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Extreme resistance to potato virus X infection in plants expressing a modified component of the putative viral replicase

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Three types of mutation were introduced into the sequence encoding the GDD motif of the putative replicase component of potato virus X (PVX). All three mutations rendered the viral genome completely non-infectious when inoculated into *Nicotiana clevelandii* or into protoplasts of *Nicotiana tabacum* (cv. Samsun NN). In order to test whether these negative mutations could inactivate the viral genome *in trans*, the mutant genes were expressed in transformed *N.tabacum* (cv. Samsun NN) under control of the 35S RNA promoter of cauliflower mosaic virus and the transformed lines were inoculated with PVX. In 10 lines tested in which the GDD motif was expressed as GAD or GED there was no effect on susceptibility to PVX. In two of four lines transformed to express the ADD form of the conserved motif, the F1 and F2 progeny plants were highly resistant to infection by PVX, although only to strains closely related to the source of the transgene. The resistance was associated with suppression of PVX accumulation in the inoculated and systemic leaves and in protoplasts of the transformed plants, although some low level viral RNA production was observed in the inoculated but not the systemic leaves when the inoculum was as high as 100 or 250 μg/ml PVX RNA. These results suggest for a plant virus, as reported previously for Qβ phage, that virus resistance may be engineered by expression of dominant negative mutant forms of viral genes in transformed cells.

Key words: GDD motif/RNA polymerase/transformed plants/virus resistance

Introduction

Several recent reports have described virus resistance resulting from transgenic expression of the putative RNA polymerase of plant RNA viruses (reviewed in Hull and Davies, 1992). The best characterized of these 'replicase-mediated resistance' effects is in tobacco transformed to express a modified form of the 54 kDa putative component of the tobacco mosaic virus (TMV) replicase in which there is extreme, but strain specific, resistance to TMV (Golemboski *et al.*, 1990). This resistance was expressed in protoplasts of the transformed plant (Carr and Zaitlin, 1991), implying that the effect operates at the level of RNA replication and can be mimicked, in part, in protoplasts expressing the viral gene from episomal DNA rather than from a transgene stably integrated into the plant genome (Carr *et al.*, 1992). Results from this transient expression system, with mutant versions of the viral gene, implicate the viral protein rather than the RNA in the resistance mechanism (Carr *et al.*, 1992). Essentially similar conclusions have been drawn from analysis of tobacco transformed to express various mutant forms of the 54 kDa homologue of pea early browning virus (PEBV) and which are consequently resistant to PEBV, although in this instance the resistance extended to broad bean yellow band virus (MacFarlane and Davies, 1992). In a further report of replicase mediated resistance, Braun and Hemenway (1992) described how transgenic expression of the intact gene encoding the putative replicase component of potato virus X (PVX) conferred resistance to PVX and how, at least in one transformed plant out of six tested, there was resistance associated with transgenic expression of the 5′ part of this gene.

However, transgenic expression of the putative replicase component of RNA viruses of plants does not always lead to resistance. In fact, with both alfalfa mosaic virus (Taschner *et al.*, 1991) and brome mosaic virus (Mori *et al.*, 1992), transgenic expression of the replicase components had a positive rather than a negative effect on virus accumulation in that there was complementation of mutations in the inoculated viruses.

Replicase-mediated resistance has also been reported from a prokaryotic system but with a modified subunit of the viral replicase: expression of the replicase component of Qβ phage in transformed *Escherichia coli* had little effect of susceptibility to Qβ unless the gene was modified in a region encoding the peptide motif Gly Asp Asp (GDD) (Inokuchi and Hirashima, 1987) which is ubiquitous in putative replicases of positive strand RNA viruses and present in a related form in other RNA polymerases (Kamer and Argos, 1984; Argos, 1988; Habili and Symons, 1989; Poch *et al.*, 1989). The GDD and similar motifs are thought to be associated with the catalytic site of the RNA polymerase (Lowe *et al.*, 1991). The resistance in transformed *E.coli* to Qβ phage was strongest when the glycine residue of the GDD motif was modified to alanine (Inokuchi and Hirashima, 1987). It was proposed that the dominant negative effect of the mutations was due to binding of the dysfunctional protein to a *cis*-acting element 5′ of the viral coat protein gene and that this binding would interfere with production of viral coat protein at the level of translation, with the arrest of phage multiplication being a secondary effect (Inokuchi and Hirashima, 1990).

