Recessive and Dominant Genes Interfere with the Vascular Transport of *Potato virus A* in Diploid Potatoes

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Accepted 15 November 1999.

Resistance to *Potato virus A* (PVA) was examined in a diploid cross involving *Solanum tuberosum* subsp. *andigena* as a resistance source. Hypersensitive resistance (HR) to PVA cosegregated with extreme resistance (ER) to *Potato virus Y* conferred by the dominant gene *Ry_{adg}* on chromosome XI. Hence, HR to PVA was controlled by a novel, dominant resistance gene closely linked to *Ry_{adg}*, or *Ry_{adg}* recognized both viruses but conferred a different type of resistance to each virus. The HR prevented systemic infection with PVA following mechanical inoculation but not following graft inoculation. Another, recessive gene, *ra*, that may be linked or even allelic with *Ry_{adg}* fully blocked vascular transport of PVA in graft-inoculated plants. Hence, a possibility exists that the genes for the three types of resistance to potyviruses may reside at the same, resistance gene-rich chromosome region syntenic in solanaceous species and might be related. The gene *ra* acted against all of the three PVA strains tested and, therefore, the avirulence determinants could not be mapped. However, also, PVA strain-specific resistance was found in the progeny. It was overcome by mutations introduced into the viral genome-linked protein and the helper component proteinase and/or the coat protein.

Potato virus A (PVA) (Bartels 1971) belongs to the genus *Potyvirus*, which contains the largest number of virus species infecting plants (Shukla et al. 1994; Fauquet and Mayo 1999). Potyviruses consist of filamentous particles containing a single-stranded, messenger-polarity RNA genome that upon infection is translated into a single polypeptide and autoproteolytically processed into 10 functional proteins (Riechmann et al. 1992; Shukla et al. 1994).

PVA and *Potato virus Y* (PVY, the type member of the genus *Potyvirus*; De Bokx and Huttinga 1981) are among the most harmful viruses infecting potatoes worldwide (Hooker 1981). Since both viruses are transmitted in a nonpersistent

manner by aphids, it is difficult to prevent infections by control of the vectors (Peters 1987). Therefore, resistant cultivars are required for the control of PVA and PVY in the field. Three types of natural resistance to potyviruses have been described in cultivated or wild potatoes (*Solanum* spp., Solanaceae): extreme resistance (ER), hypersensitive resistance (HR), and resistance to virus translocation (Cockerham 1970; Ross 1986; Jones 1990; Valkonen 1994, 1997).

ER to PVA and PVY is controlled by the dominant genes *Ra* and *Ry*, respectively. They confer a high level of resistance to different strains of the virus that is expressed as extremely low amounts of virus in plants (Cockerham 1970; Ross 1986; Barker 1996) and reduced amounts of virus in protoplasts (Barker and Harrison 1984). The only symptom that develops in infected plants is a limited necrosis (“pin-point lesions”) in the top leaves following graft inoculation of some genotypes (Ross 1986). HR to potyviruses is controlled by dominant genes that are often virus strain-specific and, in contrast to ER, provide no resistance at the protoplast level (Cockerham 1970; De Bokx and Huttinga 1981; Barker and Harrison 1984; Ross 1986; Jones 1990; Valkonen et al. 1995, 1996). HR is visualized as necrotic lesions that develop at the sites of infection in the inoculated leaves. While cell death is dispensable for the actual responses blocking virus movement, it is tightly coupled with activation of localized and systemic acquired, non-pathogen-specific resistance in the infected plant (Ross 1961a, 1961b; Ryals et al. 1996; Costet et al. 1999). Also, expression of ER can induce non-virus-specific resistance, at least at the single cell level in protoplasts (Köhm et al. 1993). The HR may or may not block vascular transport of the virus. If systemic infection occurs, necrotic symptoms develop at the top of the plant, for example following graft inoculation (Jones 1990). The leaves expressing necrotic symptoms contain detectable amounts of the virus, which is in contrast to leaves containing “pin-point lesions” in plants expressing ER (Barker 1996, 1997; Hämäläinen et al. 1997, 1998).

ER and HR may be related mechanisms since both can induce cell death observed as necrotic symptoms (Benson and Hooker 1960; Cockerham 1970). ER is epistatic to HR (Cockerham 1970; Valkonen et al. 1994) probably because the genes for ER act at an earlier stage of infection (Bendahmane et al. 1999). The genes for ER and HR to potyviruses may act

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on a gene-for-gene basis (Flor 1946) by recognizing specific viral-encoded proteins, as described for resistance genes recognizing potex- or tobamoviruses (Baulcombe et al. 1994; Culver 1997). However, no gene conferring ER or HR to potyviruses has yet been isolated and characterized from any plant species, neither has any potyvirus gene been reported as the avirulence factor (elicitor) for ER or HR.

Fewer studies have been carried out on resistance to potyvirus translocation in potato than on ER and HR. Potyviruses utilize several of their proteins to employ the active intra- and intercellular transport mechanisms for virus movement (reviewed in Carrington et al. 1998; Rajamäki and Valkonen 1999). To induce a systemic infection, the virus moves from the initially infected cell to the phloem, is loaded into the sieve elements for long distance translocation, and is unloaded from the sieve elements in the upper leaves for replication. Mutations in the interacting host or viral proteins involved in virus transport, or an induced response triggered by virus infection may restrict virus movement and block systemic infection. Slow cell-to-cell movement of PVY (Valkonen et al. 1991) and restricted movement of *Tobacco etch virus* (TEV) from epidermis to mesophyll (Valkonen and Somersalo 1996) have been described in the wild potato species *S. brevidens* Phil., but the genetic basis of resistance has not been fully resolved. In contrast, there are several examples of resistance to cell-to-cell or vascular movement of potyviruses described in other species of Solanaceae, particularly in *Capsicum* spp. (Arroyo et al. 1996; Murphy et al. 1998) and *Nicotiana* spp. (Gupton and Burk 1973; Schaad and Carrington 1996), controlled by recessive genes (Gupton and Burk 1973; Schaad and Carrington 1996; Murphy et al. 1998).

