Transgenic Resistance to PVY^O Associated with Post-Transcriptional Silencing of P1 Transgene Is Overcome by PVY^N Strains That Carry Highly Homologous P1 Sequences and Recover Transgene Expression at Infection

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Resistance to *Potato virus Y* (PVY) has been obtained in our previous studies through expression of the PVY P1 gene in sense or antisense orientation in potato cv. Pito. In the present study, the mechanism and strain specificity of the resistance were analyzed. Several features including low steady-state P1 mRNA expression in the resistant P1 plants indicated that resistance was based on post-transcriptional gene silencing (PTGS). Resistance was specific to PVY^O isolates, the PVY strain group from which the P1 transgene was derived. However, according to group analyses, there was no distinguishing characteristic between the PVY^O and PVY^N strains P1 gene sequences. Therefore, the ability of the PVY^N strains to overcome resistance could not be explained solely based on their P1 gene sequences. Infection with PVY^N of the PVY^O-resistant transgenic lines led to a recovery of expression of the P1 transgene. These data suggested that factors other than sequence homology are required in determination of the resistance specificity.

Additional keywords: pathogen-derived resistance, potyvirus.

Pathogen-derived resistance (PDR) is based on transformation of plants with genes derived from pathogens. In some cases resistance expression requires production of the protein encoded by the transgene, but in many cases resistance is obtained at the RNA level and only the expression of the transgene transcript is required (reviewed in Baulcombe 1996; Beachy 1997; van den Boogaard et al. 1998; Mäki-Valkama and Valkonen 1999). One type of RNA-mediated resistance is referred to as homology-dependent virus resistance (HDR). HDR is associated with homology-dependent gene silencing (Mueller et al. 1995). Gene silencing may occur at the transcriptional level in the nucleus or at the post-transcriptional

level in the cytoplasm (reviewed in Matzke and Matzke 1995; Meyer and Saedler 1996). HDR to RNA viruses that replicate in the cytoplasm is most likely associated with the latter mentioned, post-transcriptional gene silencing (PTGS). This is indicated by the absence of changes in transgene transcription rates in the nucleus (Lindbo et al. 1993; Mueller et al. 1995; Sijen et al. 1996) and by a reduction in transgene mRNA accumulation in the cytoplasm. The latter is explained by an increased turnover or sequence-specific degradation of the transgene mRNA. Subsequently, any RNA that shares sufficiently high sequence homology with the silenced transgene, including the cytoplasmically replicating plant viruses, will be degraded (Dougherty and Parks 1995).

A few studies have assessed the threshold sequence similarities between the transgene and the infecting virus required for the activation of HDR (Pang et al. 1993; Longstaff et al. 1993; Mueller et al. 1995; Taliansky et al. 1998; Jones et al. 1998). The threshold similarities seem to vary case by case, and the lowest reported sequence similarity that allows activation of HDR is 87% (Taliansky et al. 1998). Transgenic pea lines that expressed the NIB gene from *Pea seed-borne mosaic virus* (PSbMV, genus *Potyvirus*) isolate DPD1 were resistant to isolate L-1 of PSbMV. Sequence similarity between the NIB genes of the two isolates was 92%. Resistance to isolate NY was observed only in two experiments out of three, and the similarity (89%) between the NIB genes of isolates L-1 and NY may therefore be at the threshold level required for activation of HDR (Jones et al. 1998).

Similarity of a particular part of the sequence can be more important than overall sequence similarity for activation of HDR. In several studies the 3' end of the transgene was found to be crucial (English et al. 1996; Sijen et al. 1996; Sonoda et al. 1999). For example, in some tobacco lines transformed with the coat protein (CP) gene from *Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus*), resistance to SPFMV was most dependent on the 3' end of the mRNA. However, in some other lines both 3' and 5' ends and also the central part of the mRNA were important (Sonoda et al. 1999). It also seems that once HDR is activated based on a specific part of

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the transgene, the remainder of the transgene sequence is also able to target the degradation mechanism to homologous sequences. This was shown by silencing a green fluorescent protein (GFP) gene in a transgenic plant by bombarding the plant with a certain fragment of the GFP gene. The silencing mechanism was subsequently able to target a *Potato virus X* (PVX, genus *Potexvirus*) gene vector that carried a different fragment of the GFP gene not homologous to the sequence that activated the silencing (Voinnet et al. 1998).