There is no evidence for translational regulation of the PVX coat protein through the binding of replicase, but there are, in principle, several other routes through which dysfunctional replicase might interfere with PVX replication. For example, binding to the promoters for (+)- and (−)-

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strand RNA synthesis, which are assumed to include the 5′ and 3′ terminal sequences, might have a direct effect on the replication process. Binding of the dysfunctional protein might also have an indirect effect on virus accumulation by binding to and preventing expression from the promoter for production of the coat protein mRNA: coat protein production is a necessary requirement for efficient accumulation of PVX (+)-strand RNA and spread of PVX from the site of inoculation (Chapman *et al.*, 1992a). It is also possible, at least in principle, that a dysfunctional replicase could bind to and inactivate host components of a replicase complex. There is no direct evidence for host components of the PVX replicase but, for other plant viral replicases, it is thought that there are host proteins associated with the viral component (David *et al.*, 1992) and, in the instance of cucumber mosaic virus (CMV), there is some indication that the host component is necessary for activity of the replicase complex *in vitro* (Hayes and Buck, 1990). We have therefore introduced mutations into the 166 kDa protein of PVX, at the site of the GDD motif, in order to test whether dominant negative mutations can be used to obtain resistance in transgenic plants and analyse functions of the replicase. The phenotype of the mutations was first tested by inoculation of protoplasts with the mutant PVX genomes and subsequently by expression of the mutant forms of the gene encoding the 166 kDa protein in transformed tobacco.

Results

Mutations in the GDD-encoding motif of the 166 kDa protein inactivate the viral genome

Mutation of the viral replicase component in Qβ phage (Inokuchi and Hirashima, 1987) or the L-A double stranded RNA virus of yeast (Ribas and Wickner, 1992) at the region encoding the GDD motif, rendered the viral genome non-infectious. Similar mutations in the poliovirus replicase component reduced the activity *in vitro* to <20% that of a wild type protein (Jablonski *et al.*, 1991). To test the phenotype of similar mutations in PVX, the 166 kDa protein of PVX_{UK3}, a British isolate, was prepared as cDNA with the GDD motif intact or mutated (Figure 1a). The mutant forms had either conservative amino acid changes (GED or ADD) or a non-conservative change (GAD) and modified cDNAs were substituted into pTXS which includes a full length PVX_{UK3} cDNA (Figure 1b) from which infectious RNA could be produced by *in vitro* transcription (Kavanagh *et al.*, 1992). Inoculation of the transcripts, into either *Nicotiana clevelandii* plants or protoplasts from *Nicotiana tabacum* (cv. Samsun NN) showed that each of the mutations rendered the PVX non-infectious. The infected plants failed to accumulate viral RNA as detected by Northern analysis and did not show symptoms (data not shown). Similarly, Northern analysis of the infected protoplast failed to detect viral RNA (Figure 2) with autoradiographic exposure which would have detected accumulation of either the genomic or subgenomic mRNAs at <0.01% of the level in the samples inoculated with wild type viral RNA. These results indicated that PVX, like Qβ (Inokuchi and Hirashima, 1987) and L-A virus (Ribas and Wickner, 1992), but unlike poliovirus (Jablonski *et al.*, 1991), does not tolerate modifications to the GDD motif of the putative replicase component.

