Differential Accumulation of *Potato virus A* and Expression of Pathogenesis-Related Genes in Resistant Potato cv. Shepody upon Graft Inoculation

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ABSTRACT

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Potato (*Solanum tuberosum* L.) cv. Shepody is highly resistant to *Potato virus A* (PVA), yielding no visible symptoms after rub inoculation. In 'Shepody' rootstocks graft-inoculated by PVA-infected scions from a susceptible host, we found a resistance consisting of traces of necrosis (necrotic streaks) in stems and chlorosis in newly emerged leaves. The response was temperature dependent, appearing at 15 to 18°C but not at 28 to 31°C. Necrosis was also observed in tubers, appearing first in the bud end and spreading randomly throughout the majority of the tuber. Trace amounts of PVA in plant tissues were detected by a combination of

reverse transcription polymerase chain reaction and Southern blot procedures. The virus concentration was significantly higher in visibly necrotic areas than in nonnecrotic areas, suggesting that presence of the necrosis may be concentration dependent. Pathogenesis-related gene expression showed that chitinase A and B, glucanase B, and PR-10a were associated with chlorotic or necrotic symptoms in leaves, stems, and tubers. Gene expression was markedly more evident in tuber tissues than in leaves and stems. Furthermore, generation of oxidants was also observed within the necrotic areas. Although PVA was detected in necrotic areas of tubers, newly emerged plants from the same tubers were PVA-free.

Additional keywords: extreme virus resistance, uneven virus distribution.

The largest plant virus family, *Potyviridae*, includes several viruses that naturally infect the potato (*Solanum tuberosum* L.), namely *Potato virus A* (PVA), *Potato virus Y* (PVY), and *Potato virus V* (13,27). PVA incites a mild mosaic or no symptoms in potato depending on the cultivars and reduces yield by 10 to 40% (3). In combination with *Potato virus X* (PVX), PVA causes a severe disease known as "potato crinkle" (17). The occurrence of PVA in North America is limited to certain cultivars, with incidence rates of 9 to 11% (23,32). In recent years, the prevalence of PVA in potato production areas of Europe has also been noted (24). Because control of aphid-borne potato viruses like PVA is difficult, the most effective means of preventing virus spread is by using highly resistant potato cultivars.

The hypersensitive response (HR) is an active form of defense that is characterized by the appearance of necrosis at the site of infection and inhibition of further pathogen multiplication and spread (14). Although HR implies restriction of the initial infection, in practice it includes both local and systemic necrotic responses. For example, various tobacco cultivars and *Nicotiana* spp. containing the *N* gene express a localized necrosis when plants are rub-inoculated with *Tobacco mosaic virus* (TMV), but a systemic necrosis when plants are inoculated by grafting (5) or when rub-inoculated plants are maintained for a period of time at a temperature over 28°C but below 35°C (8,26). Similarly, HR in potato due to viruses has been expressed as the development of necrotic local lesions in inoculated leaves or, alternatively, as the development of systemic necrosis (4,36).

PVA resistance in potato can be viewed as two distinct phenotypes occurring over time. In the HR, PVA inoculation produces necrotic symptoms, the virus is easily recovered, and the resistance is attributed to *N* genes (2,7,13,36). With extreme resistance (ER), infection elicits few or no visible symptoms in what is thought to be an intensified localized hypersensitivity (25). From such plants, virus recovery is very difficult, and the resistance is attributed to *R* genes (7,20,36). Assignment of PVA-resistance types, based on phenotypes to one of the two types (ER and HR), has been difficult in certain cultivars (2,24,37). In such situations it has been speculated that *R* genes initiate responses that could result in HR, but some *R* gene responses may prevent disease so effectively that cell death is not activated (5,12).

HR resistance to PVA was originally introgressed from wild *Solanum* spp. to cultivated potato (6,7), and resulted in a vast majority of progeny of field-immune parents exhibiting HR when inoculated with PVA (38). As a result, approximately 33% of North American and European cultivars show HR to PVA (1,34). A unique phenotype between ER and HR resistance is encountered in the infection of potato cv. Shepody with PVA, which, in contrast to monogenic inheritance of HR (35), appears to be conferred by two dominant complementary genes (30). No infection takes place in 'Shepody' plants upon rub inoculation, indicating ER resistance, whereas grafted 'Shepody' plants produce chlorotic leaf symptoms with necrotic streaks or spots on stems and tubers, indicating an HR-like response. The virus is not easily detected by enzyme-linked immunosorbent assay or by reverse transcription polymerase chain reaction (RT-PCR) (29). It is, however, detected in low amounts by a combination of RT-PCR and Southern blot (RT-PCR-SB) hybridization (30).