Resistance to several potyviruses conferred by a single simple or complex locus is often encountered in potatoes (*Solanum* spp.) (Cockerham 1970) and other solanaceous species (Gupton and Burk 1973; Caranta et al. 1997; Murphy et al. 1998). The gene *Ry_{sto}* in *S. stoloniferum* Schlecht. et Bche. (Ross 1986; Barker 1996) and the gene *Ry_{adg}* in *S. tuberosum* L. subsp. *andigena* Hawkes (Valkonen et al. 1994) confer comprehensive resistance to different strains of PVY and are used in breeding programs. In different accessions of *S. stoloniferum*, ER or HR to PVA and *Potato virus V* cosegregate with ER to PVY, which is explained by a single gene recognizing several viruses, or by several tightly linked genes conferring virus-specific resistance (Cockerham 1970; Barker 1996, 1997). Also, the resistance to PVA derived from *S. tuberosum* subsp. *andigena* is linked with ER to PVY but is conferred by a different locus, according to a previous study (Hämäläinen et al. 1998). However, the genetic basis of the resistance to PVA from *S. tuberosum* subsp. *andigena* has remained unclear because the F₁ progeny segregate for two types of resistance to PVA, instead of segregating only for the expected, parental type of resistance expressed as a systemic infection followed by necrosis in graft-inoculated plants. Indeed, many progeny were not systemically infected and had no symptoms, in contrast to the parental genotypes (Hämäläinen et al. 1998). Six progeny lines that expressed the novel, non-parental type of resistance to PVA had lost *Ry_{adg}* by recombination (confirmed by a restriction fragment length polymorphism [RFLP] analysis), placing the locus controlling resistance to PVA 6.8 cM distal from *Ry_{adg}* on chromosome XI. Both types of resistance to PVA were assumed to be attributable to a single locus designated as *Ra_{adg}* (Hämäläinen et al. 1998).

The aim of this study was to characterize more closely the mechanisms of resistance to PVA in the diploid F₁ population mentioned above. The data indicate that the gene previously designated as *Ra_{adg}* is recessive and is, therefore, re-designated as *ra_{adg}*. It provides a strong resistance against vascular movement of PVA that is not overcome by graft inoculation. *Ry_{adg}*, or another gene at a locus tightly linked to *Ry_{adg}*, confers HR to PVA, which prevents systemic infection following mechanical inoculation but not following graft inoculation. The data suggest that the genes for resistance to PVY and PVA may reside at the same region of chromosome XI and may be structurally and functionally related.

RESULTS

Two types of phenotypic responses to infection with PVA in the inoculated leaves.

The 78 progeny lines of the diploid F₁ population have previously been tested for resistance to PVY and PVA by graft inoculation (Valkonen 1994; Hämäläinen et al. 1997, 1998). However, in this study, the progeny lines were sap inoculated with two PVA strains under constant conditions in a growth chamber. Two types of phenotypic responses were observed in the inoculated leaves: development of necrotic local lesions (NLLs), or no symptoms. The response was the same for both PVA strains in each tested potato line.

Fifty-one progeny lines and the parental line 2x(v-2)7 were similar in that they developed NLLs in the inoculated leaves. However, the progeny lines were placed into two phenotypic classes because some of them were systemically infected and developed necrotic symptoms in the top leaves following graft inoculation with PVA-U (class I) in contrast to the other lines that were not systemically infected (class II) (Table 1). The whole leaves containing NLLs had low but detectable amounts of PVA, as determined by enzyme-linked immunosorbent assay (ELISA; Table 1). Amounts of PVA coat protein higher than 6.4 ng per g of leaf could be quantified and, in addition, approximately 10-fold lower amounts could be reliably detected but not quantified by the ELISA used (see Table 1 for explanations).

Much higher absorbance values per sample were obtained by excising the NLLs from the leaves by a puncture with the cut tip of a 1-ml plastic pipette, including approximately 2 mm of the surrounding non-necrotic tissue, and pooling 10 to 20 disks to one sample. With four pools of NLLs, the mean amount of PVA-B11 per NLL was estimated to be 1.2 ng (SE 1.5) in the progeny lines and 1.6 ng (SE 0.8) in the potato clone "A6" that carries the gene *Na_{dms}* conferring HR to PVA (De Bokx 1972; Valkonen et al. 1995; Rajamäki et al. 1998). The areas between NLLs contained no detectable amount of PVA. No plant that developed NLLs was systemically infected at 21 days after inoculation.

In the parental line 84.194.30 and 27 progeny lines, the sap-inoculated leaves remained symptomless but contained detectable amounts of PVA, as determined by ELISA (Table 1). However, based on graft inoculation with PVA-U, these progeny lines were also placed into two classes because some of them were systemically infected (class III) in contrast to the others (class IV) (Table 1). The phenotypic responses to PVA-U and PVA-B11 were similar in the inoculated leaves of all tested genotypes, but the titers of PVA-B11 were higher than the titers of PVA-U (Table 1). The ability to accumulate in

higher titers is a trait associated with the coat protein-encoding region of PVA-B11 that differs from PVA-U (Andrejeva et al. 1999).

Amounts of virus in asymptomatic leaves following inoculation by microprojectile bombardment.

Testing the sap-inoculated leaves by ELISA resulted in rather low absorbance values. Because the infection sites in the inoculated leaves were visually not detectable in the genotypes that did not develop NLLs, inoculation was also carried out by particle bombardment on selected potato lines to verify whether there was a difference in virus accumulation between the progeny from class III and class IV. To minimize the variability between inoculations (number of initial infection sites obtained per shot), bombardment parameters were first adjusted with the potato clone A6, in which the numbers of NLLs could be taken as an indication of the number of initial infection sites (Fig. 1; see Materials and Methods).