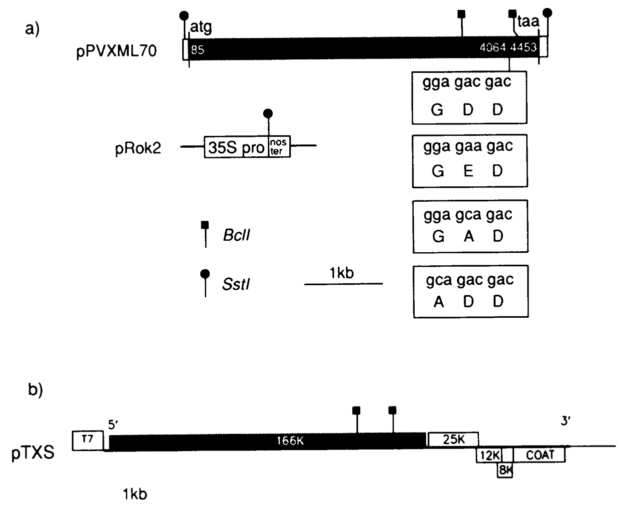

Fig. 1. PVX constructs in full length viral cDNA and the expression cassette of a transformation vector. The gene for the 166 kDa protein is shown diagrammatically in panel **a**; as a cDNA, it was mutated around position 4064 of the complete viral sequence according to the numbering of Huisman *et al.* (1988) and Skryabin *et al.* (1988). The cDNA sequences are indicated in lower case letters. The ultimate effect of the changes on the sequence of the 166 kDa protein is indicated in upper case letters. The mutant cDNAs were substituted into pPVXML70 for transfer as an *Sst*I fragment into the expression cassette of the binary transformation plasmid pRok2. The cassette comprised the 35S RNA promoter (35S pro) of CaMV and the transcriptional terminator (nos ter) of the nopaline synthase gene of *A.tumefaciens*. The mutated region was also transferred into the full length cDNA of PVX_{UK3} in plasmid pTXS (Kavanagh *et al.*, 1992) (panel **b**) using the *Bcl*I sites indicated. The diagram of pTXS illustrates the location of viral genes, identified by size or function (COAT) of the protein product and shows the promoter for T7 RNA polymerase (T7) for synthesis of RNA *in vitro*.

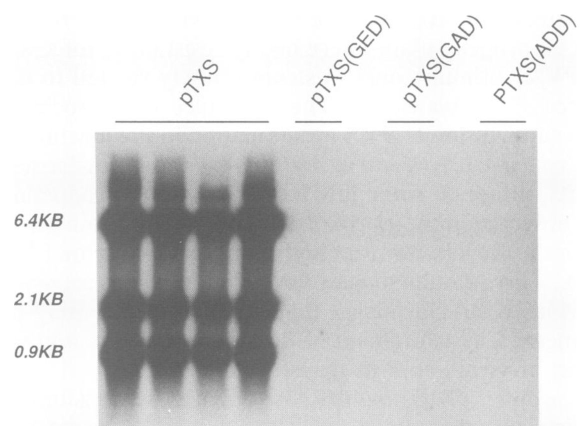

Fig. 2. Infectivity of mutant PVX in tobacco protoplasts. The *in vitro* transcripts of PVX cDNA clones (Figure 1) with either wild type (pTXS) (four samples) or mutant forms (two samples each) pTXS(GAD), pTXS(GED) or pTXS(ADD) were inoculated into protoplasts of *N.tabacum* (cv. Samsun NN) by electroporation. The figure shows Northern analysis of RNA extracted 24 h post-inoculation. In the RNA samples from protoplasts inoculated with wild type virus, these major RNA species were the genome RNA (6.4 kb) and two subgenomic mRNAs of 2.1 kb and 0.9 kb.

Expression of the PVX 166 kDa protein in infected and transgenic tobacco

An antiserum specific for the 166 kDa protein of PVX was prepared by inoculation of rats with a 166 kDa fusion