Structural and nonstructural genes of *Potato virus Y* (PVY, genus *Potyvirus*) have been used in translatable and nontranslatable forms to engineer PDR to PVY (reviewed by Mäki-Valkama and Valkonen 1999). CP gene-mediated resistance can be strain group-specific (van der Vlugt and Goldbach 1993; Malnoë et al. 1994) or broader, protecting the transgenic plants against several PVY strain groups (Farinelli and Malnoë 1993; Smith et al. 1994, 1995; Okamoto et al. 1996; Sudarsono et al. 1995). The nonstructural genes, such as the NIb replicase (Audy et al. 1994) or the P1 gene (Pehu et al. 1995; Mäki-Valkama et al. 2000), have provided only strain group-specific resistance.

The P1 protein of potyviruses is produced from the N terminus of the potyvirus-encoded polyprotein. The P1 protein contains a serine-type proteinase domain that separates the P1 protein from the helper-component proteinase (HC-Pro), the second protein of the polyprotein (Verchot et al. 1991; 1992). Furthermore, the P1 protein binds RNA (Brantley and Hunt 1993; Somounou and Laliberte 1994) and has an accessory or regulatory function in stimulating genome amplification of potyviruses (Verchot and Carrington 1995a, 1995b).

In our previous studies, potato cv. Pito was transformed with the P1 gene derived from an isolate of PVY^O (ordinary strain group of PVY) in sense (Pehu et al. 1995) or antisense orientation (Mäki-Valkama et al. 2000). In both cases, several transgenic lines were highly resistant to PVY^O, but none were resistant to PVY^N (tobacco vein necrosis strain group of PVY) or other viruses. In the present study, the basis of strain specificity of resistance was examined in these transgenic potatoes with several PVY isolates from both strain groups. The results indicated that resistance was specific to PVY^O strain group and probably based on PTGS. However, the isolates of PVY^O and PVY^N did not have distinguishing characteristics based on their P1 gene sequences that could have allowed designating them into different groups. Therefore, the ability of the PVY^N isolates to overcome resistance could not be explained solely based on their P1 gene sequences. The PTGS of the P1 transgene was suppressed in the PVY^O-resistant lines following infection with PVY^N. These results suggested that other factors are required in determining the specificity of resistance in the P1 transgenic plants.

RESULTS

Grafting analyses.

"Hybrid" potato plants were created by replacing the top of a transgenic, PVY-resistant line with a top from a nontransgenic or other PVY-susceptible control line. Alternatively, the PVY-susceptible potato lines were used as the rootstock. Subsequently, two leaves of the rootstock were sap inoculated with PVY^O-UK. This was done to assess whether the resistance was active in the inoculated leaves. Secondly, this ar-

angement would reveal accumulation of PVY in the inoculated rootstock even at low levels undetectable by enzyme-linked immunosorbent assay (ELISA), since the PVY-susceptible top part would permit virus accumulation to high, detectable levels following systemic infection.

None of the inoculated leaves were infected with PVY in any SR line (PVY^O-UK -resistant lines transformed with the P1 gene in sense orientation) according to ELISA at 14 days post inoculation (14 dpi). In contrast, all PVY-inoculated leaves of the wild-type (WT, nontransformed) plants and the transgenic control line K3 (transformed with a vector-alone plasmid) were infected (Table 1). These data indicated that resistance response was active prior to inoculation, or induced very quickly following inoculation.

Systemic infection was tested in the rootstock and the top scion at 35 dpi. Systemic infection with PVY was not detected in the inoculated SR rootstocks, in the SR top scions growing on PVY-infected susceptible rootstocks, or in the top scions from susceptible control lines grafted onto SR rootstocks. The control lines (WT and K3 grafted onto the WT line) were systemically infected (Table 1). These data showed that no systemic infection even with an accumulation of low PVY titers undetectable by ELISA occurred in the SR plants. These results on the SR lines were similar to those obtained with the ASR lines (PVY^O-UK -resistant lines transformed with the P1 gene in antisense orientation) in a previous study (Mäki-Valkama et al. 2000).

Strain group specificity of resistance.