The numbers of NLLs increased with increased acceleration pressure. The pressure of 200 lb/in² and the distance of 0 cm between the spacer and leaf resulted in the largest number of NLLs, differing significantly ($P < 0.01$) from the other pressures tested. Higher pressures caused a ribbed appearance of the leaves and longer distance between the leaf and the spacer reduced the number of NLLs, and, therefore, neither was used. The polyvinyl pyrrolidone (PVP) concentration of 0.05 mg (per 1 ml of 99.5% ethanol) resulted in more NLLs than no PVP or 1.0 mg of PVP. The latter results indicated that binding of the microprojectiles (gold particles) to the tubing was too weak or too tight, respectively. The numbers and size of NLLs were similar irrespective of the amount of cDNA used for coating the microprojectiles (0.2 to 10.0 µg of viral cDNA delivered on 0.5 mg of gold per shot). Bombardment with large (diameter 1.6 µm) microprojectiles resulted in significantly more NLLs than bombardment with the smallest microprojectiles (0.6 µm) tested ($P < 0.01$), but the numbers of

NLLs obtained with the middle-size (1.0 µm) microprojectiles were not significantly different. Thus, the diploid progeny were inoculated with the optimized parameters given in Materials and Methods. The center of the shot area invariably died due to the mechanical damage caused by bombardment. Therefore, the initial infection sites were confined to a well-defined, symmetric area (Fig. 1) that could be excised by a cork borer and tested for virus concentration by ELISA.

The bombarded areas were observed under a light microscope prior to testing by ELISA. No symptoms developed in the parental line 84.194.30 and the progeny lines tested (Fig. 1). The mechanical damage caused by bombardment was readily distinguishable from the necrosis resulting from the PVA-induced HR (Fig. 1). The amounts of PVA accumulated at a similar rate in the inoculated leaves of all tested progeny from class III and class IV. Virus titers increased four- to 22-fold from the sixth to the eleventh day after inoculation (Table 2). In contrast, NLL developed and the titers of PVA were low with no significant increase over time in the bombarded leaves of line A6 and the parental line 2x(v-2)7 (Table 2).

Lack of induction of systemic acquired resistance.

A few progeny lines and the parental lines were sap inoculated again 10 days later with the same PVA isolate to the third and fourth leaves above the previously inoculated leaves. The response in the later inoculated upper leaves was no different from the response observed in the previously inoculated lower leaves (Table 3). During the study it was observed that PVA-B11 did not infect systemically the potato lines from class III albeit they were systemically infected with PVA-U. This observation confirmed that the virus detected in the challenge-inoculated upper leaves (Table 3) was not present as a result of systemic infection by the virus inoculated 10 days earlier.

The parental clone 2x(v-2)7 was also tested by inoculating the lower leaves with PVA-U or PVA-B11 and challenge-

Table 1. Amount of *Potato virus A* (PVA) in the sap-inoculated leaves of progeny lines grouped into phenotypic classes

Plants	n	Sap inoculation					
		Graft inoculation symptoms		PVA-U		PVA-B11	
		PVY	PVA-U	Symptoms	Amount	Symptoms	Amount
Progeny phenotypes							
Class I	29	NSI ^a	SVN	NLL	46.3 (1.2) ^b	NLL	55.8 (2.1)
Class II	22	NSI	NSI	NLL	ND	NLL	82.5 (51.7)
Class III	21	H/S	SMM	NS	55.7 (14.2)	NS	264.8 (188.2)
Class IV	6	H/S	NSI	NS	44.4 (12.6)	NS	125.1 (20.9)
Parental lines							
84.194.30	S	SMM	NS	43.7 (0.8)	NS		133.8 (23.4)
2x(v-2)7	NSI	SNL, SVN	NLL	68.8 (2.4)	NLL	87.93 (12.4)	
Control line A6	VN	NLL	ND	NLL	86.7 (13.7)		

^a Systemically infected leaves of plants graft-inoculated with *Potato virus Y* (PVY) or PVA: NSI, no detectable systemic infection; H/S, a hypersensitive response not controlled by the locus *Ry^{adg}*, or mosaic symptoms; SMM, mild mosaic symptoms; SNL, necrotic lesions; SVN, vein necrosis (see Hämmäläinen et al. 1997; 1998). Sap-inoculated leaves: NLL, necrotic lesions; NS, no symptoms; ND, not determined.

^b Amounts of PVA antigen (ng/g leaf; SE of mean in parentheses) at 10 (PVA-U) or 11 (PVA-B11) days after inoculation. Comparison to known amounts of purified virions of PVA-B11 (200, 100, 20, 4.0, 0.8, and 0.16 ng) was used to estimate amounts of PVA antigen in the samples. Absorbances were read when 200 ng of virions reached the A_{405} value 2.500 (30–45 min after adding the substrate). Values for buffer and healthy plants were similar (0.04–0.06) and values twice as high as those of the healthy plants were considered virus-positive. Plates were monitored for up to 5 h after adding the substrate but the A_{405} values of healthy plants did not increase in any test. Detection threshold of the antigen was estimated to be approximately 10-fold lower than the quantifiable amounts of antigen (A_{405} values higher than those for 0.16 ng virions). There were a few inoculated but apparently noninfected leaves in each experiment that generated A_{405} values similar to those of the noninoculated leaves (<0.06), while all leaves considered virus-infected had A_{405} values >0.040. The residual inoculum on the inoculated leaves therefore had no significant effect on the data. Numbers are means of 1–8 leaves (two measurements from each).

inoculating the upper leaves with *Potato virus X* (PVX, genus *Potexvirus*) 10 days later, which resulted in systemic infection by PVX at a similar rate in all the previously PVA-inoculated and mock-inoculated plants (data not shown).

The data suggested that no detectable levels of systemic resistance to PVA or PVX was acquired in the tested diploid potato lines following infection with PVA, including in those that expressed an HR. The levels of systemic acquired resistance can vary depending on the plant genotype (Ross 1961b; Ryals et al. 1996), and our data may indicate poor or defective signaling for the induction of systemic resistance (Ryals et al. 1996) in the potato lines used in this study.

Virus strain-specific resistance to systemic infection with PVA.