Studies with bacterial, fungal, and viral pathogens have shown that in a classical HR several physiological alterations occur in the plant cell (12,33). Invariably, transcription of pathogenesis-related (PR) genes is induced in neighboring cells (12). The *N* gene from

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tobacco, which is responsible for HR against TMV, has been cloned (39). It induces production of both acidic and basic PR proteins in inoculated and systemically infected leaves (16). Rapid generation of oxidants is characteristic of the HR in many plant-pathogen systems (15). Such an *N* gene-mediated oxidative burst has been detected following TMV infection (10). However, these observations are based on systems where phenotypic HR expression is rapid and symptoms develop within days and not weeks.

We examined the delayed HR-like response (weeks versus days after grafting) to PVA in 'Shepody'. We report that PVA-induced necrosis is distributed in discrete locations throughout the PVA-grafted 'Shepody' plants. The degree of necrosis and PVA concentration at these sites are enhanced by low temperature, in contrast to the enhanced accumulation of TMV at elevated temperature in *N* gene containing tobacco. Furthermore, we show that other characteristics of the HR, i.e., induction of PR gene expression and oxidative burst are also present in necrotic areas of PVA-resistant 'Shepody' plants.

MATERIALS AND METHODS

Potato virus culture, potato cultivars, and graft inoculation.

PVA was a local isolate obtained from a commercial potato field and used in previous studies (30,32). It belongs to strain group four among the categories described by Rajamäki et al. (24). The virus was maintained in PVA-susceptible potato cv. Goldrus in a greenhouse with 12-h light ($90 \mu\text{m}^{-2} \text{s}^{-1}$) at 15 to 18°C to optimize virus symptoms (28). Virus-free potato cv. Shepody plantlets were obtained from the Plant Propagation Centre (New Brunswick Department of Agriculture and Rural Development, Fredericton, Canada). The plantlets were transplanted in 12.5-cm pots and maintained in a greenhouse. Prior to PVA graft inoculation, the plantlets were routinely confirmed to be PVA-free by RT-PCR-SB (30,31). When 'Shepody' plantlets (rootstocks) were approximately 6-weeks old, they were cleft-grafted with shoots of PVA-infected potato plants of cv. Goldrus (scions). Visual observations were made and virus detection was carried out in scions and rootstocks at 30-, 40-, and 90-days postgrafting (dpg) by RT-PCR-SB (30). Five control plants were grafted with healthy 'Goldrus' shoots. At 90 dpg, tubers were harvested and stored at 20 to 25°C. After approximately 3 months of storage, when sprouts began emerging, tubers were planted in the greenhouse to study the presence of virus in the second generation. Graft inoculation and symptom observation of 'Shepody' plants were carried out at two temperatures (15 to 18°C and 28 to 31°C) in adjacent rooms of a greenhouse. In each experiment, 15 to 20 plants were grafted, and the experiment was repeated using growth rooms at 15 to 28°C.

Nucleic acid extraction. For virus detection only, RNA was isolated from different tissues as previously described (30,31). The RNA was quantified by measuring the absorbency at 260 nm (A_{260}). For mRNA as well as virus detection, RNA was isolated

according to Mohapatra et al. (19). Five grams of leaf, stem, or tuber tissue was ground in liquid N_2 with a pestle and mortar, incubated with 10 ml of preheated (60°C) extraction buffer (0.2 M sodium acetate, 10 mM EDTA, 0.2% sodium dodecyl sulfate [SDS], and 0.1 M β -mercaptoethanol at pH 5.2), and 10 ml of water-saturated phenol. After centrifugation at $6,000 \times g$ at 4°C for 15 min, the aqueous phase was extracted with 1 vol of phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol), followed by 1 vol of chloroform (chloroform/isoamyl alcohol, 24:1, vol/vol). Nucleic acids were precipitated by incubation in 2 M LiCl at 4°C overnight and collected by centrifugation at $10,000 \times g$ at 4°C for 30 min. The pellet was air dried, dissolved in 400 μl of distilled sterile H_2O , treated with RNase-free DNase I at 37°C for 10 min, and extracted with phenol (1 vol) once, then phenol/chloroform/isoamyl alcohol (25:24:1), and finally chloroform/isoamyl alcohol (24:1). The aqueous phase was collected and RNA was precipitated with 2 vol of ethanol in the presence of 0.1 vol of 3 M sodium acetate (pH 5.2) overnight at -20°C. The RNA was collected after centrifugation at $12,000 \times g$, dissolved in 100 μl of distilled sterile H_2O , and quantified spectrophotometrically.