Resistance in the SR and ASR lines was previously tested with only one isolate of PVY^O (PVY^O-UK) and PVY^N (PVY^N-UK) and the resistance was found to function only against PVY^O-UK (Pehu et al. 1995, Mäki-Valkama et al. 2000). In this study, three additional isolates of PVY^O (PVY^O-803, PVY^O-Loimaa, PVY^O-Viikki) and one additional isolate of PVY^N (PVY^N-RUS) were included to test whether the resistance was isolate specific to PVY^O-UK or strain group spe-

Table 1. Mean absorbance values of control and transgenic lines after grafting onto the PVY^O-inoculated resistant sense plant or the PVY^O-inoculated nontransgenic Pito plant, respectively

Rootstock ^a	Top scion	Inoculated leaf		Rootstock		Top scion	
		14 dpi ^b		35 dpi		35 dpi	
		A _{405±}	SE ^c	A _{405±}	SE	A _{405±}	SE
S1 1002-1	WT ^d	0.01	0.00	0.01	0.00	0.01	0.01
SI11 1113-1	WT	0.01	0.01	0.00	0.00	0.01	0.00
SI 0308-3	WT	0.01	0.00	0.01	0.00	0.01	0.01
K3 ^e	WT	1.25	0.05	1.02	0.26	1.14	0.05
WT	WT	0.45	0.16	0.93	0.20	0.96	0.22
WT	S1 1002-1	0.32	0.14	0.64	0.20	0.01	0.01
WT	SI11 1113-1	0.70	0.24	0.64	0.14	0.01	0.01
WT	SI 0308-3	0.82	0.26	0.71	0.24	0.03	0.01
WT	K3	1.19	0.15	0.80	0.21	0.92	0.04
WT	WT	0.50	0.09	0.94	0.05	0.92	0.04
Non-inoculated controls		0.01	0.00	0.01	0.00	0.01	0.00

^a Sap-inoculated with PVY^O-UK.

^b Days post inoculation.

^c Mean enzyme-linked immunosorbent assay absorbance values for six plants ± standard error.

^d WT, nontransgenic potato cultivar Pito.

^e K3, empty plasmid-transformed control line.

cific against different isolates or strains of PVY^O. When the resistant plants were mechanically inoculated with the PVY^O isolates they did not develop any symptoms and did not contain detectable amounts of PVY according to ELISA at 28 dpi. In contrast, systemic infection was readily detected by ELISA at 21 dpi in all tested transgenic potato lines inoculated with the two PVY^N isolates.

Correlation of resistance with the numbers of the transgene loci and with expression of the transgene.

The numbers of loci of the P1 transgene were determined in the SR lines, and a few P1 sense-lines not resistant to PVY^O (SNR) (Pehu et al. 1995) were included for comparison. Southern analysis carried out on total DNA digested with *Eco*RI (Fig. 1), *Hind*III, or *Pst*I suggested that the SR and SNR lines both contained one to three transgene loci, except for the SNR line SI 0305-2, that contained five to seven loci. The sizes of the fragments (approximately 2.7 kb) detected with the probe in the SR lines SI 1002-1, SI 0308-3, and SIII 1113-1 (Fig. 1, lanes 1, 4, and 5, respectively) may indicate an inverted-repeat arrangement of the transferred DNA. These data revealed no systematic difference in the number of transgene loci between the SR and SNR lines, similar to a previous study, in which no systematic difference was detected between the numbers of transgene loci in the P1 antisense-lines resistant or susceptible to PVY^O (Mäki-Valkama et al. 2000).

Expression of the P1 transgene transcript was tested by Northern (RNA) analysis. A fragment of approximately 1.0 kb was detected in the SR plants as well as in the SNR plants. However, the steady-state expression levels of the P1 mRNA in the SR lines (Fig. 2, lanes 6 to 8) were much lower than in the SNR lines (Fig. 2, lanes 1 to 5). Thus, low levels of steady-state P1 mRNA expression were associated with resistance to PVY.

Expression of the P1 protein was analyzed by Western blotting (immunoblotting). No P1 protein could be detected in the SR lines (Fig. 3, lanes 2 to 4), whereas the SNR lines (Fig. 3, lanes 5 to 7) and the PVY-infected control plants (Fig. 3, lanes 8 to 9) contained detectable amounts of P1 protein. Hence, resistance to PVY was not associated with detectable P1 protein expression.

Accumulation of P1 mRNA and viral RNA in infected plants.

The steady-state P1 mRNA expression levels were also tested in the leaves of PVY^O-resistant SR lines that were grown from tubers infected with the PVY^N isolates. The level of P1 transcripts was higher in the PVY^N-infected plants (Fig. 4, lanes 1, 3, and 5) than in the healthy control plants of the SR lines (Fig. 4, lanes 2, 4, and 6). These data indicated that infection with PVY^N increased the steady-state P1 mRNA levels.

