Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7089–7093, August 1993
Genetics

Broad-spectrum virus resistance in transgenic plants expressing pokeweed antiviral protein

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Communicated by Myron K. Brakke, April 12, 1993

ABSTRACT **Exogenous application of pokeweed antiviral protein (PAP), a ribosome-inhibiting protein found in the cell walls of *Phytolacca americana* (pokeweed), protects heterologous plants from viral infection. A cDNA clone for PAP was isolated and introduced into tobacco and potato plants by transformation with *Agrobacterium tumefaciens*. Transgenic plants that expressed either PAP or a double mutant derivative of PAP showed resistance to infection by different viruses. Resistance was effective against both mechanical and aphid transmission. Analysis of the vacuum infiltrate of leaves expressing PAP showed that it is enriched in the intercellular fluid. Analysis of resistance in transgenic plants suggests that PAP confers viral resistance by inhibiting an early event in infection. Previous methods for creating virus-resistant plants have been specific for a particular virus or closely related viruses. To protect plants against more than one virus, multiple genes must be introduced and expressed in a single transgenic line. Expression of PAP in transgenic plants offers the possibility of developing resistance to a broad spectrum of plant viruses by expression of a single gene.**

Ribosome-inhibiting proteins (RIPs), which have been isolated from many different plant species, inactivate eukaryotic ribosomes. There are two classes: type I RIPs have a single polypeptide chain, and type II RIPs have two polypeptides, an A (active) chain and a B chain, which is a galactose-binding lectin (1). RIPs deglycosylate a specific base in the 28S rRNA and prevent binding of elongation factor 2. Type I RIPs, which do not possess a binding domain, do not bind easily to cells and consequently have a relatively low mammalian cytotoxicity (1).

RIPs have been of considerable interest recently due to their therapeutic potential as chimeric toxins, which can be targeted to a particular cell type, such as a cancer cell (1). In addition, type I RIPs and the A chains of type II RIPs have antiviral activity. Recently, it has been reported that several RIPs inhibit the replication of human immunodeficiency virus 1 in T cells and macrophages *in vitro* (1).

Three distinct antiviral proteins with similar activities have been identified in pokeweed (*Phytolacca americana*). PAP, PAPII, and PAP-S are the forms of pokeweed antiviral protein (PAP) that appear in spring leaves, summer leaves, and seeds, respectively (2–4). They are active against plant and animal viruses (5, 6). Exogenous application of small amounts of PAP to the surface of plant leaves completely prevents mechanical transmission of unrelated viruses to several different host plants (5). PAP is stored in the cell wall matrix of leaf mesophyll cells and can readily be obtained from water extracts of macerated leaf tissue (7).

The fact that PAP is a general inhibitor of virus infection makes it an ideal candidate for developing virus-resistant plants. We cloned the cDNA encoding PAP from pokeweed leaves and used it to transform tobacco and potato plants. We show that expression of PAP confers resistance to unrelated viruses. Virus resistance, previously observed in transgenic plants expressing coat protein genes or the read-through component of the tobacco mosaic virus replicase gene, has been specific for the virus from which the genes are derived or closely related viruses (8, 9). The results of the experiments described here provide a way of producing transgenic plants that may be resistant to a broad spectrum of plant viruses.

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MATERIALS AND METHODS

Isolation of a PAP cDNA Clone. A probe to identify the cDNA for the PAP gene was made by using PCR to amplify the DNA that encodes the first 30 amino acids of the mature protein (10). The template DNA in the reaction was cDNA made from poly(A)$^+$ RNA from pokeweed leaves and a 5′ primer (5′-GGGGTCTAGAATTCGTNAAYACNATHAT-HTAYAAYGT) that was designed to match the sense strand of putative DNA sequence encoding amino acids 1–8 and a 3′ primer (5′-GGRTCYTTYGCYTCRTT) that was designed to match the antisense strand of the putative DNA sequence encoding amino acids 25 through 30, where N = A, G, C, or T; Y = T or C; R = G or A; and H = A, C, or T. A 100-bp fragment obtained from PCR was used to isolate the 5′ half of the coding sequence from a λZAP library (Stratagene), and this fragment of the gene was then used to isolate the remainder of the coding sequence from a separate cDNA library.

