolates of several potyviruses such as *Potato virus Y* (PVY), *Tobacco vein mottling virus* (TVMV), or TuMV. In addition, experiments involving three different isolates of TEV failed to reveal variability in their behavior towards the RTM genes (Whitham et al. 2000). By contrast, the results reported here demonstrate that the long-distance movement of both PPV-EA and LMV-AF199 is restricted by the RTM genes in the Col-0 accession. This interpretation is indirectly supported by the observation that these isolates are able to systemically infect *Arabidopsis* accessions known to carry naturally permissive *rtm* alleles such as C24 or *Ler*. The RTM-mediated resistance, therefore, appears to have a significantly broader spectrum of action than was initially hypothesized and could even potentially be envisioned as a broad-specificity, *Potyvirus*-specific, long-distance movement restriction system. In such a hypothesis, potyviruses such as PVY, TVMV, and TuMV, which are able to systemically invade accessions carrying restrictive alleles, such as Col-0, would be interpreted as being able to overcome the RTM-mediated resistance. A similar situation applies to the NAT, PS, and R isolates of PPV, which are able to systemically spread on RTM-restrictive accessions such as Col-0 and Wassilewskija (Ws and Ws-2). Despite the cloning of the *RTM1* and *RTM2* genes (Chisholm et al. 2000; Whitham et al. 1999, 2000), the one or more mechanisms by which the *RTM* genes exert their restrictive effect on the potyvirus movement are still a matter of speculation. The discovery that some potyviruses such as PPV are able to overcome this resistance in an isolate-specific fashion opens the way to the identification, through reverse genetics approaches, of one or more viral resistance-breaking determinants. This information should, in turn, help us to understand the functioning of this original resistance system.

Another question that remains open is whether the block of the long-distance movement of RTM-breaking isolates observed in the Kas-1, Shahdara (PPV-R), and Cvi (PPV-PS) accessions is related to the RTM system or represents yet other genetic systems controlling potyviral invasion of *A. thaliana*. In the case of the Cvi-1 accession, however, the inheritance pattern reported here is consistent with a single recessive gene (*rpv1*), which was mapped away from the known positions of *RTM1* and *RTM2*. Although *RTM3* has not yet been cloned, preliminary mapping data indicate that it is located in a different genome position than *rpv1* (J. C. Carrington, *personal communication*). Therefore, it appears very unlikely that the *rpv1* resistance could be related to the RTM-mediated resistance. Given the recessive nature of the *rpv1* resistance, the

most likely hypothesis is that, similar to the eIF4E-mediated resistance, the *RPV* gene encodes a plant factor strictly required by the virus, in this case for infection of uninoculated tissues. Such a situation would be similar to that reported by Lartey and associates (1998), who described an *A. thaliana* recessive mutant, *vsm1*, defective for *Turnip vein clearing tobamovirus* long-distance spread. The preliminary investigations of the *rpv1* resistance-breaking determinant carried by the PPV-R isolate indicate that it is located either in the C-terminal end of the NIB or in the CP. The results obtained so far do not rule out, however, the possibility that multiple determinants located in either the small genomic region identified, the 5' part of the PPV genome, or both might be involved in this resistance-breaking phenomenon. The CP is known to be essential for potyvirus long-distance movement (Carrington et al. 1996; Dolja 1994; Urcuqui-Inchima et al. 2001). Since *RPV1* is likely to encode a host factor involved in PPV long-distance movement and therefore interacting, directly or indirectly, with viral proteins involved in this process, it seems reasonable to hypothesize that CP is the viral resistance-breaking determinant. So far, very little is known about plant factors contributing to viral long-distance movement. The positional cloning of the *rpv1* gene, which is currently underway in our laboratory, should provide original information on this largely unknown process of the potyvirus infectious cycle.

One key question that will remain once *Arabidopsis* resistance and susceptibility factors to PPV infection are identified is whether this new knowledge will be transferable to other pathosystems and, in particular, to its natural *Prunus* hosts. Because of the limited number of proteins encoded by the pathogen, the virus completely depends on the host factor to complete its life cycle. Studying recessive resistance genes provides a good opportunity to reveal host factors required for susceptibility. Indeed, results obtained with eIF4E and its isoform (Decroocq et al. 2005) suggest that searching for recessive genes that condition critical steps of the potyvirus infectious cycle allow the identification of key factors that are required for PPV infection, both in its herbaceous and perennial hosts.

MATERIALS AND METHODS

Plant and virus materials.

All plants were grown under greenhouse conditions. Initial seed stocks were obtained from the Nottingham Arabidopsis Stock Centre (Loughborough, U.K.) or from the Institut National de la Recherche Agronomique (INRA) Versailles collection. Each accession was grown and self-pollinated for one or two generations before screening. PPV isolates were propagated on *N. benthamiana* prior to *Arabidopsis* inoculation. The LMV isolate LMV-AF199 described by Krause-Sakate and associates (2002) was routinely propagated on the lettuce cultivar Trocadéro.

The *Arabidopsis RTM2* T-DNA insertion line in the Col-0 background (SALK_010448) was obtained from the Nottingham Arabidopsis Stock Centre. Insertion mutant information was obtained from the Salk Institute Genomic Analysis Laboratory website. The T-DNA insertion site was confirmed by PCR, using the T-DNA left border-specific primer LBa1 5'-TGGTTCACGTAGTGGCCATCG-3' and *RTM2*-specific primers LP10448 5'-TGATGACCTGAGACAAAAGAAGAG A-3' and RP10448 5'-TTCTTGAAGCTTCTTTGCCGC-3'.