These findings prompted us to test whether, in general, the isolates of the two PVY strain groups replicated to different titers in potato cv. Pito (WT plants). The amounts of RNA for PVY^O-UK, PVY^O-803, PVY^O-Loimaa, and PVY^O-Viikki (Fig. 5, lanes 4 to 7, respectively) were higher than the amounts for PVY^N-UK and PVY^N-RUS (Fig. 5, lanes 8 and 9, respectively) in the systemically infected leaves of sap-inoculated plants 21 dpi (primary infection). Similarly,

Fig. 1. Southern analyses of transgenic Pito plants expressing P1 gene from PVY^O-UK in sense orientation. **A**, Transfer-DNA fragment that was transferred into plants. Locations of restriction sites are indicated above and position of 35S promoter and P1 transgene below in boxes. RB, right border; LB, left border; nos-nptII, neomycin phosphotransferase II gene under nopaline synthase promoter. **B**, Southern analyses of resistant (R) and susceptible (S) plants digested with *Eco*RI restriction enzyme. M = DNA molecular weight marker (fragments in descending order: 23.13, 9.41, 6.56, 4.36, 2.32, 2.03, 0.56 kb). Lane 1, SI 1002-1; lane 2, Pito; lane 3, SI 0305-2; lane 4, SI 0308-3; lane 5, SIII 1113-1; lane 6, SI 1646-1; lane 7, SI 1010-1; lane 8, SI 2001-1; and lane 9, SIII 1130-1.

the amount of RNA was higher for PVY^O-UK (Fig. 5, lane 1) than for PVY^N-UK or PVY^N-RUS RNA (Fig. 5, lanes 2 and 3, respectively) in the leaves of the WT plants grown from infected tubers (secondary infection). These data showed that PVY^N accumulated in lower amounts than PVY^O in potato cv. Pito.

Fig. 2. Northern (RNA) analysis of transgenic Pito plants expressing PVY^O-UK P1 gene in sense orientation. **A**, Steady-state levels of P1 mRNA in Pito lines expressing the P1 gene in sense orientation. Total RNA was blotted onto a nylon membrane and hybridized with digoxigenin-dUTP labeled P1 RNA specific to P1 coding strand of PVY^O-UK. M = RNA molecular weight marker (fragments in descending order: 1.5, 0.58, 0.44, 0.31 kb). Lane 1, SIII 1130-1; lane 2, SI 2001-1; lane 3, SI 1010-1; lane 4, SI 1646-1; lane 5, SI 0305-2; lane 6, SIII 1113-1; lane 7, SI 0308-3; lane 8, SI 1002-1; and lane 9, Pito. **B**, Photograph from Northern membrane to show loading and transfer of RNA samples. Order of samples is the same as from lane 1 to lane 9 in **A**.

Fig. 3. Western (immunoblot) analysis of resistant and susceptible Pito lines expressing P1 gene in sense orientation. Lane 1, Pito; lane 2, SI 1002-1; lane 3, SI 0308-3; lane 4, SIII 1113-1; lane 5, SI 0305-2; lane 6, SI 1646-1; lane 7, SI 1010-1; lane 8, PVY^O-infected Pito; lane 9, PVY^O-infected tobacco. Arrow indicates position of P1 protein. Antibody raised against PVY^O-UK P1 protein (Arbatova et al. 1998) cross-reacted with some potato proteins.

P1 gene sequences of PVY^O and PVY^N.

The P1 gene sequences were determined from the PVY isolates used for inoculations. Sequence identities among the P1 genes of the four PVY^O isolates, including PVY^O-UK (transgene sequence), ranged between 96 and 99%. Sequence identities between the P1 genes of the two PVY^N isolates and the P1 sequence of PVY^O-UK ranged from 98 to 99%.

The nucleotide substitutions in PVY^N and PVY^O isolates were similarly distributed over the P1 sequence. There was no nucleotide sequence that was unique to the PVY^N isolates as a group that could differentiate them from the group of PVY^O isolates. The P1 sequences were also analyzed by phylogenetic analysis, which could not distinguish the two PVY strain groups, since no cluster contained only PVY^O or PVY^N isolates (see Materials and Methods; data not shown).

While being indistinguishable as two strain groups based on the P1 sequences, each PVY isolate contained unique nucleotide changes and, subsequently, unique amino acid residues at 1 to 4 positions of the deduced P1 protein sequence (Table 2).