Construction of Plant Transformation Vectors. PCR was used to engineer convenient restriction sites (*Bgl* II at the 5′ end and *Sma* I at the 3′ end) into the full-length cDNA encoding PAP for inserting into plant transformation vectors. pMON8443 has the PAP gene expressed from the enhanced 35S RNA promoter from cauliflower mosaic virus (CaMV E35S) (11) and pMON8484 has the PAP gene expressed from the 35S RNA promoter from figwort mosaic virus (FMV) (12). pMON8442 has the variant PAP gene expressed from the 35S RNA promoter from FMV (12).

Plant Transformations. *Agrobacterium tumefaciens* containing the plant transformation vectors was used to transform tobacco (*Nicotiana tabacum* cv. Samsun and *Nicotiana benthamiana*) by the leaf disc method (13) and potato (*Solanum tuberosum* cv. Russet Burbank) by transformation of stem sections (14).

Abbreviations: RIPs, ribosome-inhibiting proteins; PAP, pokeweed antiviral protein; NPTII, neomycin phosphotransferase II; GUS, β-glucuronidase; BMV, brome mosaic virus; PVX and PVY, potato viruses X and Y; PLRV, potato leafroll virus; CMV, cucumber mosaic virus.

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Virus Resistance Analysis. Leaf samples from transgenic plants were analyzed for expression of PAP by ELISA. One disc (15 mm in diameter) was sampled from each plant and homogenized in 500 μl of phosphate-buffered saline (PBS), 0.05% Tween-20, and 0.2% ovalbumin (PBSTO). Plates were coated with 1:1000 dilution of a 1 mg/ml solution of anti-PAP IgG. The plant extract (250 μl) was added and the plates were incubated overnight at 4°C. The bound protein was detected with alkaline phosphatase-conjugated anti-PAP IgG (1:5000). To estimate virus levels in plants, two discs were sampled from the second systemically infected leaf at various times after inoculation and antigen levels were determined by ELISA as previously described (15).

RIP Activity of PAP and Variant PAP. Leaf disks from *N. tabacum* plants expressing PAP or variant PAP were ground in PBS and spun for 5 min in a microcentrifuge. Several dilutions of the extracts were made and the amount of PAP in each dilution was measured by ELISA and by Western blotting. Two and one-half microliters of each extract dilution was added to a modified rabbit reticulocyte lysate. Titrations were done to determine lysate conditions which were saturating for amino acids and RNA but were limiting for ribosomes. The modified lysate contained 20 μl of lysate (Promega), 15 μl of the S-100 fraction of the same lysate, 5.7 μl of H_2O, 1 μl of a mixture containing each amino acid (minus methionine) at 1 mM, 0.8 μl of ^{35}S-labeled methionine (Amersham), and 4.0 μl of brome mosaic virus (BMV) RNA at 0.25 mg/ml (added after the PAP containing extract). After 30 min at 30°C, 5-μl aliquots were spotted onto filter paper and washed in 5% trichloroacetic acid, ethanol, and ether, and their radioactivities were measured.

Vacuum Infiltration. Two leaves were excised from each plant, rinsed in water, and vacuum infiltrated, using 100 mM sodium citrate buffer, pH 5.0. Intracellular fluid was recovered by centrifugation (16). Protein concentration was determined by using the Pierce BCA assay according to the manufacturer's instructions. PAP and neomycin phosphotransferase II (NPTII) levels were determined in the vacuum infiltrate and the whole tissue by ELISA. Plates were coated with 1:1000 dilution of anti-PAP IgG at 1 mg/ml or anti-NPTII IgG at 1 mg/ml, and 250 μl of each extract was loaded on ELISA plates. The bound protein was detected with either alkaline phosphatase-conjugated anti-PAP IgG (1:5000) or horseradish peroxidase-conjugated anti-NPTII IgG (1:6000).

RESULTS AND DISCUSSION

Exogenously applied PAP protected tobacco and potato plants from mechanical inoculation by potato virus X (PVX) or potato virus Y (PVY), but it did not protect potato plants against aphid transmission of PVY or potato leafroll virus (PLRV) (Table 1). It has been proposed that exogenously applied PAP enters a damaged cell along with the invading virus and inhibits translation of viral gene products (17). If this is the mechanism, PAP applied to the surface of a leaf may not be sufficient to enter cells during aphid feeding.