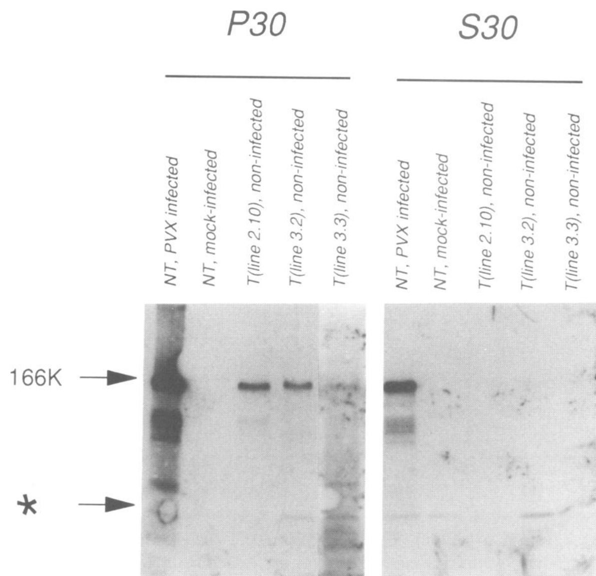

Fig. 3. Expression of mutant 166 kDa protein in transgenic plants. Tobacco plants were transformed with the derivatives of pRok2 containing the mutant forms of the gene for the 166 kDa protein. Leaf extracts of the plants were fractionated by centrifugation. The 1000 *g* pellet (P1), 30 000 *g* pellet (P30) and the 30 000 *g* supernatant (S30) fractions were analysed by Western blotting, using antisera prepared against the 166 kDa protein of PVX_{UK3} (M.Longstaff, unpublished). There was little or no signal from the P1 samples (not shown). For comparison, the analysis is also shown of the same fractions of non-transformed (NT) plants either mock inoculated or inoculated with PVX_{UK3}. The figure shows the analysis of transformed (T) lines 3.2 and 3.3 expressing the ADD mutation and line 2.10 expressing the GAD mutation. The other transformed lines expressing the mutant 166 kDa protein all produced the 166 kDa protein at levels higher than in line 3.3 or lower than in 3.2 (Table I). The immunoreactive protein with electrophoretic mobility predicted for the 166 kDa protein is indicated as 166K. A protein of 116 kDa (indicated as ★) was also detected by the pre-immune serum and is therefore not thought to have a viral origin. The immunoreactive proteins migrating between 166 and 116 kDa in the sample from the NT, PVX-infected plant may be degradation products of the 166 kDa protein, as they were present variably in different samples and were more prevalent in samples that had been stored. The track showing the P30 sample of the 3.3 plant is a longer exposure than for the other samples.

protein produced in *E.coli* transformed with a derivative of the plasmid pET (Stanley and Luzio, 1984) in which the gene encoding the 166 kDa protein was fused in the same reading frame as *lacZ*. Western analysis of this antiserum confirmed that infected plants contained a single major immunoreactive protein (Figure 3) of the predicted M_r, and that this protein fractionated by differential centrifugation in the 10 000 *g* supernatant (data not shown). Further fractionation of the 10 000 *g* supernatant into 30 000 *g* pellet (P30) and supernatant (S30) fractions produced a pellet enriched for the 166 kDa protein (Figure 3). However, allowing for the proportion of the two fractions analysed, we estimate that the protein partitioned equally between the P30 and S30 fractions.

The P30 was also the most enriched fraction for the 166 kDa protein in the transformed plants (Figure 3) although, because the levels of expression were much lower in the transformed than in the infected plants, it was not possible to determine the relative amounts of the protein in the P30 and S30 fractions or whether there were small amounts of 166 kDa protein in 1000 *g* and 10 000 *g* pellet fractions. The range of expression levels in the transgenic plants is illustrated in Figure 3 and in Table I there is a summary of the expression data with each of the transformed lines expressing the mutant forms of the 166 kDa protein.

Table I. Expression of the 166 kDa protein in transformed tobacco

Plant line	Mutation[a]	Level of 166 kDa protein[b]
1.4	GED	+
1.5	GED	++
1.7	GED	+++
1.8	GED	++
1.11	GED	++
1.13	GED	++
2.1	GAD	+++
2.4	GAD	+
2.5	GAD	++
2.6	GAD	+++
3.1	ADD	++
3.2	ADD	+++
3.3	ADD	+
3.8	ADD	++

[a]Indicates the sequence of the encoded peptide in the region of the GDD motif.
[b]The expression level indicated as + is the level shown by 3.3 in Figure 3 and the level +++ as shown by 3.2 in Figure 3.