As mentioned above, both PVA-U and PVA-B11 failed to systemically infect the progeny lines of class IV, but, unexpectedly, and in contrast to PVA-U, PVA-B11 also failed to systemically infect the potato lines from class III. A third PVA

isolate (PVA-M, the type isolate of PVA strain group 2; Valkonen et al. 1995) was sap inoculated to the progeny line v2-134 (class III) and line v2-51 (class IV) and the resulting phenotypic responses were the same as those observed for PVA-U. These data indicated that there was a gene or genes conferring PVA strain-specific resistance to systemic infection with PVA-B11.

Since the resistance to systemic infection in line v2-51 was not virus strain-specific, the viral determinants responsible for the lack of systemic infection could not be mapped. However, mapping for the viral determinant was done in PVA-B11 regarding the virus strain-specific resistance with the progeny line v2-134. The differences in the deduced amino acid sequences of the proteins involved in potyvirus movement (reviewed by Carrington et al. 1998; Rajamäki and Valkonen 1999) were compared between the three PVA strains. Subsequently, amino acid substitutions were introduced into the proteins of PVA-B11 to make them similar to PVA-U and/or PVA-M (Andrejeva et al. 1999; Kekarainen et al. 1999; Rajamäki and Valkonen 1999). The helper component-proteinase

Fig. 1. Symptoms on leaves inoculated with PVA-B11 by particle bombardment. **A**, Three shots on the same leaf of the control line A6 under the adjusted bombardment conditions. **B**, A necrotic lesion in the parental line 2x(v-2)7. **C** and **D**, Mechanical damage and absence of virus-induced symptoms in bombarded leaves of progeny lines v2-23 (lacks *Ry_{adg}* and contains *ra_{adg}*) and v2-22 (lacks both resistance genes). Symmetric round lesions (white arrowheads in **B–D**) indicate mechanical damage caused by microprojectiles. Magnification in **B–D** is approximately 10-fold.

(HC-Pro; recombinant virus B11-U_{HC}), the viral genome-linked protein (VPg; B11-M_{VPg}), the N terminus of the coat protein (CP) (B11-U_{CP}), both the HC-Pro and the CP (B11-U_{HC}U_{CP}), or all of the three proteins (B11-U_{HC}M_{VPg}U_{CP}) were mutated in the viral cDNAs used (Fig. 2).

PVA-U, PVA-M and the mutant virus B11-U_{HC}M_{VPg}U_{CP} infected the line v2-134 systemically, in contrast to PVA-B11 and the remaining four mutant viruses, in which amino acid substitutions were introduced into either the HC-Pro or the CP, or into both the HC-Pro and the CP but not into the VPg (Table 4). These data showed not only that the mutations in VPg were crucial for overcoming the resistance but also that additional mutations in the HC-Pro or the CP, or in both pro-

teins, were required. The more specific mapping of the significant amino acid substitutions was not done in this study. The ability of the viruses to infect line v2-134 systemically was not positively correlated with the level of virus accumulation in inoculated leaves since PVA-B11 and B11-U_{HC} had the highest titers in the inoculated leaves, as reported previously (Andrejeva et al. 1999), but were unable to infect the plants systemically. As expected, none of the three PVA strains or mutant viruses, including B11-U_{HC}M_{VPg}U_{CP}, were able to infect line v2-51 systemically.

The lines v2-51 and v2-134 were systemically infected with PVY, *Potato virus V*, and PVX (J. P. T. Valkonen, *unpublished data*), which showed that the resistance to systemic infection affects PVA only.

The mechanisms and the genetics of resistance to PVA.

All of the 51 progeny lines and the parental line, 2x(v-2)7, that expressed HR to PVA also express ER to PVY (Hämäläinen et al. 1998). In contrast, the remaining 27 progeny lines and the parental line 84.194.30 did not express HR to PVA and ER to PVY. Thus, the HR to PVA cosegregated with ER to PVY, indicating that either a novel, dominant, PVA-specific resistance gene, *Na_{adg}*, is tightly linked to *Ry_{adg}*, or *Ry_{adg}* confers resistance to two viruses (ER to PVY and HR to PVA). *Ry_{adg}* has been mapped to the short arm of chromosome XI (Hämäläinen et al. 1997).

The progeny lines were placed in two phenotypic groups according to the responses observed in the inoculated leaves (NLLs, or no symptoms). Both of these groups were further divided into two response groups following graft inoculation with PVA-U because some progeny lines were systemically infected, in contrast to the others (Table 1). The HR prevented systemic infection following sap inoculation but not following graft inoculation [progeny class I and the parental line 2x(v-2)7]. There were six progeny lines (class IV) that did not develop NLLs, albeit they were resistant to systemic infection with PVA-U.

Table 2. Symptoms and *Potato virus A* (PVA) concentration in leaf areas inoculated by particle bombardment

Potato line	Phenotypic class ^a	Symptoms ^b	Virus amounts ^c	
			6 days	11 days
Progeny				
v2-22	III	NS	436 (116)	6110 (2310)
v2-39	III	NS	1120 (629)	9850 (1460)
v2-48	III	NS	1030 (773)	22600 (10400)
v2-54	III	NS	1120 (762)	9710 (5720)
v2-23	IV	NS	1410 (963)	5870 (5640)
v2-51	IV	NS	1240 (528)	9880 (8120)
Parental lines				
84.194.30	III	NS	702 (273)	4110 (2160)
2x(v-2)7	I	NLL	273 (72)	316 (94)
Control line A6		NLL	425 (110)	310 (129)

^a See Table 1.

^b Symptoms in the bombarded leaf area: NS, no symptoms (except mechanical damage); NLL, necrotic lesions similar to those induced by sap inoculation.

^c Mean amount of PVA coat protein antigen per gram of infected leaf (SE) detected in the bombarded leaf area (1–10 leaf disks per potato line, two measurements from each) by enzyme-linked immunosorbent assay. For details of determination of virus amounts, see Table 1.