The gene encoding PAP was isolated by probing a cDNA library made from RNA from pokeweed leaves, with a 90-bp PCR-derived fragment from the coding sequence of the first 30 amino acids. This gene encodes a 313-amino acid protein, which has the same amino acid sequence as a PAP gene recently reported (18). The first 22 amino acids presumably contain the signal sequence required to translocate the protein to the cell wall, where it has been localized in pokeweed, and is not present in the mature protein (18). The coding sequence for the entire preprotein was cloned in plant transformation vectors, using PCR to introduce convenient restriction sites (10). During this process, a variant PAP gene was created which had three nucleotide changes that resulted in two amino acid changes in the mature protein. Leucine at position 20 of the mature protein was changed to arginine and tyrosine at position 49 was changed to histidine. Fig. 1 shows the maps of the transformation vectors and the two amino acid changes of the variant PAP. Tobacco leaf disks and potato stem sections were transformed by cocultivation with *A. tumefaciens* containing the binary plant transformation vectors (14, 15). Regenerated plants were screened by ELISA for expression of NPTII and PAP. Western blots of transgenic tobacco and potato plants, expressing PAP or variant PAP, showed a band which comigrated with PAP from pokeweed (data not shown). Transgenic *N. tabacum* plants which accumulated high levels (above 10 ng/mg protein) of wild-type or variant PAP tended to have a stunted, mottled phenotype, which was related to the level of PAP expressed. The plants that accumulated the highest levels of wild-type or variant PAP were sterile. *N. tabacum* plants that had lower levels (1–5 ng/mg protein) of wild-type PAP were fertile and normal in appearance. Similarly, transgenic *N. benthamiana* lines that accumulated 1–5 ng of variant PAP per mg of protein were phenotypically normal and fertile.

N. tabacum transformation frequencies, defined as the number of transgenic plants obtained per initial leaf disk, times 100, were greatly reduced for pMON8443 (0.7%) and

Table 1. Protection against virus infection with exogenously applied PAP

Virus	Group	Host	% infected: Without PAP	% infected: With PAP
		Mechanically transmitted*		
PVX	Potexvirus	*N. tabacum*	100	0
PVX	Potexvirus	*S. tuberosum*	100	0
PVY	Potyvirus	*N. tabacum*	100	0
PVY	Potyvirus	*S. tuberosum*	30	0
		Aphid transmitted†		
PVY	Potyvirus	*S. tuberosum*	80	60
PLRV	Luteovirus	*S. tuberosum*	80	85

*PAP from seeds was obtained from Calbiochem. Six to 20 plants were inoculated on two leaves per plant with PVY at 20 μg/ml or PVX at 5 μg/ml, in the absence of PAP or in its presence at 1 μg per leaf. Two weeks after inoculation, each plant was analyzed by ELISA (16).

†Five aphids, which were fed for 1–2 min on a PVY-infected plant, were transferred to each potato plant after the leaves had been coated with PAP (15 μg per leaf). For PLRV experiments, 10 viruliferous aphids were transferred to each potato plant after the leaves had been coated with PAP (15 μg per leaf). Aphids were killed 5 days later and plants were analyzed by ELISA 4 weeks after inoculation.

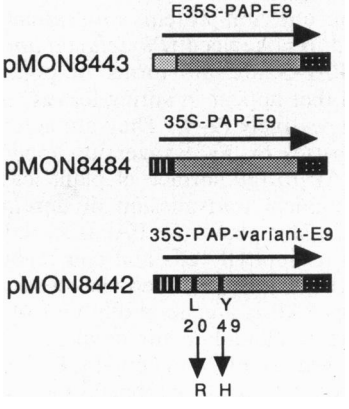

FIG. 1. Plant transformation vectors. Three plant transformation vectors containing the PAP gene were used to transform tobacco and potato plants. The amino acid changes in the variant PAP gene at residues 20 and 49 are shown below the diagram.