PVX resistance in plants expressing the 166 kDa protein of PVX

The transgenic lines referred to in Table I were all tested for resistance to PVX infection by inoculation with an RNA extract of PVX-infected plants. The titre of the extract, based on lesion production on *Chenopodium amaranticolor*, was equivalent to an inoculum of 1 μg/ml PVX RNA. In the primary transformants, one of the lines (3.3) expressing the ADD form of the 166 kDa protein was completely resistant to infection, as assessed by symptom production and ELISA measurement of virus accumulation. In the selfed F1 progeny, with at least 40 plants of each transformed line being tested with an inoculum equivalent to 1 μg/ml PVX RNA, there was resistance in the 3.3 line and in a second line expressing the ADD mutation (line 3.1). There was no resistance in the 10 lines expressing the GAD or GED mutations or the two remaining lines expressing the ADD mutation of the 166 kDa protein (lines 3.2 and 3.8). The resistance in the 3.1 and 3.3 lines was subsequently characterized in detail by inoculation of more progeny plants and by analysis of PVX accumulation in the inoculated and systemic leaves.

The analysis of the 3.3 F1 progeny confirmed the extreme PVX resistance in this line: all of the transformed progeny were free of PVX symptoms on both inoculated and the systemic leaves, even 30 days post-inoculation, when the experiment was terminated (Figure 4a). The Northern analysis of the PVX RNA from these transformed plants showed that the absence of symptoms was due to suppression of virus accumulation. In the inoculated and systemic leaves of these plants there was $<0.01\%$ the level of PVX RNA in the inoculated leaf of a non-transformed plant (Figure 5a). The 3.3 plants were also resistant to high levels of purified

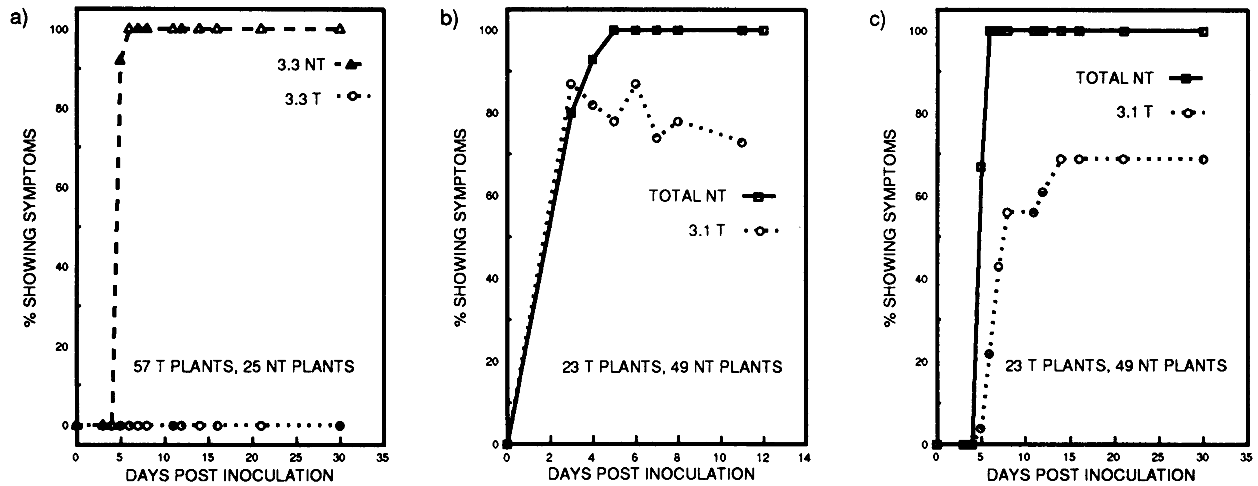

Fig. 4. PVX_{UK3} symptoms in transformed plants expressing the ADD variant of the 166 kDa protein. The F1 or F2 seedlings of transformed tobacco lines 3.1 or 3.3 were inoculated with PVX_{UK3} RNA and monitored for symptom production on the inoculated and systemically infected leaves. The data are presented separately for the transformed (T) and non-transformed (NT) progeny and the number of plants tested is indicated in each panel. Panel **a** illustrates the time course of systemic symptom development on 3.3 plants; panels **b** and **c** show the timing of symptom production on the inoculated (b) and systemic (c) leaves of the 3.1 plants. In panels b and c, the NT lines include NT progeny of lines other than 3.1 so that the analysis included a sufficiently large number of plants.