RT-PCR. For PVA analysis, PVA antisense primer (Table 1) was used for RT. The RT was performed as described previously (31) with the following modifications. Briefly, 50 ng of total RNA was denatured at 65°C for 5 min and chilled on ice and combined with the RT mixture to a final volume of 10 μl . The RT reaction contained 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl_2 , 1.5 μl of phosphate-buffered saline, 0.1 M dithiothreitol (Gibco BRL, Life Technologies, Gaithersburg, MD), 0.5 mM each of dNTPs (Promega Corp., Madison, WI), 0.1 $\mu\text{g}/\mu\text{l}$ of PVA antisense primer, 20 units of RNasin (Promega), and 200 units of cloned *Moloney murine leukemia virus* RT (Gibco BRL). The reaction mix was incubated at 42°C for 1 h, and the reaction was terminated by heating to 95°C for 2 min.

For mRNA and viral RNA analysis together, RT was carried out using oligo(dT)₂₁ as antisense primer as described (21). One microgram of total RNA in 5 μl was mixed with 1 μl (1 μg) of (dT)₂₁. The mixture was incubated at 65°C for 5 min, chilled on ice for 3 min, and added to 14 μl of the RT mixture, as described previously. After RT was terminated, 80 μl of sterile H_2O was added to make a final volume of 100 μl . Four microliters of the cDNA solution was added to 21 μl of PCR mixture for amplification.

PCR was carried out by both sense and antisense primers for PVA or PR genes (Table 1). PCR was performed in a volume of 25 μl containing 4- μl aliquots of the cDNA mixture, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 100 μM each of dATP, dCTP, dGTP, and dTTP (Promega), 0.1 μg each of the sense and antisense primers, and 0.625 units of *Taq* polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA). Samples were amplified in 15 to 30 cycles (Peltier Thermal Cycler, PTC-200; MJ Research, Watertown, MA) with denaturation at 94°C, primer annealing at 55 or 60°C and primer extension at 72°C, each for

TABLE 1. The primer pairs specific for *Potato virus A* and several host messenger-RNAs used in reverse transcription-polymerase chain reaction

Virus/genes	Sequence	Polarity	Nucleotide in genome	Fragment (bp)
<i>Potato virus A</i>	5' GTTGGAGAATTCAAGATCCTGG 3'	Sense	183-204	255
	5' TTTCTCTGCCACCTCATCG 3'	Antisense	437-419	
Potato chitinase (<i>Cht</i>) A2 and A3	5' ACTACGAACGAGCTGGACAAGG 3'	Sense	396-417	386
	5' ATGTAACGACGCTTAGCCCTGG 3'	Antisense	781-760	
Potato chitinase (<i>Cht</i>) B2 and B3	5' GCTGCTTTCCTTGCCCAAAC 3'	Sense	373-392	446
	5' TGACCACATTCCAAGCCACC 3'	Antisense	818-799	
Potato glucanase (<i>Glu</i>) B2 & B3	5' TGCTGCGATGGAACGAACAG 3'	Sense	754-773	297
	5' TCCCAAACCTCTCTCAGACACCC 3'	Antisense	1050-1029	
Potato <i>PR-10a</i>	5' GAAGGAGATGTTCTTGAGACAACTTG 3'	Sense	320-347	217
	5' AGCGTAGACAGAAGGATTGGCG 3'	Antisense	538-517	
Potato cytochrome	5' GGTCGGACATACCCTGAAAC 3'	Sense	1210-1229	332
C oxidase subunit I (<i>coxI</i>) gene	5' CAAAAGTATGAAAAGCTGGAG 3'	Antisense	1541-1520	

1 min. A total of 10 µl of the amplification product was fractionated by electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and photographed under UV illumination. A low mass DNA ladder (Gibco-BRL) was used in the gel to determine the size of the amplified products. After the electrophoresis, the gel was blotted to Hybond N+ (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

Southern blotting. The blotted membrane was rinsed briefly in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) and air-dried. The DNA was fixed by UV cross-linking under UV illumination for 1 to 2 min. The membrane was prehybridized in a solution containing 5× SSC, 50% formamide, 0.1% sodium-lauryl sarcosine, 0.02% SDS, and 2% blocking reagent (Boehringer GmbH, Mannheim, Germany) at 42°C for 1.5 h. The solution was replaced with fresh prehybridization solution plus denatured digoxigenin (DIG)-labeled probe and incubated at 42°C overnight. After hybridization, the filter was washed twice with 2× SSC plus 0.5% SDS at 65°C, and twice with 0.5× SSC plus 0.1% SDS at 65°C, for 15 min each time. The filter was incubated with anti-DIG alkaline phosphatase (AP), followed by incubation with chemiluminescent substrate disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate according to the manufacturer's instructions (GmbH). The chemiluminescent signals were detected by exposure to X-ray film (X-AR; Kodak, Rochester, NY) at room temperature.