pMON8484 (0%), which contains the wild-type PAP gene, and somewhat reduced for pMON8442 (3.7%), which contains the variant PAP, as compared with other vectors without the PAP gene (7–18%). Similarly, transformation frequencies of *N. benthamiana* were reduced with pMON8443 (1.0%), but not with pMON8442 (8.6%), as compared with vectors without the PAP gene (7–18%). This result was confirmed in potato, where only one transgenic plant which expressed the variant PAP was obtained and no plants which expressed the wild-type PAP were obtained. To further test the transformation efficiencies of these vectors, a β-glucuronidase (GUS) gene containing an intron to prevent expression in *A. tumefaciens* (19) was inserted into the vectors. Leaf disks were squashed and stained for GUS activity at two times after cocultivation (20). Blue spots indicative of transformed tissue were counted, and the results (Fig. 2) showed that fewer spots were obtained with vectors containing the PAP genes than with the vector alone. The spots on the PAP-transformed disks were also much smaller than those on discs transformed with vector alone. These data are consistent with a reduction in either the number or the recovery of transformation events when PAP is expressed, as well as an inhibition of growth of transformed tissue expressing PAP. The inherent difficulty in introducing the PAP gene into a heterologous plant is likely due to its potent RIP activity. PAP is an abundant protein in pokeweed and is active on pokeweed ribosomes *in vitro* (21). Pokeweed may protect its own ribosomes either by compartmentalizing the mature PAP to the cell wall (7) or by some other as-yet-unidentified mechanism. A direct comparison of identical vectors containing the wild-type PAP (pMON8484) or the variant PAP (pMON8442) in the various transformation assays suggests that it is more difficult to transform plants with the wild-type PAP than with the variant PAP. The most obvious explanation for the differences in the transformation frequencies is that the variant PAP is not as potent a RIP as the wild-type PAP.

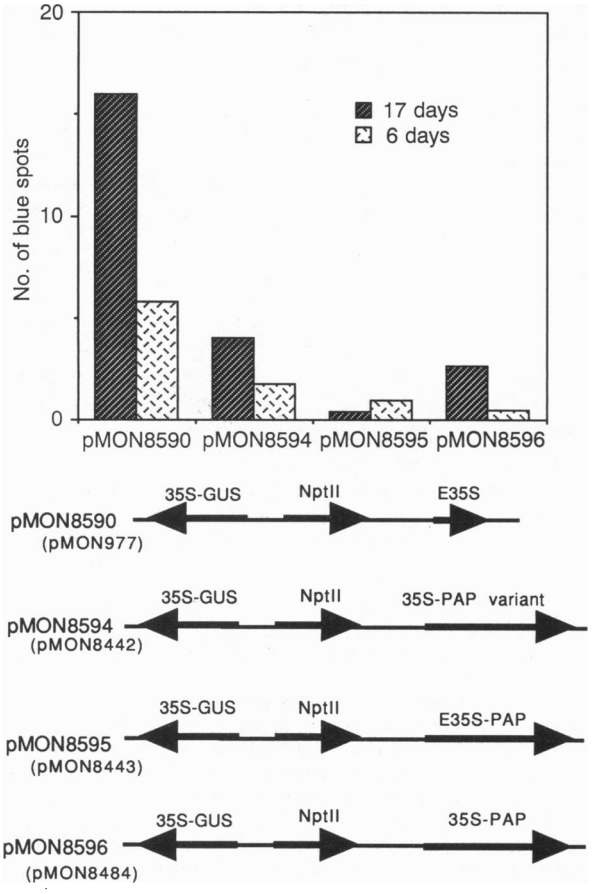

FIG. 2. GUS expression during the transformation process. A chimeric gene consisting of the coding region of the GUS gene containing an intron to prevent its expression in *A. tumefaciens* (19) was introduced into the plant transformation vectors whose numbers are shown in parentheses. A schematic drawing of the GUS vectors is shown below the histogram. *A. tumefaciens* carrying these vectors were cocultivated with tobacco leaf disks. At 6 days and 17 days after cocultivation, 10 leaf disks per vector were squashed and stained for GUS activity (20). Blue spots were counted, and the average number of spots per disk is shown.

The RIP activities of wild-type and variant PAP were tested in an *in vitro* rabbit reticulocyte translation assay. Leaf disks from tobacco plants expressing similar levels of PAP or variant PAP were ground in buffer and various dilutions were added to a modified rabbit reticulocyte lysate containing [^{35}S]methionine and BMV RNA. The activities of the variant PAP expressed in tobacco, wild-type PAP expressed in tobacco, and wild-type PAP isolated from pokeweed and spiked into nontransgenic tobacco extract were indistinguishable from each other (Fig. 3). Similar dilutions of untransformed tobacco extract were added to lysates as a control. The most concentrated dilution had a slight effect on translation, but the rest of the dilutions had no effect.