PVX RNA. In a test with 26 transformed plants inoculated with 250 μg/ml PVX RNA and 10 plants inoculated with 100 μg/ml PVX RNA, all of the plants were free of symptoms on both the inoculated and systemic leaves for 30 days post-inoculation, when the experiment was terminated. The Northern analysis confirmed the absence of viral RNA in the systemic leaves (Figure 5b) although in two out of three plants inoculated with 250 μg/ml PVX RNA and analysed by Northern blotting, there was accumulation of PVX RNA in the inoculated leaf (Figure 5b) at levels between 0.1% and 0.01% that in a non-transformed plant. Similarly, Northern analysis of four plants inoculated with 100 μg/ml PVX RNA showed two plants in which there was PVX RNA accumulation in the inoculated leaf at ~0.01% the level in a non-transformed plant, but no detectable accumulation in the systemic leaf (data not shown).

The results for symptom production on the 3.1 line are summarized in Figure 4b and c and show that there was symptom suppression on both the inoculated and systemic leaves in some of the transformed plants. This resistance was more pronounced on the systemic leaves, on which there was complete suppression of symptoms in ~25% of the transformed progeny from the F1 generation. A similar proportion was symptomatic, but displayed ameliorated symptoms, characterized by isolated chlorotic lesions rather than the confluent mosaic produced on the non-transformed progeny (Figure 6).

In contrast to the 3.3 progeny in which the transformed (neomycin phosphotransferase):non-transformed phenotype was inherited in a ratio of 3:1, there was ~15:1 inheritance (52:3) of the transformed phenotype in the F1 progeny of the 3.1 line, indicating that the primary transformant had two or more unlinked transgenic insertions. Consequently, it was possible that the partial resistance in this line was associated with only one of the transgenes which was absent from the susceptible progeny. However, it was also possible that incomplete penetrance of the resistance phenotype could also explain the partial resistance in the 3.1 plants. These alternatives were assessed by analysis of the selfed F2