Table 3. Examination of systemic induced resistance to *Potato virus A* (PVA) in plants sap-inoculated with PVA-U or PVA-B11

Plants	Phenotypic class	First inoculated leaves		Challenge-inoculated upper leaves ^a	
		Symptoms	Virus ng/g	Symptoms	Virus ng/g
PVA-U					
Progeny lines					
v2-23	IV ^b	NS ^c	49.2 (16.5) ^d	NS	46.5 (0.5)
v2-51	IV	NS	41.3 (7.8)	NS	55.6 (1.4)
Parental lines					
2x(v-2)7	I	NLL*	68.8 (2.4)	NLL*	44.5 (0.8)
PVA-B11					
Progeny lines					
v2-44	I	NLL*	55.84 (1.2)	NLL*	276.6 (186.4)
v2-48	III	NS	409.8 (230.4)	NS	58.7 (6.0)
v2-54	III	NS	168.1 (15.8)	NS	51.0 (2.5)
v2-23	IV	NS	140.6 (19.7)	NS	311.5 (227.3)
v2-51	IV	NS	109.73 (3.5)	NS	< 6.4
Parental lines					
84.194.30	III	NS	133.8 (23.4)	NS	63.9 (13.8)
2x(v-2)7	I	NLL*	87.9 (12.4)	NLL**	169.4 (74.4)
Control line A6		NLL**	86.7 (13.7)	NLL***	577.5 (322.4)

^a The third and fourth leaf above the first inoculated leaves were challenge-inoculated 10 days after the first inoculation.

^b See Table 1.

^c Symptoms in inoculated leaves: NS, no symptoms; NLL*, <10 necrotic local lesions; NLL**, 10–100 necrotic local lesions; NLL***, >100 necrotic local lesions.

^d Mean amount of PVA coat protein antigen per gram of infected leaf (SE) detected in the sap-inoculated leaves by enzyme-linked immunosorbent assay (1–4 leaves per potato line, two measurements from each). For details of determination of virus amounts, see Table 1.

Therefore, the data indicated that the gene conferring a block against vascular transport, and which was not overcome by graft inoculation, was different from the gene conferring HR to PVA (i.e., *Ry_{adg}* or another gene coupled with it). This was consistent with an RFLP analysis with markers linked to *Ry_{adg}* (Fig. 3).

Since the virus accumulation in the inoculated leaves was not reduced (Tables 1–3), the resistance did not restrict virus multiplication or movement in the mesophyll tissue but only blocked the transport of PVA in the vascular tissue. The resistance against vascular transport was very strong as it was not overcome by graft inoculation, which delivers virus from the infected scion directly to the vascular system. A similar resistance was not observed in the parental lines. The line 84.194.30 was systemically infected following mechanical inoculation with PVA-U. The HR induced in the mechanically inoculated leaves of line 2x(v-2)7 prevented systemic infection with PVA-U, but systemic infection occurred following graft inoculation (Fig. 4). Furthermore, stem necrosis, necrotic lesions, and vein necrosis (Fig. 4), and the detectable amounts of PVA that developed in line 2x(v-2)7 following graft inoculation, showed that PVA unloaded from the sieve elements and replicated in the surrounding tissues. Thus, the resistance to vascular transport of PVA was only observed in new combinations of parental alleles in the progeny lines of class II and class IV.

DISCUSSION

In a previous study (Hämäläinen et al. 1998), the resistance to PVA was assumed to be controlled by a single gene, *Ra_{adg}*, linked to the locus *Ry_{adg}* (ER to PVY) on potato chromosome XI. According to the model, the genes were separated by 5.5 cM. The fact that after graft and sap inoculation four phenotypic resistance classes could be distinguished in the progeny of the cross 2x(v-2)7 with 84.194.30 indicates, however, that resistance to PVA may be controlled by two independent genes, one designated as *Na_{adg}* and controlling HR to PVA, and another one designated as *ra* that blocks vascular transport of PVA. The dominant gene *Na_{adg}* is tightly linked to *Ry_{adg}*, as both phenotypes, ER to PVY and HR to PVA, cosegregated in 51 resistant progeny lines. The possibility also exists that *Ry_{adg}* recognizes both viruses but triggers a different type of resistance response to each virus. These two alternatives may be resolved in the future by isolating *Ry_{adg}* and testing its function and virus specificity.

Resistance to vascular transport conferred by the gene *ra* is suppressed in the parental genotypes but is activated in progeny classes II and IV, where parental alleles are newly combined. Suppression of a resistance phenotype depending on the genetic background of the potato line has been reported

Fig. 2. Schematic presentation of polyprotein encoded by infectious cDNA of PVA-B11, and the mutant cDNAs of PVA-B11 containing amino acid substitutions introduced into the HC-Pro, VPg, and/or CP according to the genomic sequences of PVA-U or PVA-M. Those positions that in PVA-B11 contain a unique amino acid residue not found in PVA-U and PVA-M, and the corresponding amino acid substitutions introduced to the cDNA of PVA-B11, are indicated for each of the mutant viruses. The proteins autocatalytically processed from the polyprotein: P1, proteinase; HC-Pro, helper component-proteinase; P3, the third protein; 6K1 and 6K2, 6-kDa proteins 1 and 2, respectively; CI, helicase; VPg, the genome-linked protein; Nla-Pro, proteinase; NIb, replicase; CP, coat protein.

Table 4. Amounts of *Potato virus A* (PVA) isolates and mutants in two diploid progeny lines

Virus	dpi ^a	Line v2-134		Line v2-51	
		IL ^b	SL	IL	SL
PVA-B11	13	85.1 (1.5) ^c	0.0	109.7 (3.5)	0.0
	21	6250 (230)	0.0	ND	0.0
	44	> 8000	0.0	...	...
PVA-U	13	ND	ND	88.3 (11.6)	0
	21	69.0 (22.6)	53.8 (7.2)	55.3 (9.2)	0
	44	ND	859 (460)	87.3 (8.7)	0
PVA-M	13	96.3 (5.7)	171.3 (9.0)	82.8 (2.8)	0
	21	ND	151.4 (5.8)	ND	0
B11-U _{HC}	21	782 (62)	0	2748 (800)	0
	44	>8000	0	1350 (151)	0
B11-M _{VPg}	13	83.4 (4.8)	0	...	...
	21	ND	0	...	...
	44	1108.8 (83.3)	0	...	...
B11-U _{CP}	13	80.8 (4.3)	0	<12.8	0
	21	ND	0	ND	0
B11-U _{HC} U _{CP}	13	ND	ND	...	...
	21	41.2	0	...	...
B11-U _{HC} M _{VPg} U _{CP}	13	183.8 (60.6)	0	81.3 (22.6)	0
	21	...	93.8	ND	0
	44	...	...	3759 (284)	0

^a Days post inoculation.