Experiments using β-globin mRNA as the translation template had similar results (data not shown). These results suggest that the difference in transformation frequency between vectors containing wild-type or variant PAP is not due to inherent differences in RIP activity.

To determine whether transgenic seedlings could suppress viral infection, R_1 (first recombinant) progeny expressing PAP or the variant PAP were challenged with three unrelated viruses: PVX, which is the type member of the potexvirus group, PVY, which is the type member of the potyvirus group, and cucumber mosaic virus (CMV), which is the type member of the cucumovirus group. These are all RNA viruses and share no nucleic acid homology or structural similarity.

ELISA analysis of R_1 progeny from self-fertilized plants of five different *N. tabacum* lines showed that progeny of each line were segregating for PAP expression. Progeny of *N. tabacum* lines that express PAP and rooted cuttings from transgenic *S. tuberosum* line 555 were inoculated with PVX

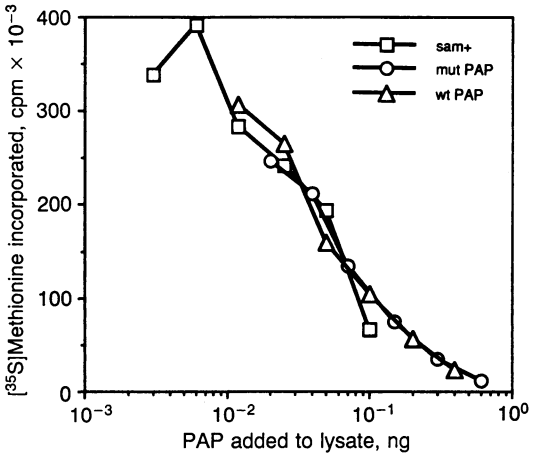

FIG. 3. Comparison of RIP activity of wild-type and variant PAP. The amount of PAP or variant PAP in each dilution was calculated from the amount in the original extract and plotted against the cpm of [^{35}S]methionine incorporated into protein in the translation assay. Each point represents three lysate reactions, each assayed in triplicate. sam+ = the nontransgenic *N. tabacum* extract spiked with known amounts of PAP isolated from pokeweed; mut PAP = transgenic *N. tabacum* expressing the variant PAP; and wt PAP = transgenic *N. tabacum* expressing the wild-type PAP.

at 1 and 5 μg/ml, respectively. R_1 progeny of untransformed tobacco plants and rooted cuttings of untransformed potato plants served as controls. Table 2 shows that the transgenic lines 33617 and 31634, expressing wild-type PAP, had fewer lesions on their inoculated leaves than the tobacco control plants, suggesting that there were fewer initial infection events. However, once a lesion was formed, the plant became infected systemically, indicating that PAP inhibits viral infection at a very early stage. It was difficult to count lesions on the inoculated leaves of the plants expressing the variant PAP because of the mottled appearance of these leaves. ELISA analysis of upper leaves demonstrated that plants from transgenic lines 29509, 26139, 29472, and 555, which contain the variant PAP, and lines 33617 and 31634, which contain the wild-type PAP, showed a significant reduction in incidence of PVX infection compared with controls (Table 2). Viral antigen levels were similar in infected transgenic plants and in infected controls (data not shown). PVX experiments were repeated three times, and all transgenic lines tested showed resistance even when 100% of the untransformed control plants were infected with PVX.

Some of the plants from line 29509 that expressed low levels of PAP looked like healthy control tobacco plants, while the high expressors were mottled and stunted. All of these plants were resistant to PVX infection. Plants from lines 33617 and 31634 that expressed low levels of wild-type PAP and plants from transgenic potato line 555 that expressed the variant PAP were indistinguishable from healthy plants as judged by growth rates, physical appearance, and fertility. These results indicate that the PVX resistance observed in lines 29509, 33617, 31634, and 555 is due to expression of PAP. Transgenic plants were observed up to 8 weeks after inoculation. The plants that did not show symptoms or accumulate virus at 2 weeks after inoculation did not show symptoms or accumulate virus at 8 weeks after inoculation.