For quantitative analysis of PVA RNA in sample tissues, a concentration series ranging from 0 to 2 ng of purified PVA RNA was used as a standard for RT-PCR-SB, as previously described. The resulting bands were quantified using the EagleSight software (version 3.2, Stratagene Inc., La Jolla, CA). The concentration of PVA RNA from different samples was determined by comparison with the purified PVA RNA standards.

Preparation of probes for PVA and PR genes. DIG-labeled probes were prepared using PCR-amplified fragments produced in the presence of DIG-dUTP (GmbH). For PVA, purified viral RNA was used as template. For cytochrome c oxidase subunit I (*coxI*), total RNA from potato leaf was extracted according to Mohapatra et al. (19) and used as template for RT-PCR. The 255-bp fragment of PVA and 332-bp fragment of *coxI* were excised from the gel, purified by phenol/chloroform extraction, and used as a template for DIG labeling by PCR, according to the manufacturer's instructions (GmbH). However, for chitinase A and B, glucanase B, and PR-10a, the cloned portions of these genes were used directly as templates for DIG labeling of their corresponding probes.

The probes were precipitated by 2 vol of ethanol in the presence of 0.1 vol of 8 M LiCl at -20°C overnight, pelleted by centrifugation at 13,000 × g for 30 min, and dissolved in 200 µl of Tris-EDTA buffer (pH 8.0). Probe quality was checked by electrophoresis in 1.5% agarose. Addition of the DIG label to the PVA fragment was demonstrated by its reduced migration rate.

Lipid peroxidation assay. Lipid peroxidation was determined by the thiobarbituric acid (TBA) method, according to May et al. (18). Five hundred milligrams of tissue was ground in 1 ml of distilled water. To the 500-µl homogenate were added 125 µl of 3% SDS (wt/vol), 250 µl of 3% TBA in 50 mM NaOH, and 250 µl of 25% HCl (vol/vol), with thorough mixing after each addition. The mixture was heated at 80°C in a water bath for 20 min and snap-cooled on ice. TBA-reactive species (TBARS) were extracted with 1.5 ml of butanol. The low nonspecific A_{600} values were subtracted from the high specific A_{532} values of the organic phase, and the measurements were expressed as A_{532} g⁻¹ fresh weight.

RESULTS

Infection and reaction of 'Shepody' plants by PVA at two temperatures. During the summer months in greenhouse, when temperature (28 to 31°C) and light intensity (135 µm m⁻² s⁻¹) were

high, 20 rootstocks of 'Shepody' plantlets grafted with PVA-containing scions failed to develop chlorotic spots in leaves and necrotic streaks in stems or produce high levels of PVA as analyzed by RT-PCR-SB. Forty-seven tubers produced by these plants did not show necrotic symptoms nor was PVA detected in any plant subsequently grown from these tubers. PVA-infected scions from 'Goldrus' from the same grafted plants, tested at 30, 40, and 90 dpv contained a high level of PVA throughout the testing period (Fig. 1, lanes 30 to 33). Twenty 'Shepody' rootstocks, grafted during October to December at low temperatures (15 to 18°C) and light intensity (60 µm m⁻² s⁻¹), developed chlorotic and necrotic symptoms and PVA was detected in leaf, stem, and tuber tissues. Of the 45 tubers produced, 15 showed various amounts of necrosis. However, none of these tubers produced PVA-containing plants when planted. Greenhouse observations on symptom expression and virus concentration were confirmed when experiments were repeated using growth rooms at 15 and 28°C (data not shown).

At 15 to 18°C, tiny isolated necrotic streaks first occurred in the stems close to the graft union on the rootstock approximately 2 to 3 weeks after grafting. With time, the necrotic streaks increased in size and number. Chlorotic spots appeared in some newly emerged leaves 3 to 4 weeks after grafting. However, as these leaves enlarged, chlorotic areas became fainter and eventually disappeared. Concurrently, chlorotic spots continued to appear in other newly emerged leaves. Necrotic streaks in the stems occurred mainly at the nodes and only occasionally in the internodes. At later developmental stages, some necrotic streaks were as long as 2 cm. Necrotic symptoms also appeared on the stolons but not on the roots. Necrosis in tubers appeared in the bud end first, and later spread randomly throughout the tuber. The necrotic symptoms continued internally, and the tuber tissues became hard and dry (30). Only one-third of the tubers produced by a grafted plant developed necrotic symptoms.

RT-PCR-SB analysis revealed that in graft-inoculated 'Shepody' plants more PVA accumulated in plants maintained at 15 to 18°C (Fig. 1, lanes 11 to 20) than at 28 to 31°C (Fig. 1, lanes 1 to 10). However, for susceptible cv. Goldrus, the virus accumulation was unaffected by temperature (Fig. 1, lanes 26 to 29 and 30 to 33).