DISCUSSION

Resistance to PVY^O in the Pito lines transformed with the PVY^O P1 gene in sense or antisense orientation showed several features indicating that the resistance mechanism was based on PTGS (Mueller et al. 1995; Baulcombe 1996; Beachy 1997; van den Boogaard et al. 1998). The present study and the previous study (Mäki-Valkama et al. 2000) re-

Fig. 4. Northern (RNA) analysis of steady-state P1 transgene transcript levels in transgenic Pito plants that contain the P1 transgene in sense orientation and that were grown from PVY^N-infected tubers. **A**, Lane 1, PVY^N-infected SI 1002-1; lane 2, SI 1002-1; lane 3, PVY^N-infected SIII 1113-1; lane 4, SIII 1113-1; lane 5, PVY^N-infected SI 0308-3; and lane 6, SI 0308-3. M = RNA molecular weight marker (fragments in descending order: 1.5, 0.58, 0.44, 0.31 kb). Upper arrow indicates position of PVY genomic RNA; lower arrow, the transgenic P1 transcript. **B**, Photograph from Northern membrane to show loading and transfer of RNA samples. Order of samples is the same as from lane 1 to lane 6 in **A**.

vealed that in all PVY-resistant lines (SR and ASR) the resistance is active prior to inoculation, or is induced very quickly following infection, and is associated with low steady-state P1 mRNA accumulation. Therefore, the resistance does not belong to the induced recovery type of resistance that requires detectable virus accumulation prior to induction (Lindbo and Dougherty 1992a,1992b; Jones et al. 1998). Resistance was not associated with detectable production of the P1 protein or correlated with any particular number of transgene loci since a single locus was sufficient for high levels of resistance. It is not overcome with high inoculum dose, e.g., by graft inoculation (Pehu et al. 1995; Mäki-Valkama et al. 2000).

Resistance in the transgenic lines of this study is virus specific because it is effective against PVY and not other viruses such as PVA and PVX (Pehu et al. 1995; Mäki-Valkama et al. 2000). Furthermore, resistance was specific to PVY^O isolates, i.e., the PVY strain group from which the P1 transgene was derived. Strain group-specific resistance to PVY has also been reported with the CP and N1b genes of PVY for transformation (van der Vlugt and Goldbach 1993; Audy et al. 1994; Malnoë et al. 1994; Mäki-Valkama and Valkonen 1999).

The reason why resistance was effective against PVY^O can be explained according to the concept of HDR (Mueller et al. 1995) since the P1 gene sequences of the PVY^O isolates were

almost identical (>96%) to the P1 transgene sequence. Also, as suggested previously (Mäki-Valkama et al. 2000), the natural strain group-specific resistance to PVY^O conferred by the gene *Ny* in potato cv. Pito (Valkonen et al. 1998) may contribute to targeting resistance to PVY^O, even though the natural resistance is not fully expressed under the temperatures used in our study. In contrast, the reason why resistance was not effective against PVY^N is less obvious and cannot be fully explained with the data obtained in this study. However, hypotheses directly or indirectly supported by the data may be presented.

Determination of the P1 sequences of the PVY^O and PVY^N isolates excluded the possibility that the PVY^N isolates escaped the resistance mechanism simply because their P1 gene sequences were significantly different from those of PVY^O. In contrast, the sequences showed high identity (>96%) and further analyses revealed no systematic difference distinguishing PVY^O from PVY^N. Furthermore, sequence analyses excluded the possibility that the nucleotide differences were located in different parts of the P1 gene in PVY^O and PVY^N and that a specific part of the PVY^O P1 sequence was recognized by the homology-dependent gene silencing mechanism (English et al. 1996; Sijen et al. 1996). There was no nucleotide that was unique to the PVY^N isolates as a group that could have differentiated them from the PVY^O strain group. However, these data do not completely exclude the possibility that the differences in the P1 nucleotide sequence are important. For example, the PVY^N P1 sequences might be folded into secondary structures not recognized and targeted for RNA degradation, or which resist degradation by RNases (Hellwald and Palukaitis 1995; Metzloff et al. 1997).