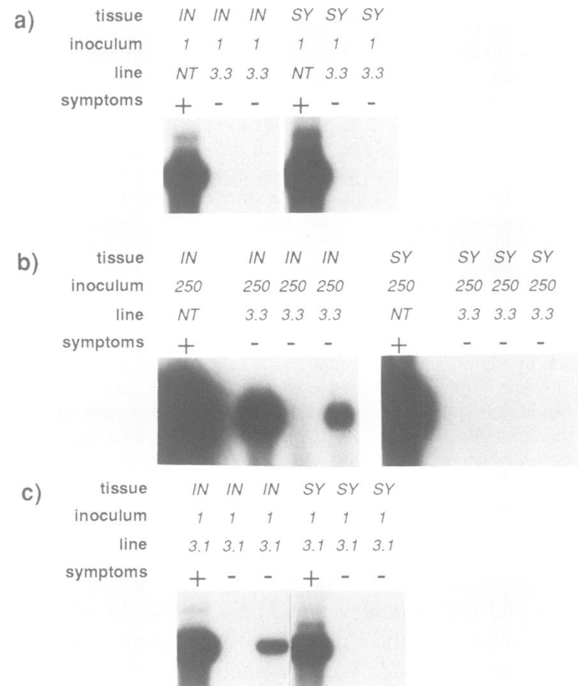

Fig. 5. Northern analysis of PVX RNA in inoculated or systemic leaves of the 3.3 or 3.1 transformed lines. RNA samples were isolated from the inoculated (IN) and systemic (SY) leaves of transformed (T) and non-transformed (NT) progeny of both 3.3 (panels **a** and **b**) and 3.1 (panel **c**) lines. Inoculated leaf samples were taken at 1 week and from the systemic leaves at 2 weeks post-inoculation with PVX_{UK3}. The inoculated and systemic leaf samples were taken from the same plants. Aliquots of 1 μg RNA were analysed by Northern blotting using a ^{32}P-labelled RNA probe specific for the 3′ end of the PVX_{UK3} genome and which detected viral RNA but not the RNA product of the transgene. The panel shows only the genomic length RNA on the autoradiograph: the sub-genomic RNAs were present only as minor components in the inoculated leaf samples and not detectably in the samples from systemic leaves. The sensitivity of the Northern analysis was assessed by analysis of RNA from infected leaves diluted in RNA from healthy leaves which showed that 10^{-4} the level (0.01%) of PVX RNA in non-transformed leaves was readily detectable. Panels a and c are from the same experiment and 2 h autoradiographic exposure. Panel b shows a 3 h exposure of the autoradiograph.

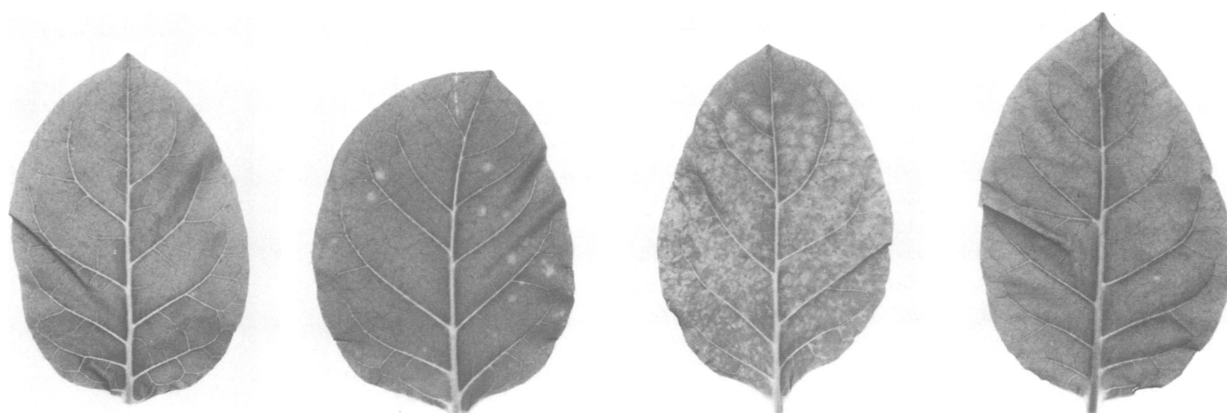

Fig. 6. Attenuated symptoms in the 3.1 line of transformed plants. The symptoms shown on systemic leaves of the PVX_{UK3}-infected T and NT progeny of line 3.1 are shown at 2 weeks post-inoculation. The examples shown include an NT plant with full symptoms of PVX, and T plants with either no systemic symptoms or attenuated symptoms. A mock inoculated plant is shown for comparison.

Table II. PVX infection of F1 progeny of tobacco lines 3.3 expressing the ADD variant of the PVX 166 kDa protein

Inoculum	Experiment 1		Experiment 2	
	Transformed[a]	Non-transformed[a]	Transformed[a]	Non-transformed[a]
PVX_{UK3} SAP ≡ 40 μg/ml PVX[b]	0/11[c]	8/8[d]	0/31[c]	9/9[d]
PVX_{DX} SAP ≡ 10 μg/ml PVX[b]	0/10[c]	8/11[d]	0/13[c]	8/8[d]
PVX_{EX} SAP ≡ 10 μg/ml PVX[b]	20/20[d,e]			
PVX_{CP2} ≡ 2 μg/ml PVX RNA[c]	6/6[d]	4/4[d]		
PVX_{HB} SAP[g]	20/20[e]		17/17[e]	