Association of chlorotic spots in graft-inoculated 'Shepody' leaves with the presence of PVA. A previous study (30) indicated that significantly lower amounts of PVA were present in graft-inoculated 'Shepody' leaves compared with leaves in susceptible cv. Goldrus. In order to understand the resistance of 'Shepody' plants to PVA, we isolated RNA separately from the chlorotic spots and surrounding green areas of leaves showing typical chlorotic mosaic in PVA-grafted 'Shepody' plants. RT-PCR was carried out to amplify PVA RNA in the extracts. RNA from isolated chlorotic spots produced a barely discernible PVA-specific band (255 bp) (Fig. 2A, lanes 3 to 6). No such bands were produced by the extract prepared from the whole leaf showing chlorotic mosaic symptoms (Fig. 2A, lanes 1 to 2) or the isolated green regions (Fig. 2A, lanes 7 to 10) of a chlorotic mosaic leaf. Southern blot analysis of the same preparations showed strong virus-specific bands in samples from chlorotic spots (Fig. 2B, lanes 3 to 6). Although these bands were also detected in the samples of green regions and the samples of whole leaf (Fig. 2B,

Fig. 1. Southern blot of reverse transcription-polymerase chain reaction (RT-PCR) products of *Potato virus A* from extracts of potato plants grown at two temperatures. At 28 to 31°C, 'Shepody' rootstocks (lanes 1 to 10), and 'Goldrus' scions at 90-days postgrafting (lanes 26 to 29). At 15 to 18°C, 'Shepody' rootstocks (lanes 11 to 20), and 'Goldrus' scions at 90-days postgrafting (lanes 30 to 33); lanes 21 to 25 contain products from uninfected plants and PCR mix control.

lanes 1 to 2 and 7 to 10), the intensity of the bands was much lower than that in the samples from chlorotic spots, indicating that the latter possessed a higher concentration of virus, whereas the green areas of chlorotic mosaic leaves had lower concentrations of PVA.

Association of necrosis in grafted ‘Shepody’ stems with PVA. To investigate possible causes of the discrete necrotic streaks in stems, RT-PCR-SB was carried out to investigate the occurrence of PVA in these samples, including stems of healthy grafted (rootstock/scion) ‘Shepody’ plants and visibly necrotic and nonnecrotic areas of stems of graft-inoculated ‘Shepody’ plants. Strong PVA bands were observed in the necrotic samples (Fig. 3, lanes 6 to 10), whereas fainter viral bands were present in the nonnecrotic samples (Fig. 3, lanes 11 to 15). No PVA-specific bands were observed in the healthy stem samples.

Fig. 2. Southern blot of reverse transcription-polymerase chain reaction (RT-PCR) products from extracts of various parts of stems grafted to *Potato virus A* (PVA)-infected tissue ‘Shepody’ leaves. **A**, Analysis of RT-PCR products by agarose gel electrophoresis. Lane M, molecular size markers; lanes 1 and 2, extracts from chlorotic mosaic whole leaf; lanes 3 to 6, chlorotic areas from a chlorotic leaf; lanes 7 to 10, green areas from the chlorotic leaf; lanes 11 and 12, negative uninfected control; and lane 13, purified virus A RNA control. Note only very faint bands at PVA position. The higher band is unknown nonspecific band. **B**, Southern blot of the gel shown in **A**. Arrow indicates the location of PVA fragment.

Because the virus appeared to be transported from the scion to various tissues including the leaves and stems, the occurrence of PVA in other tissues was investigated by RT-PCR-SB. Strong PVA bands were detected in the stolon (necrotic) samples (Fig. 3, lanes 20 to 23), and fainter bands appeared in the petiole (non-necrotic) samples (Fig. 3, lanes 16 to 19). Although unevenly distributed in petioles and stolons, the presence of virus in these two key organs connecting the leaves and the tubers, respectively, indicates that the virus may be translocated to all parts of the plants.

Association of necrosis in tubers of grafted ‘Shepody’ plants with PVA. ‘Shepody’ rootstock grafted with PVA-infected scions produced tubers with various degrees of necrosis, ranging from surface necrosis to internal necrotic hard and dry tissues (30). To investigate the presence of PVA in these tissues, RT-PCR-SB was carried out on extracts of tubers from graft-inoculated ‘Shepody’ plants and also nonnecrotic tubers from susceptible ‘Goldrus’ plants. The tubers were sampled shortly after harvesting and after 3 months of storage at 20 to 25°C.