Besides nucleotide differences, the two PVY^N isolates each contained two different, unique amino acid residues in their deduced P1 protein sequence, the significance of which could not be determined. However, assuming that an interaction between the P1 protein and the HC-Pro may be important for the suppression of PTGS (mediated by the HC-Pro of PVY^N; Brigneti et al. 1998), the unique amino acid residues in the P1 proteins of the PVY^N isolates may affect this interaction and possibly enhance suppression of PTGS. Indeed, PVY^N may overcome the P1 gene-mediated resistance by suppressing

Fig. 5. Northern (RNA) analysis of the PVY^O and PVY^N RNA levels in nontransformed Pito. Total RNA was blotted onto a nylon membrane and hybridized with digoxigenin-dUTP labeled P1 RNA probe made according to P1 sequence of PVY^O-UK. **A**, Levels of PVY^O and PVY^N RNA in leaves of plants grown from infected tubers of nontransformed Pito (secondary infection; lanes 1 to 3) and in systemically infected leaves of nontransformed Pito plants mechanically inoculated 3 weeks earlier (primary infection; lanes 4 to 9). Lane 1, PVY^O-UK; lane 2, PVY^N-UK; lane 3, PVY^N-RUS; lane 4, PVY^O-UK; lane 5, PVY^O-803; lane 6, PVY^O-Loimaa; lane 7, PVY^O-Viikki; lane 8, PVY^N-UK; and lane 9, PVY^N-RUS. Arrow indicates position of PVY RNA. Dashes correspond to location of molecular weight markers (fragments in descending order: 1.5, 0.58, 0.44, 0.31 kb). **B**, Photograph from Northern membrane to show loading and transfer of RNA samples. Order of samples is the same as in **A**.

Table 2. The unique amino acid residues in the deduced P1 protein sequences of the six *Potato virus Y* (PVY) isolates included in this study

Isolate ^a	Unique amino acid		Amino acid in other isolates
	Position	Name	
PVY ^N -RUS	65	Aspartic acid	Glycine
	159	Proline	Serine
PVY ^N -UK	196	Phenylalanine	Leucine
	200	Leucine	Valine
PVY ^O -803	183	Threonine	Alanine
PVY ^O -Viikki	137	Histidine	Arginine
	269	Alanine	Threonine
PVY ^O -Loimaa	86	Lysine	Arginine
	87	Asparagine	Lysine
	93	Asparagine	Histidine
	189	Glutamine	Arginine
PVY ^O -UK	160	Glycine	Glutamic acid
	170	Arginine	Lysine

^a Nucleotide sequence databank accession numbers: X82848 (PVY^O-UK), AJ245554 (PVY^O-Loimaa), AJ245555 (PVY^O-803), AJ245556 (PVY^O-Viikki), AJ245557 (PVY^N-RUS), and AJ245558 (PVY^N-UK).

PTGS, as suggested by the recovery of P1 transgene expression during PVY^N infection (Fig. 4).

The amino acids unique to the PVY^N isolates might also be important in determining the level of enhancement of genome amplification mediated by the P1 protein (Verchot and Carlington 1995a, 1995b). Hence, they might make genome amplification less efficient, consistent with accumulation of lower amounts of viral RNA in potatoes infected with PVY^N than in potatoes infected with PVY^O (Fig. 5). Accumulation to lower levels may allow the RNA of the PVY^N isolates to remain below a threshold concentration required for recognition and targeting for the RNA degradation machinery. This would allow establishment of infection and thereafter production of sufficient amounts of HC-Pro to suppress PTGS. Threshold levels of viral RNA have been reported for initiation of the recovery type of resistance (Lindbo et al. 1993; Swaney et al. 1995; Jones et al. 1998). A recovery from infection might also occur in the resistant potato lines of our study, but if so it probably takes place at the level of the initially infected cells since no detectable levels of PVY^O were found in the inoculated resistant lines.

The data presented in this study show that the P1 gene-mediated resistance to PVY in the transgenic lines of potato cv. Pito possesses many characteristics of PTGS and is therefore likely to be based on PTGS. Furthermore, the resistance acts against isolates of the strain group PVY^O consistent with the concept of HDR. However, isolates of the strain group PVY^N overcome the resistance and, subsequently, suppress PTGS. The differential response to the two PVY strain groups, despite their highly similar P1 gene sequences, suggests that other factors besides sequence homology between the transgene sequence and the infecting virus play a role in determining whether or not resistance will be effective.

MATERIALS AND METHODS

Plants and viruses.