Self-fertilized R_1 progeny from five different independently transformed *N. tabacum* lines and one transgenic *N. benthamiana* line that expressed PAP and rooted cuttings from potato line 555 were inoculated with PVY at 5 and 20 μg/ml, respectively. In addition to wild-type tobacco and potato controls, progeny of transgenic *N. tabacum* lines that did not accumulate detectable levels of PAP (below 0.2 ng/mg of protein) were included as controls. As shown in Table 2, plants from each control line were 100% infected with PVY. All of the transgenic lines tested, except for line 31634, were highly resistant to mechanical transmission of PVY, and three transgenic *N. tobacum* lines, 26139, 29472, and 33617, were resistant to aphid transmission as well. Of the two lines that expressed similar levels of the wild-type PAP, 33617 and 31634, one was highly resistant to PVY and the other line was susceptible. PVY experiments were repeated twice, and in both tests plants from lines 29509, 26139, 29472, and 33617 were resistant to PVY and plants from line 31634 were susceptible. Transgenic tobacco lines 33617 and 30230 and the potato line 555 that expressed PAP and looked normal were resistant to PVY, while the transgenic lines that did not express PAP and the wild-type lines were highly

Table 2. Susceptibility of transgenic tobacco and potato plants to infection by PVX, PVY, and CMV

Vector/line	PAP,* ng/mg	Phenotype†	PVX‡ No. of lesions	PVX‡ % infected	CMV,§ % infected	PVY (mechanical),¶ % infected	PVY (aphid),‖ % infected
N. tabacum							
Wild type	0	Normal	8.2 ± 5.75	85	100	100	69
8443/31635	0	Normal	—	—	—	100	—
8443/31636	0	Normal	—	—	—	100	—
8442/29509	9.0 ± 2.2	Mixed	ND	0***	33***	0***	—
8442/26139	10.2 ± 1.0	Mottled	ND	42***	79	0***	12***
8442/29472	12.4 ± 1.8	Mottled	ND	0***	33***	0***	0***
8443/33617	1.6 ± 1.0	Normal	1.0 ± 1.0	36***	100	0***	22**
8443/31634	1.6 ± 1.6	Normal	0.2 ± 1.0	36***	100	100	—
N. benthamiana							
Wild type	0	Normal	—	—	—	90	—
8442/30230	3.2 ± 3.0	Normal	—	—	—	30***	—
S. tuberosum							
Wild type	0	Normal	—	86	—	86	41
8442/555	3.6 ± 1.1	Normal	—	14***	—	14***	24

*Eleven to 34 plants from R_1 progeny of each tobacco line were analyzed by ELISA for expression of PAP. Mean values ± SD are shown, expressed as ng of PAP per mg of total plant protein.

†Phenotype of each transgenic line is indicated. Some plants from line 29509 were mottled, others were normal.

‡Ten to 20 plants expressing PAP from R_1 progeny of each transgenic tobacco line and 20 wild-type tobacco plants were inoculated with PVX at 1 μg/ml. Twelve days after inoculation, systemically infected leaves were sampled from each plant and PVX levels were determined by ELISA. Seven plants propagated as cuttings from transgenic potato line 555 and seven control potato plants were inoculated with PVX at 5 μg/ml. Twenty-seven days after inoculation, PVX levels were determined by ELISA in each plant. Numbers of lesions indicated as ND (not determined) could not be determined due to mottled appearance of tobacco leaves. χ^2 analysis for differences between percentages (22) was used to determine if resistance in transgenic lines was significantly different from the controls. Levels of significance are indicated by footnotes.

§Twelve plants expressing PAP from R_1 progeny of each line were challenged with CMV (Italian strain) at 50 μg/ml. Ten days after inoculation the second systemically infected leaf was sampled and CMV levels were determined by ELISA in each plant.

¶Ten to 20 plants expressing PAP from R_1 progeny of each tobacco line, 10 plants from progeny of transgenic lines that did not express PAP, and 10 wild-type tobacco plants were inoculated with purified PVY at 5 μg/ml. Fifteen days after inoculation, the second systemically infected leaf was sampled from each plant, and PVY levels were determined by ELISA. Seven plants propagated as cuttings from transgenic potato line 555 and seven control potato plants were inoculated with PVY at 20 μg/ml. Twenty-seven days after inoculation, PVY levels were determined by ELISA.