Although PVA-specific bands were detected in samples from both necrotic (Fig. 4A, lanes 1 to 5) and nonnecrotic areas (Fig. 4A, lanes 6 to 10) of freshly harvested tubers of graft-inoculated ‘Shepody’ plants, the intensity of bands from the necrotic areas was markedly stronger than that from the nonnecrotic areas. PVA bands were present in samples of nonnecrotic tubers from susceptible ‘Goldrus’ plants (Fig. 4A, lanes 11 to 16). After 3-month storage of necrotic tubers at room temperature, PVA was detected only from necrotic areas (Fig. 4B, lanes 6 to 10) of tubers of graft-inoculated ‘Shepody’ and from the whole nonnecrotic tubers of PVA susceptible ‘Goldrus’ (Fig. 4B, lanes 16 to 20). PVA was not detected in nonnecrotic areas of tubers of graft-inoculated ‘Shepody’ plants (Fig. 4B, lanes 11 to 15) or in tubers from healthy ‘Shepody’ plants (Fig. 4B, lanes 1 to 5).

Induction of PR genes by PVA in ‘Shepody’. The relative expression of several PR genes was examined in different tissues of ‘Shepody’ plants and tubers. Samples included leaves and stems with chlorotic and necrotic symptoms from 10 graft-inoculated plants, 15 necrotic tubers collected from these plants and 10 leaves, stems, and tubers from plants grafted with healthy scions. The PR genes included were chitinase A and B (*ChA* and *ChB*), glucanase B (*GluB*), *PR-10a*, and the *coxI* gene, which is not a PR gene but was used as control. Although differing in degree, all tested PR genes were induced by PVA in leaves, stems, and tubers of graft-inoculated ‘Shepody’ plants (Fig. 5A, lanes 1, 2, 5, and 7;

Fig. 3. Southern blot of reverse transcription-polymerase chain reaction products from extracts of various parts of grafted and healthy ‘Shepody’ plants. Lanes 1 to 5, extracts of stem from healthy plant; lanes 6 to 10, extracts of necrotic areas of grafted plant; lanes 11 to 15, extracts of nonnecrotic areas of grafted plant; lanes 16 to 19, extracts of petioles (symptomless) of grafted plant; lanes 20 to 23, extracts of stolons (showing necrosis) of grafted plant; lanes 24 and 25, negative controls; and lane 26, purified *Potato virus A* (PVA) RNA. Arrow indicates the location of PVA fragment.

Fig. 5B, lane 2). Detection of PVA-specific RNA was faint from the symptomless whole leaf (Fig. 5A, lane 1), stronger from whole leaf extracts showing chlorotic spot symptoms (Fig. 5A, lane 2), and strongest from the leaves of the infected scions (susceptible plants) (Fig. 5A, lane 3). The *cox1* gene was expressed at the same level in all leaf samples (Fig. 5A, *cox1*).

Expression of *Cht A* and *Cht B* did not vary significantly between whole symptomatic and symptomless leaves (Fig. 5A, lanes 1 to 2), chlorotic spots (lane 5), and green areas (lane 4). In contrast, *Glu B* and *PR-10a* genes were expressed more strongly in symptomatic whole leaf (Fig. 5A, lane 2) than in symptomless whole leaf in graft-inoculated 'Shepody' (Fig. 5A, lane 1).

When chlorotic spots (Fig. 5A, lane 5) and green areas (Fig. 5A, lane 4) in symptomatic leaves were tested for PVA as well for PR gene expression, there was a close association between all PR gene expression and PVA accumulation in the chlorotic spots. Both *PR-10a* and *Glu B* were induced to a greater level in chlorotic spot and green areas than *Cht A* and *Cht B* genes.

In stems, all the PR genes were expressed at much higher levels in necrotic areas (Fig. 5A, lane 7) than in the nonnecrotic areas (Fig. 5A, lane 6). Similarly, PVA concentration was higher in the necrotic areas than in the nonnecrotic areas (Fig. 5A, lanes 6 and 7), but *cox1* expression did not vary.

In tubers from graft-inoculated 'Shepody' plants, all four PR genes (*Cht A*, *Cht B*, *Glu B*, and *PR-10a*) were expressed more strongly in necrotic areas (Fig. 5B, lane 2) accompanied by enhanced accumulation of PVA (Fig. 5B, lane 2, PVA) than in nonnecrotic areas (Fig. 5B, lane 1, PVA). In contrast, although a strong PVA band was detected in susceptible 'Goldrus' tubers (Fig. 5B, lane 3, PVA), the PR gene expression remained at a

relatively low level (Fig. 5B, lane 3). The level of expression of *cox1* was the same in all samples (Fig. 5B, *cox1*).