Three transgenic lines of Pito containing the P1 gene in sense orientation (SR lines SI 1002-1, SI 0308-3, and SIII 1113-1) (Pehu et al. 1995) and five transgenic lines containing the P1 gene in antisense orientation (ASR lines AI 0306-3, AI 0623-2, AII 1814-1, AII 1813-1, AI 1139-1) (Mäki-Valkama et al. 2000) were included in this study. The SR and ASR lines are highly resistant to the isolate PVY^O-UK from which the transgene sequence had been derived. Resistance to PVY^O-UK is expressed as no symptoms and no detectable amounts of PVY antigen measured by ELISA in the upper, noninoculated leaves after mechanical and side-graft inoculation (Pehu et al. 1995, Mäki-Valkama et al. 2000). Nontransformed Pito (WT) plants, and plants of transgenic lines of Pito (K3) transformed with a vector-alone plasmid were used as controls. A few additional lines carrying the P1 transgene in sense orientation but not expressing resistance to PVY were included in the Southern, Northern, and Western analyses for comparison. Propagation and maintenance of the potato lines in vitro and cultivation and growth conditions in the greenhouse were similar to those of previous studies (Pehu et al. 1995, Mäki-Valkama et al. 2000).

Isolates PVY^O-UK and PVY^N-UK originate in the United Kingdom and have been previously described (Gibson et al.

1990). PVY^O-UK is the PVY isolate from which the P1 gene was cloned for transformation of cv. Pito (Pehu et al. 1995; Mäki-Valkama et al. 2000). PVY^O-803 was provided by A. Hassi (Agricultural Research Centre, Finland). PVY^O-Loimaa is the reference isolate used at the Seed Testing Institute, Finland, and was provided by H. Kortemaa. PVY^O-Viikki was isolated from a plant of cv. Pito infected in the field in Viikki, Helsinki, Finland. PVY^N-RUS is from Russia and was provided by M. Saarma (Institute of Biotechnology, University of Helsinki). All PVY^O isolates produce mosaic symptoms in the leaves of tobacco (*Nicotiana tabacum* cv. Samsun) and Pito, in which they were propagated. The PVY^N isolates induce mild or no symptoms in Pito, in which they were propagated, whereas they induce vein necrosis in the systemically infected leaves of tobacco.

Virus inoculation and detection.

In the grafting analysis, the top of the SR line (resistant to PVY^O) was replaced by grafting with a scion from a nontransformed (WT) Pito plant, or vice versa. Six plants from each transgenic and control potato line were grafted with both arrangements. Graft union was allowed to form for 3 weeks before two lower leaves of the rootstock part were sap inoculated with PVY^O-UK. Inoculum was prepared by grinding PVY^O-UK-infected leaves (tobacco or Pito) in distilled water at 1 g / 3 ml. Inoculated leaves were tested by ELISA 14 dpi. Noninoculated, upper leaves of the rootstock and the leaves of the scion grafted on the top were tested by ELISA 35 dpi. Similar experiments have been previously carried out with the ASR lines (Mäki-Valkama et al. 2000).

For experiments with different PVY isolates to determine the specificity of resistance, three plants from each transgenic and control potato line were propagated by taking cuttings in the greenhouse. Plants were sap inoculated with PVY^O-Loimaa, PVY^O-803, PVY^O-Viikki, PVY^N-RUS, or PVY^N-UK. Systemic infection was tested in the uppermost fully expanded leaves by double antibody sandwich (DAS)-ELISA at 14, 21, and 28 dpi as previously described (Pehu et al. 1995). Leaves were weighed, then ground in ELISA sample buffer at 1 g / 3 ml, and aliquots of sap (100 µl) transferred to two wells of a microtiter plate (Greiner Labortechnik, Frickenhausen, Germany) coated with polyclonal antibodies to PVY (Boehringer Mannheim, Mannheim, Germany).

Southern analysis.

Total DNA was extracted from potato leaves as described by Pehu et al. (1995). Extracted DNA was digested with a restriction enzyme (*Eco*RI, *Hind*III, or *Pst*I). The restriction sites of these enzymes within the transferred DNA are shown in Figure 1A. Digested DNA samples were separated in an agarose gel and transferred onto a nylon membrane (Boehringer Mannheim) (Sambrook et al. 1989). The blot was prehybridized at 50°C for 2 h and hybridized at 50°C in a DIG Easy hybridization solution (Boehringer Mannheim) for 16 h with a digoxigenin-dUTP labeled RNA probe (50 ng/ml) (DIG RNA Labeling Kit; Boehringer Mannheim) specific to the P1 coding sequence of PVY^O-UK. After the hybridization, the blots were washed twice at room temperature (5 min) in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)+0.1% SDS (sodium dodecyl sulfate) and twice (15 min) at 65°C in 0.5× SSC + 0.1 % SDS. Chemiluminescent detection

with a CDP-Star substrate was carried out according to the manufacturer's instructions (Boehringer Mannheim).