^b IL, sap-inoculated leaves; SL, noninoculated upper leaves; ND, not determined; ... = not tested.

^c Mean amount of PVA coat protein antigen per gram of infected leaf (standard error) detected by enzyme-linked immunosorbent assay (2–4 leaves per potato line). For details of determination of virus amounts, see Table 1.

(El-Kharbotly et al. 1996, Valkonen et al. 1998). These data suggest a genetic model in which the parental line 84.194.30, which is homozygous susceptible to PVY, is heterozygous for one of the multiple recessive alleles of the *ra* gene (*ry/ry* *Ra1/ra1*), whereas the parental line 2x(v-2)7, which is heterozygous resistant to PVY, is homozygous for a recessive allele different from *ra1* (*Ry/ry* *ra2/ra2*). According to this model, the genotype of progeny in class I is *Ry/ry* *Ra1/ra1*, in class II *Ry/ry* *ra1/ra2*, in class III *ry/ry* *Ra1/ra1*, and in class IV *ry/ry* *ra1/ra2*.

The model of two independent genes controlling resistance to PVA predicts equal proportions of the four genotype classes in the progeny. The proportions observed were, however, 29, 22, 21, and 6 progeny in classes I, II, III, and IV, respectively (Table 1). Hence, class IV contained fewer progeny than expected. Distorted segregation ratios are frequently observed in potato hybrid families (Gebhardt et al. 1991; Ritter et al. 1991; Leister et al. 1996; Oberhagemann et al. 1999). The reason for distorted segregation ratios might be reduced viability of gametes or zygotes having particular allele combinations at some loci. This might be due to the highly interspecific pedigree of parental line 84.194.30 (Valkonen et al. 1994; Watanabe et al. 1994).

In contrast to *Na_{adg}*, the position of the *ra* locus in the potato genome is currently not known. The locus controlling resistance to vascular movement of PVA may be allelic with or tightly linked to the loci *Ry_{adg}* and *Na_{adg}*, or it is unlinked to the *Ry_{adg}* and *Na_{adg}* loci. A single resistance gene or a resistance gene cluster on potato chromosome XI with multiple alleles expressing the various resistance phenotypes (ER to PVY, HR to PVA, resistance to vascular movement) is compatible with the

Fig. 3. Southern analysis of eight progeny lines and the parental lines 2x(v-2)7 (v-2) and 84.198.30 (84) with two DNA markers. **A**, The probe TG508 identifies a flanking marker for *Ry_{adg}* located 1.3 cM distal from *Ry_{adg}*. **B**, The probe TG523 identifies a restriction fragment length polymorphism (RFLP) marker that cosegregates with extreme resistance (ER) to PVY (controlled by *Ry_{adg}*) and hypersensitive resistance (HR) to *Potato virus A* (PVA) (controlled by *Ry_{adg}*, or a novel gene coupled with *Ry_{adg}*) [(progeny lines v2-28 and v2-44, and parental line 2x(v-2)7]. It does not identify the lines 23 and 51 that express resistance (R) to vascular movement of PVA (class IV, Table 1), which, therefore, is controlled by a gene (*ra*) different from *Ry_{adg}*.

observed segregation data. To prove linkage of *ra* with the loci *Ry_{adg}* and *Na_{adg}*, construction of a molecular linkage map for the parental line 84.194.30 is required, but is not yet available.

Recessive resistance to potyviruses has been described in several species of Solanaceae (Gupton and Burk 1973; Arroyo et al. 1996; Schaad and Carrington 1996; Murphy et al. 1998) and a common feature seems to be that mutations in the VPg allow the viruses to overcome the resistance. Examples include resistance to the cell-to-cell movement of several potyviruses in the mesophyll of tobacco conferred by the gene *va* (Nicolas et al. 1997), the multigenic resistance that blocks vascular transport of TEV in tobacco plants (Schaad et al. 1997), and the resistance to accumulation of *Pea seed-borne virus* in inoculated leaves conferred by the gene *sbm-1* in pea plants (*Pisum sativum* L.) (Keller et al. 1998). Also, the resistance to vascular movement of PVA in *Nicandra physaloides* L., controlled by yet unidentified genes, is overcome by mutations in the VPg (Rajamäki and Valkonen 1999). The region crucial for overcoming the resistance conferred by *ra* was not mapped in the PVA genome for the reasons explained, whereas the virus strain-specific resistance to vascular transport of PVA-B11 was overcome by mutations in the VPg (and the HC-Pro and/or the CP). It is common for all of the above mentioned resistant plants that no local or systemic responses resembling HR have been observed following infection with the avirulent virus isolates. However, a recessive gene in *Capsicum chinense* L. PI 152225 seems to confer a TEV isolate-specific local response resulting in chlorotic lesions (Murphy et al. 1998). Thus, the possibility remains that the recessive resistance genes may induce a response different from HR but sufficient to block virus movement, and that the VPg may play a role as an elicitor of such a resistance response.