Association of lipid peroxidation with the necrosis response. To investigate the possible involvement of active oxygen species in PVA resistance in 'Shepody', lipid peroxidation was measured. Lipid peroxidation was highest in the necrotic areas of tubers and stems (Table 2), where PVA accumulation was greatest (Figs. 3 and 4).

Estimation of virus titer in necrotic streaks of tubers and stems. It appears that the development of necrosis in stems may be the result of a delayed or failing HR response in these tissues compared with the intensive necrosis observed in tubers, but both were directly associated with the presence of PVA. To determine the extent of variation in virus titer in these tissues, the PVA concentration in different tissues was estimated by a quantitative RT-PCR with purified PVA RNA as a standard. After RT-PCR-SB analysis, virus concentration in the tissues was calculated from the scanned data in comparison with the standards. On the basis of three experiments and four replicates of each sample, PVA RNA concentration in stem tissue was estimated to be 10 pmol in the necrotic areas and only 0.5 pmol in symptomless areas; similarly in the tubers, 26 pmol of RNA occurred in the necrotic areas and unquantifiable band intensity in nonnecrotic areas.

Absence of PVA in new plants from tubers of graft-inoculated 'Shepody' plants. After confirmation of the presence of PVA in the necrotic areas of graft-inoculated 'Shepody' plants by RT-PCR-SB, 38 tubers were planted in a greenhouse, with 15 tubers from healthy (nonnecrotic) 'Shepody' plants and 15 tubers from susceptible 'Goldrus' plants infected with PVA. The PVA bands were detected only in plants emerging from tubers of susceptible

Fig. 4. Southern blot of reverse transcription-polymerase chain reaction products of tubers. **A**, Tissue extracted immediately after harvest: lanes 1 to 5, necrotic tissue; lanes 6 to 10, nonnecrotic tissue; and lanes 11 to 16, nonsymptomatic tuber tissue from susceptible 'Goldrus'. Arrow indicates the location of *Potato virus A* (PVA) fragment. **B**, Tissue extracted after 3 months in storage. Lanes 1 to 5, healthy tuber tissue from symptomless tubers; lanes 6 to 10, PVA-infected necrotic tuber tissue; lanes 11 to 15, nonnecrotic tuber tissue; and lanes 16 to 20, PVA-infected 'Goldrus' tuber tissue.

plants. We tested sprouts from necrotic areas of graft-inoculated ‘Shepody’ tubers as they emerged and found 10 of 38, that originated at the border of necrotic and nonnecrotic areas, were positive for PVA. However, no PVA was detected in plants that grew from tubers of either grafted ‘Shepody’ or healthy ‘Shepody’ including the positive sprouts. This indicated that the second generation of PVA-grafted ‘Shepody’ plants were free from PVA. We considered the possibility that a trace amount of PVA may have existed in plants grown from tubers with necrotic symptoms. Therefore, RNA from leaves, stems, and new tubers at several developmental stages was tested in RT-PCR-SB. None were infected with PVA, confirming that the virus was lost during the storage and sprouting process.

DISCUSSION

In this study, temperature had a profound effect on the appearance of chlorotic and necrotic symptoms in PVA graft-inoculated ‘Shepody’ rootstock. The necrosis was greater at 15 rather than at 28°C. PVA causes more necrotic local lesions at 15°C than at higher temperatures in indicator plant *Physalis angulata* (28), which indicates that lower temperatures are more favorable for PVA infection and replication. The *N* gene-mediated HR has long served as the model for plant–pathogen systems. Because Samuel (26) demonstrated that TMV induced small local lesions in *N. glutinosa* at 21°C, whereas larger local lesions at 28°C and no local lesions at 35°C, temperature studies are used as a criterion of HR characterization. The temperature at which HR phenotypes are altered (i.e., local to systemic expression) differs in response to different TMV isolates (8) and TMV mutants (22), suggesting that temperature sensitivity of HR phenotypes in these instances

may not be a property of the host but a property of the virus (22). It is possible that our PVA strain activates the chlorotic and necrotic response in ‘Shepody’ at 15°C. Altering growth temperature can modify movement and systemic accumulation pattern of viruses with minimal effects at the initial sites of infection (9,11). Therefore, the precise temperature at which a HR fails to localize virus accumulation may differ among viruses and may be linked to the temperatures at which optimal viral replication occurs. It is also possible, however, that the lower temperature has affected the hosts response to virus.