Construction of pGPPV-R, pGPPV-PS, and the corresponding recombinant clones was described previously (Sáenz et al. 2000). The recombinant GFP-tagged PPV isolates used in this work derived from the PPV-R cDNA clone pICPPV-NK-

GFP (Fernandez-Fernandez et al. 2001) and from the PPV-PS cDNA clone pGPPVPS-RnGFPs (B. Salvador, P. Sáenz, J. B. Quiot, C. Simón-Mateo, and J. A. García, *unpublished results*).

PPV inoculation procedure.

Before inoculation, inflorescences of 4- to 6-week-old *Arabidopsis* plants were removed. Four plants of each accession were hand-inoculated twice, two days apart, on the upper side of three rosette leaves. Inoculum was prepared by homogenizing infected *N. benthamiana* leaves with three volumes of cold citrate buffer (Na₃Citrate 0.05 M, EDTA 0.5 mM, diethyldithiocarbamic acid 0.02 M, pH 7.8). After each inoculation, plants were rinsed with water and were maintained in as S3 level containment greenhouse. Samples of inoculated leaves and new inflorescences were collected 9 dpi, and inflorescence tissues were collected again at 21 dpi. Additional sampling was also initially performed at 30 dpi, but since this sampling time did not provide any additional information, this strategy was not continued in later experiments (data not shown). For infection with the PPV-R/PPV-PS chimeras, *Arabidopsis* plants were inoculated with fresh sap from *N. clevelandii* plants infected with progeny virus derived from RNA obtained by *in vitro* transcription of the corresponding recombinant plasmids (Sáenz et al. 2000).

PPV detection.

PPV replication in inoculated rosette leaves was assayed by RT-PCR using the P1 and P2 primers (Wetzel et al. 1991b). Total RNA was prepared following the method of Bertheau and associates (1998). For RT-PCR reactions, 2.5 µl of total RNA were added to 22.5 µl of a reaction mixture (TrisHCl, pH 9, 10 mM, KCl 50 mM, bovine serum albumin 0.2 mg/ml, Triton X100 0.3%, MgCl₂ 1.5 mM, dNTP 0.25 mM, each primer 1 µM, ABgene *Reverse-iT*reverse transcriptase blend 1.5 U, Invitrogen *Taq* polymerase 0.5 U). A reverse transcription of 15 min at 42°C was followed by 40 cycles of denaturation at 92°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 40 s. Infection on uninoculated inflorescence tissue at 9 and 21 dpi was assayed by double antibody sandwich-ELISA using PPV capsid antibody (M+D antibody, LCA Laboratory, Blanquefort, France) and, if needed, was confirmed by RT-PCR. Symptoms were scored visually from 15 to 21 dpi.

Three replicate measurements were made for each accession. In each replicate, leaves and inflorescences were collected from four distinct plants of the same accession. F2 populations, RIL, and mutants were tested similarly to the parental lines. The *AteIF(iso)4E-1* transposon-disrupted mutant line (Duprat et al. 2002; Lellis et al. 2002) was used as a negative control and, at the time of sampling 9 days after inoculation, the inoculum was detected neither by ELISA nor RT-PCR on inoculated leaves of this line.

Mapping of the *rpv1* locus.

The mapping population consisted of 162 RIL developed from a cross between the Cape Verde Islands (Cvi-1) and *Ler* accessions (Alonso-Blanco et al. 1998). The genetic linkage map used contains 99 amplification and restriction fragment length polymorphism markers. The chromosomal location of the *rpv1* locus was determined using the MAPMAKER program version 3.0 (Lander et al. 1987), and markers were positioned on the linkage map at a minimum LOD = 3. The genetic map was drawn with MapChart software (Voorrips 2002). To confirm the occurrence of one or more genomic regions, a quantitative trait analysis using MultiQTL software was also performed (Korol et al. 2001). Information on the DNA markers was obtained from the *Arabidopsis* Information Resource (TAIR) database and the European "Natural" cooperative database.

Fluorescence detection of GFP-tagged PPV.

Cvi-1 plants 4 to 5 weeks old were mechanically inoculated with GFP-tagged PPV isolates on the tip of four to five rosette leaves as described above. Green fluorescence was scored on inoculated leaves 4 to 14 dpi and at 19 to 21 dpi in uninoculated organs. Fluorescence was observed using a fluorescence stereomicroscope MZFL III (Leica Microsystems, Wetzlar, Germany), equipped with a filter with an excitation window at 470/40 nm for GFP3 and B filters, a barrier filter at 525/50 nm for GFP3, and 515 nm for the B filter.

NOTE ADDED IN PROOF

After partial sequencing, the authors noticed that the PS isolate maintained on *N. benthamiana* in Bordeaux and used initially to screen *A. thaliana* accessions (Table 1) does not correspond to the PS sequence used to generate the infectious GFP-tagged PS PPV and PS/R recombinants (Sáenz et al. 2000). However, data presented in this paper are still relevant since both isolates were compared on the Cvi-1 and *Ler* accessions and displayed similar behavior (resistance versus susceptibility).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The European "Natural" cooperative database:
www.dpw.wau.nl/natural/resources/populations.htm
- The INRA Versailles website: dbgap.versailles.inra.fr/publiclines
- MAPMAKER software: www-genome.wi.mit.edu/genome_software
- MultiQTL software: esti.haifa.ac.il/~poptheor and www.multiqtl.com
- The Nottingham Arabidopsis Stock Center: nasc.life.nott.ac.uk
- United States Department of Agriculture public health security and bioterrorism website:
www.aphis.usda.gov/ppq/permits/agr_bioterrorism
- The Salk Institute Genomic Analysis Laboratory website: signal.salk.edu
- TAIR database: www.arabidopsis.org