According to the genetic models of this study, the genes conferring the three types of resistance, namely ER to PVY, HR to PVA, and the resistance against vascular transport of PVA, may all reside in the same region of potato chromosome XI. This region is a "resistance hot spot," i.e., a syntenic cluster of genes that confer resistance to many diseases in different potato species and other species of Solanaceae (Brown et al. 1996; Leister et al. 1996; Brigneti et al. 1997; Hämäläinen et al. 1997, 1998; Wolters et al. 1998; Hehl et al. 1999). In potato, several gene fragments, cloned based on the sequence homology to the isolated and characterized resistance genes, have been mapped to this region of chromosome XI (Leister et al. 1996). They include homologues of the gene *N*, which confers HR to *Tobacco mosaic virus* (TMV; genus *Tobamovirus*) in tobacco (Whitham et al. 1994). Two homologues of potato cosegregate with *Ry_{adg}* (Hämäläinen et al. 1998; Sorri et al. 1999). Hence, consistent with the theories explaining evolution of resistance gene clusters on plant chromosomes by mispairing, recombination, and gene duplication (Keen 1990; Baker et al. 1997; Hammond-Kosack and Jones 1997), the different resistance genes in the same region of chromosome XI may be structurally and functionally related. It seems that there are few reports on recessive and dominant genes for virus resistance residing in the same chromosome region, whereas there are many examples of clustered recessive resistance genes, e.g., in the chromosomes of pea (Provvidenti 1990) and *Capsicum* spp. (Murphy et al. 1998).

Structural and functional relatedness of the genes for ER and HR to viruses are indicated by the studies of the previous authors. The recent reports show that the gene *N* for HR to TMV in tobacco (Whitham et al. 1994) and the gene *Rx* conferring ER to PVX in potato (Bendahmane et al. 1999) share

Fig. 4. Symptoms in parental line 2x(v-2)7, 18 days after side-graft inoculation with PVA-U. Grafted scion was on basal part of stem and is not visible. **A**, Leaf-drop symptoms due to death of petioles. **B**, Necrosis developing along side of stem to which scion was grafted. **C**, Necrotic spots and vein necrosis in a leaf from upper part of stem. Symptoms in top leaves developed approximately 2 days before stem necrosis had reached position of the leaf.

similar structures. Functional relatedness of the genes for ER and HR has been suggested in the earlier studies because both types of genes can induce necrotic symptoms in plants (Benson and Hooker 1960; Cockerham 1970) and acquired resistance in protoplasts and plants, respectively (Köhm et al. 1993; Valkonen 1997). Indeed, the latest studies show that a high concentration of the CP elicitor permits *Rx* to induce cell death, which is normally not observed (Bendahmane et al. 1999). A level of relatedness seems evident also because ER to PVX (Cockerham 1970) and PVY (Valkonen et al. 1994) is epistatic to HR to the same virus, suggesting that the genes for ER act at an earlier stage of infection than the HR genes. Comparison of the resistance responses triggered by *N* and *Rx* in transgenic tobacco plants expressing both genes shows that *Rx* induces resistance earlier than *N* (Bendahmane et al. 1999). Thus, in the presence of the gene for ER, the virus cannot multiply to titers sufficiently high for triggering the gene for HR, which explains the interaction of the two resistances described as epistasis in the literature (Cockerham 1970; Valkonen et al. 1994; Bendahmane et al. 1999).

Comparison of the recessive resistance conferred by *ra* to the well-described ER and HR to potyviruses in potato shows that because *ra* did not reduce virus multiplication, the resistance is different from the ER but similar to HR. On the other hand, because *ra* did not induce cell death, the resistance is also different from HR. Nevertheless, both *ra* and the gene for HR were able to block the vascular transport of PVA from the sap-inoculated leaves. It has been recognized since the early studies of Ross (1961a, 1961b) that HR and cell death are dispensable for blocking virus transport, which occurs by an unknown mechanism (Murphy et al. 1999) in the living tissue surrounding the necrotic lesion (Ross 1961a, 1961b; Allison and Shalla 1974). Therefore, *ra* may activate the mechanisms essential for blocking virus translocation in phloem, analogous to HR, but does not induce HR, cell death, and acquired resistance (Ross 1961a, 1961b; Ryals et al. 1996; Costet et al. 1999). Hence, the resistance conferred by *ra* may represent a third type of resistance controlled by a gene structurally resembling the genes previously shown to confer HR or ER to viruses in Solanaceae (Whitham et al. 1994; Hammond-Kosack and Jones 1997; Bendahmane et al. 1999).

MATERIALS AND METHODS

Plant material and virus isolates.

The diploid potato lines 84.194.30 and 2x(v-2)7 (Valkonen et al. 1994; Watanabe et al. 1994), the progeny produced by crossing these lines (Valkonen et al. 1994; Hämäläinen et al. 1997, 1998), and the potato line A6 (*S. demissum* Lindl. × *S. tuberosum* L. Aquila) (De Bokx 1972) have been described. Plants were maintained in tissue culture. They were planted in soil for virus experiments that were carried out in a growth chamber (8.8 m²; Weiss Umwelttechnik, Reiskirchen, Germany) under highly constant conditions. The photoperiod was 18 h, light intensity was 250 µE s⁻¹ m⁻², temperatures were 19/17°C at day/night, and relative humidity was 40%.

The isolates PVA-B11, PVA-U, and PVA-M represent three strain groups of PVA (Valkonen et al. 1995), each of which was described previously (Puurand et al. 1994; Valkonen et al. 1995; Rajamäki et al. 1998; Kekarainen et al. 1999; Rajamäki and Valkonen 1999).

Construction of the PVA mutants.

An infectious cDNA of PVA-B11 was developed in a previous study (Puurand et al. 1996). Construction of the mutant cDNA clones B11-U_{HC} and B11-U_{CP} placed under the T7 promoter (Andrejeva et al. 1999) and B11-M_{VPg} (originally designated as B11-M) under the 35S promoter (Rajamäki and Valkonen 1999) have been described elsewhere. The mutant cDNA B11-U_{HC}M_{VPg}U_{CP}, placed under the T7 promoter, was made for this study by first recombining the cDNA clones B11-U_{HC} and B11-U_{CP} (construct B11-U_{HC}U_{CP}; Andrejeva et al. 1999), with the central region then replaced with a sequence from PVA-M defined by unique *Sma*I and *Apa*I restriction sites, as described previously for B11-M_{VPg} (Rajamäki and Valkonen 1999). The mutant viruses contained a number of amino acid substitutions, compared with the sequence of PVA-B11 (Puurand et al. 1994). Those positions that in PVA-B11 contain a unique amino acid residue not found in PVA-U or PVA-M are indicated in Figure 2.