It has been suggested that an interaction between elicitor molecule, generated by a pathogen gene, and a receptor, encoded by a resistance gene of the host plant, leads to the activation of a signal transduction cascade that eventually results in a HR (33). However, at present, the precise sequence of HR development is unknown. The recent isolation and cloning of *N* and *Rx* genes from plants has shown that the appearance of a necrotic HR at the initial site of infection may not be a crucial effect of the genes that elicit HR expression. The *Rx*-mediated ER against PVX in potato does not produce HR at the initial site of mechanical inoculation but does so in graft-inoculated plants (5). Despite this difference, the *Rx*-gene product is structurally similar to the *N*-gene product, and it confers ER and has the potential to initiate cell death (5). The PVA–‘Shepody’ system has striking similarity to that of the *Rx*-mediated resistance (5), in that ‘Shepody’ plants do not exhibit necrosis when rub-inoculated with PVA but do so upon graft inoculation when the plants are maintained at lower temperature. The lack of HR expression at the initial infection site may depend on virus concentration and translocation to certain “receptor” sites. In graft-inoculated plants, a continuous supply of virus from susceptible scions would result in its translocation through the resistant rootstock, as in *Rx*-mediated resistance such that PVX translocation through the phloem of resistant plants is not suppressed (5). Therefore, once viral accumulation has reached an appropriate concentration and at a specific tissue site a HR-like response would be initiated. We have shown that the concentration of PVA observed in chlorotic leaves and necrotic stem and tuber tissues was consistently higher than that observed in non-symptomatic plant parts (Fig. 2).

The PVA–‘Shepody’ system demonstrates the expression of PR genes and the generation of oxidative reactive species, which are typical of HR production in other plant–pathogen systems. However, it varies from the classical HR type of plant–virus interaction in its response to low temperatures and the absence of a localized necrotic response. In addition, ‘Shepody’ plants appear to mount an effective PVA restriction response in tubers. The tubers showing severe necrosis and detectable PVA yield second generation plants with no detectable PVA. This indicates virus movement, replication or both are arrested in some way during tuber storage and sprouting (Fig. 4). Virus arrest continues through the sprouting stage, because some sprouts originating at the junction of necrotic/nonnecrotic area contained PVA, but virus was not detected when the leaves from such plants were tested. The mechanism of virus loss in infected tuber/sprout is not known and further studies are needed.

The PVA–‘Shepody’ system shows features characteristic of a failed HR interaction. First, the accumulation of PVA in grafted

Fig. 5. Southern blot of reverse transcription-polymerase chain reaction products of various pathogenesis-related (PR) protein mRNAs from extracts representing **A**, leaves and stems of grafted ‘Shepody’ and **B**, tuber. **A**, PR gene expression in leaves and stems. Whole leaf (lanes 1 to 3); symptomless leaf (lane 1); symptomatic leaf (lane 2); and leaf of scions containing virus (lane 3). Green areas (lane 4) and chlorotic spots (lane 5) of symptomatic leaf. Nonnecrotic areas (lane 6) and necrotic areas (lane 7) of stems of grafted plants. **B**, PR gene expression in tubers: nonnecrotic tissue (lane 1) and necrotic tissue (lane 2) of symptomatic tubers; and tuber tissue of *Potato virus A* (PVA)-infected susceptible ‘Goldrus’ (lane 3). Each gene is identified on the right side.

TABLE 2. Lipid peroxidation in tissues of ‘Shepody’ plants grafted with scions containing *Potato virus A*

Tissues	$\Delta A_{532-600}$ ($A_{532} \text{ g}^{-1} \text{ fwt}^a$)
Nonnecrotic areas of stems	0.400 ± 0.032
Necrotic areas of stems	0.886 ± 0.118
Stolons (nonnecrotic)	0.479 ± 0.078
Nonnecrotic areas of tubers	0.329 ± 0.048
Necrotic areas of tubers	0.680 ± 0.048

^a Absorbance per gram fresh weight.

'Shepody' plants is localized, but in discrete places distant from the inoculation site, and the virus fails to multiply in second generation plants grown from diseased necrotic tubers. Second, the increased expression of PR protein genes, characteristically associated with other HR (16), is induced in chlorotic or necrotic areas of the leaves, stems, and particularly the tubers of grafted 'Shepody' (Fig. 5). Third, the generation of oxidants was characteristic of PVA-'Shepody' interactions, where high concentrations of TBARs were observed in the necrotic areas that PVA accumulated (Table 2, Figs. 3 and 4).

We concluded that response of potato cv. Shepody to PVA shares many of the characteristics of HR-like responses demonstrated by viral, fungal, and bacterial pathogens (14,22). The slow appearance of symptoms, low virus concentration, and induction of PR protein genes indicate that cv. Shepody is highly resistant to PVA. We propose that the continuous supply of virus from infected scions under experimental conditions forces the plant to react by the production of visible symptoms. Thus, it is a unique model system to elucidate the various steps of a systemic necrosis response in a vegetatively propagated crop.

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