Northern analysis.

Total RNA was extracted (RNeasy Kit; Qiagen, Hilden, Germany), and 10-µg samples of the extracted RNA were run in a formaldehyde-containing agarose gel and blotted onto a nylon membrane (Boehringer Mannheim). The blot was pre-hybridized for 2 h and hybridized for 16 h at 68°C in a DIG Easy hybridization solution with a digoxigenin-dUTP labeled RNA probe (50 ng/ml) (DIG RNA Labeling Kit; Boehringer Mannheim) specific to the P1 coding sequence of PVY^O-UK. After hybridization, the blot was washed twice at room temperature (5 min) in 2× SSC + 0.1% SDS and twice (15 min) at 68°C in 0.2× SSC + 0.1% SDS. Chemiluminescent detection of the hybridized fragments was as indicated in the manufacturer's instructions (Boehringer Mannheim).

Western analysis.

Potato leaves were ground in an extraction buffer (50 mM Tris-Cl pH 7.5, 10 mM KCl, 20% glycerol, 5 mM MgCl₂, 5 mM PMSF (phenylmethylsulfonyl fluoride), 5 mM DTT (dithiothreitol), 0.072% β-mercaptoethanol) (1:10 vol/vol), mixed with an equal volume of the sample buffer (Sambrook et al. 1989) and 10-µl samples were run in a 12% SDS polyacrylamide gel at 100 V (Sambrook et al. 1989). Proteins were transferred onto a PVDF (polyvinylidene difluoride) membrane (Immobilon-P; Millipore, Bedford, MA) with a semi-dry blotting system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions, and detected with the ECL system (Amersham, Arlington Heights, IL). The P1 protein was detected with the anti-P1 rabbit antibodies (Arbatova et al. 1998) provided by T. Pehu, Department of Plant Production, University of Helsinki (dilution 1:2500). Anti-rabbit Ig coupled with a horseradish peroxidase linked F(ab')₂ fragment (from donkey) (Amersham) was used as the second antibody (dilution 1:5000).

Cloning of P1 genes from PVY.

Total RNA was extracted from PVY-infected leaves of cv. Pito as described by Verwoerd et al. (1989) and using RNeasy Kit (Qiagen). RT (reverse transcription)-PCR was carried out with the Titan One-Tube RT-PCR system (Boehringer Mannheim) with the primers specific to the PVY P1 gene (Pehu et al. 1995). The primers are designed to the 5' end and 3' end of the P1 transgene that lacks 24 nucleotides from the 3' end of the P1 gene of PVY^O-UK. Amplified fragments were cloned into pCR-Script plasmid (Stratagene, La Jolla, CA) and the nucleotide sequences were determined by the cyclic DNA sequencing method at the A. I. Virtanen Institute, University of Kuopio, Finland. Two independent clones from each PVY isolate were sequenced to both directions. The P1 gene sequences were deposited to a sequence data bank (Table 2).

Phylogenetic analysis of the P1 gene sequences.

The P1 gene nucleotide sequences were analyzed with the computer programs of the Wisconsin package version 9.1. Multiple sequence alignments were done with the PileUp program. A tree was constructed from the multiple sequence alignments with the PAUP program (Sinauer Associates, Sunderland, MA), based on the Kimura nucleotide distance matri-

ces and the neighbor-joining method. Bootstrap analysis with 100 bootstrap replications was done with the same program.

Analysis of amounts of viral RNA in potato cv. Pito infected with PVY^N or PVY^O.

Plants were grown from tubers infected with PVY^O-UK, PVY^N-UK, or PVY^N-RUS (secondary infection). Alternatively, plants were sap inoculated with PVY^O-UK, PVY^O-803, PVY^O-Viikki, PVY^O-Loimaa, PVY^N-UK, or PVY^N-RUS and RNA was extracted from the upper systemically infected leaves 21 dpi (primary infection). Northern analysis, hybridization, and detection were carried out as described above. The level of sequence difference (1 to 4%) between the P1 gene sequences of the PVY isolates does not affect the ability of the PVY^O-UK specific probe for the P1 gene to detect the P1 sequences of the PVY isolates.

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