Virus inoculation by particle bombardment.

Infectious transcripts were produced from the viral cDNAs placed under the T7 promoter as described previously (Puurand et al. 1996). The transcripts, or the 35S promoter-driven cDNA clones linearized by *Age*I digestion, were precipitated on to gold particles (Bio-Rad Laboratories, Hercules, CA) by addition of 1/10 volume of 3 M NaAc (pH 7.4) and 3 volumes of 99.5% ethanol. Cartridges were prepared according to the Helios Gene Gun System Instruction Manual (Bio-Rad). Success in coating the microcarriers with cDNA was checked by resuspension from the prepared cartridges and by subsequent measurement of nucleic acid concentration with a spectrophotometer.

Bombardment conditions were adjusted for potato leaves with the development of NLLs in the leaves of line A6 as an indicator of the number of successful infections per shot. Three cartridges of the PVA cDNA placed under the 35S promoter were bombarded to potato leaves with each combination of test parameters, and each experiment was carried out at least three times. Differences between the means of the numbers of NLLs were tested by analysis of variance and calculation of the least significant differences. The best parameters used in subsequent experiments were as follows: large microprojectiles (1.6 µm); 2 µg of viral cDNA per mg of microprojectiles; 0.05 mg of PVP per ml of ethanol; 0.5 mg of gold per shot; pressure 200 lb/in²; 0 cm distance between the leaf and the spacer. The initial parameters were as follows: 2 µg of nucleic acid per 1.0 mg of gold; 0.05 mg of PVP per 1 ml of 99.5% ethanol; 1.0-µm microprojectiles; and 0.5 mg of gold per shot. Helium pressures (80, 100, 150, 200, 250, and 300 lb/in²); the distance between the spacer and the leaf (0 or 2 cm); PVP concentrations (0, 0.05, and 0.1 mg per ml of 99.5% ethanol); amounts (µg) of PVA icDNA per mg of microprojectiles (ratios 0.2 [0.1/0.5], 1.33 [1.0/0.75], 2 [1.0/0.5], 8 [1.0/0.125], and 10 [5.0/0.5]); and sizes of microprojectiles (0.6, 1.0, and 1.6 µm), were varied.

Plants were conditioned for inoculation by shading them overnight before and after inoculation. Two mature leaves of each plant were inoculated by one shot to the left and right side of the midvein under the adjusted bombardment conditions, after which the leaves were sprayed with water. Experiments on each potato line were carried out twice.

Sap inoculation with viruses.

The PVA isolates and the recombinant viruses were first inoculated to tobacco plants (*N. tabacum* cv. Samsun nn), the latter by particle bombardment, and a systemic infection by each was confirmed by ELISA. The systemically infected leaves were collected and amounts of virus determined by ELISA with purified particles of PVA-B11 diluted in ELISA sample buffer as a standard (Table 1). The rest of the leaves were frozen at -20°C to be used as an inoculum for the sap inoculation of potatoes. The leaves were ground with a mortar and a pestle, and sap was diluted with distilled water to obtain similar concentrations of inoculum from each virus mutant and the three virus isolates. Two fully expanded, Carborundum-dusted leaves of each potato plant conditioned for inoculation as above were sap inoculated. Experiments were carried out at least two times on each potato line.

Detection of viruses.

Viruses were detected by double antibody sandwich (DAS)-ELISA as described previously (Rajamäki et al. 1998). The monoclonal antibody 58/0 (Adgen, Ayr, UK) was used for detection of PVA. This detection system is very sensitive and recognizes PVA-U, PVA-B11, and PVA-M equally well (Rajamäki et al. 1998; Andrejeva et al. 1999). Polyclonal antibodies (Boehringer, Mannheim, Germany) were used for the detection of PVX. Samples were weighed and ground in ELISA sample buffer at 1 g per 3 ml. Two aliquots (100 μl each) were transferred to two wells of a microtiter plate coated with the appropriate antibodies. Absorbances were measured at 405 nm with a Benchmark microtiter plate reader with Microplate Manager software (Bio-Rad Laboratories, Hercules, CA). Leaf disks (ϕ 20 mm) containing the entire bombarded area were sampled 6 or 11 days after bombardment and observed under light microscope (Olympus SZH-ILLD; Olympus Optical, Tokyo) before testing by ELISA.

Southern (RFLP) analysis.

DNA was extracted from the leaves of plants grown in a growth chamber as described by Bernatzky and Tanksley (1986) except that sodium bisulfite (5 g/liter) was used instead of mercaptoethanol. DNA samples were digested with *Dra*I or *Eco*RI (Fermentas, Vilnius, Lithuania) and 6 μg of the digested DNA was subjected to electrophoresis through a 1.0% agarose gel. Southern analysis was carried out according to Sambrook et al. (1989). Tomato genomic clones TG508 and TG523 were used as probes (Tanksley et al. 1992). Each probe was labeled with digoxigenin and hybridized to potato DNA, and the signal was detected by chemiluminescence according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

ACKNOWLEDGMENTS

We are grateful to Minna-Liisa Rajamäki for providing the construct B11- $\text{U}_{\text{HC}}\text{M}_{\text{VPg}}\text{U}_{\text{CP}}$, and to her and Sabina Vidal for critically reading the manuscript. This work was supported by stipends to J. H. from the Finnish Cultural Foundation (Alma ja Jussi Jalkasen rahasto), Ella och Georg Ehrnrooths Stiftelse, August Johannes ja Aino Tiuran maatalouden tutkimussäätiö, and Kemira Oyj:n säätiö, and grants to J. V. from The Academy of Finland (grants 34529 and 36256), Stiftelsen för Strategisk Forskning (SSF), Forestry and Agricultural Research Council (SJFR, grant 32.0667/97), and Carl Tryggers Stiftelse.

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