‖Five green peach aphids that had been allowed to probe a PVY-infected tobacco plant for 5 min were placed on 6–17 transgenic and 13 control plants and allowed to feed for 1 hr. The aphids were then killed by an insecticide and PVY levels were determined by ELISA at 22 days after inoculation.

**Significantly different from control at 5% level.

***Significantly different from control at 1% level.

susceptible, indicating that PAP expression is responsible for resistance.

Progeny of two independently transformed *N. tabacum* lines, 29509 and 29472, which were resistant to mechanical inoculation of PVX and PVY were also resistant to mechanical inoculation of CMV (Table 2). Progeny of lines 26139, 33617, and 31634 were not resistant to the highly infectious Italian strain of CMV used in these experiments. Since these lines were only partially resistant to PVX, the high levels of CMV inoculum used might have overcome the resistance. CMV inoculation experiments were repeated twice, and progeny of lines 29509 and 29472 were resistant in each test.

Although expression of PAP is essential for resistance, we did not observe a good correlation between the level of expression of PAP and the level of resistance to viral infection. This observation is typical of other virus-resistance studies with transgenic plants expressing viral coat protein (15, 23) or replicase genes (24, 25), and it suggests that in addition to level of expression, other unknown factors might be critical for resistance.

We do not yet understand the mechanism of PAP-mediated resistance. One possibility is that PAP enters the host cell along with the virus and prevents translation of viral RNA. Alternatively, PAP might be binding to the virus or to a component of the cell wall and preventing virus from entering cells. These mechanisms would imply that PAP in transgenic plants, as in pokeweed, is localized in the cell wall.

To determine if PAP and the variant PAP are enriched in the apoplastic space, vacuum infiltration was used to recover intercellular proteins from transgenic *N. tabacum* plants (16). The selectable marker NPTII, which is encoded by the same vector as PAP, was used as the cytoplasmic marker. Buffer was vacuum infiltrated into the apoplastic space of tobacco plants expressing the variant PAP. The levels of PAP and NPTII were measured by ELISA in the infiltrate and in intact tissue (Table 3). The high ratio of PAP to NPTII in the infiltrate compared with the low ratio in the whole tissue indicates that the PAP is enriched in the intercellular fluid.

Thus, during viral infection PAP could enter the cell along with the virus. Once internalized it could inactivate ribosomes and cause inhibition of viral protein synthesis.

Low levels of PAP expression are needed to obtain virus-resistant plants that are phenotypically normal. Tissue-specific expression of PAP may be required for resistance to different viruses. Mechanical transmission of a virus probably occurs in the cells close to the surface, whereas aphid transmission can occur in the epidermal layer for nonpersistent transmission of viruses such as PVY or in the vascular layer for persistent transmission of viruses such as PLRV. It is possible that protection against certain viruses could be enhanced by targeting the expression of PAP to specific tissues. Further studies on PAP will contribute to a better understanding of the mechanism of its antiviral action and facilitate development of broad spectrum virus-resistant plants.

Table 3. PAP and NPTII levels in apoplastic space vs. intact tissue

	Infiltrate			Whole tissue		
Plant no.	PAP, ng/mg	NPTII, ng/mg	PAP/ NPTII	PAP, ng/mg	NPTII, ng/mg	PAP/ NPTII
29509-3	2.4	0.24	10.0	0.3	0.92	0.3
29491-42	6.5	0.2	32.5	3.6	2.7	1.3
29472-49	4.0	0.6	6.6	2.5	1.6	1.56
26139-38	2.8	0.4	7.0	2.4	1.1	2.2

One leaf was excised from each plant containing pMON8442 and vacuum infiltrated (16). PAP and NPTII levels were determined in the vacuum infiltrate and the whole tissue by ELISA. Levels of PAP or NPTII are expressed as ng of PAP or NPTII per mg of total protein.

We acknowledge Keith O'Connell for purification of PAP from pokeweed leaves, Jerry Anderson and Leslie Wilson for assistance with PAP expression analysis, Tim Coombe for growth and maintenance of plants, Dr. Larry Holden for statistical analysis, and Dr. Ganesh Kishore for helpful discussions and review of the manuscript.

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