[a]Number of symptomatic plants/number of plants inoculated.
[b]Crude sap extracted from *N.clevelandii* infected with the indicated strain of PVX. The titre of the sap was determined by inoculation into the local lesion host *Chenopodium amaranticolor*.
[c]Result at the end of the experiment (25 days post-inoculation).
[d]Systemic mosaic symptoms developed within 6 days post-inoculation.
[e]The inoculated plants were the F1 progeny of line 3.3 unselected for the transformed phenotype.
[f]The inoculum contained 500 μg/ml total cell RNA of *N.clevelandii* infected with PVX_{CP2}. The titre on the local lesion host *Chenopodium amaranticolor* indicated that the inoculum was equivalent to 2 μg/ml PVX RNA.
[g]PVX_{HB} infection was determined by sap spot hybridization, as this strain is almost symptomless on *N.tabacum*.

progeny from four of the PVX-resistant plants in the F1 generation. In each instance these F2 lines were completely resistant to infection, as determined by the absence of symptoms following inoculation of 80 plants from each line with RNA equivalent to 1 μg PVX RNA, indicating complete penetrance of the resistance phenotype in these lines. It is therefore most likely that the incomplete resistance in the F1 generation was due to segregation of transgenes with different resistance phenotypes.

The fully symptomatic F1 progeny of the 3.1 line accumulated PVX RNA at the same level as the non-transformed plants (Figure 5a and c). In some instances, the asymptomatic 3.1 plants were free of PVX RNA as detected by Northern analysis of both the inoculated and systemic leaves. However, in two out of four samples analysed, there was a low level of PVX RNA accumulation in the inoculated, but not the systemic leaf of asymptomatic plants. These data are shown in Figure 5c for one of the plants which failed to accumulate and one which accumulated low level PVX RNA in the inoculated leaf. This result is parallel to the analysis of symptoms which showed that in the 3.1 line (Figure 4b and c), the resistance was less pronounced in the inoculated than in the systemic leaves.

The results in Table II summarize data obtained with different types of inoculum and with strains of PVX other than PVX_{UK3} which was the source of the transgene. The resistance in the 3.3 plants was effective with inocula containing either PVX_{UK3} RNA or particles. The 3.3 plants were also resistant to infection by PVX_{DX} but were not resistant (Table II) to PVX_{EX}, PVX_{CP2} or PVX_{HB}. The available sequence data are not complete for all of these strains but the indications are that the strains able to accumulate on the 3.3 plants are less related to PVX_{UK3} than PVX_{DX}, which did not accumulate in the 3.3 plants. Both PVX_{CP2} (Orman *et al.*, 1990) and PVX_{HB} (M. Goulden, unpublished), which have been sequenced in entirety, are 80% similar to PVX_{UK3} with the heterogeneity distributed evenly through the genome, except for a region of 500 nucleotides in the gene for the 166 kDa protein which is 50% similar to PVX_{UK3}. The other resistance breaking strain PVX_{EX} is 80% similar to PVX_{UK3} in the region of the coat protein gene, whereas PVX_{DX} is 98% similar to PVX_{UK3} in that region (S.Santa Cruz, unpublished).

PVX resistance in protoplasts from transgenic plants expressing the PVX 166 kDa protein with the ADD mutation

Protoplasts were prepared from non-transformed plants or from the transformed progeny of the 3.3 line and were

inoculated with PVX RNA by electroporation. Northern analysis of RNA samples taken at 24 h post-inoculation showed that both types of protoplast were equally susceptible to infection by PVX_{HB} but that only the non-transformed protoplasts were susceptible to PVX_{UK3} (Figure 7). These non-transformed protoplasts accumulated the genomic and the two major sub-genomic RNAs of PVX, whereas in the 3.3 protoplasts there was no detectable PVX RNA, with a limit of detection at 0.01% of the level in non-transformed protoplasts. A minor RNA component of 1.8 kb in the extracts of the PVX_{UK3}-infected protoplasts which hybridized with the PVX probe was not thought to be viral in origin as the same RNA species was also detected in samples inoculated with a mutant form of PVX RNA (designated dUK) in which there was a large deletion in the gene for the 166 kDa protein (Figure 7).

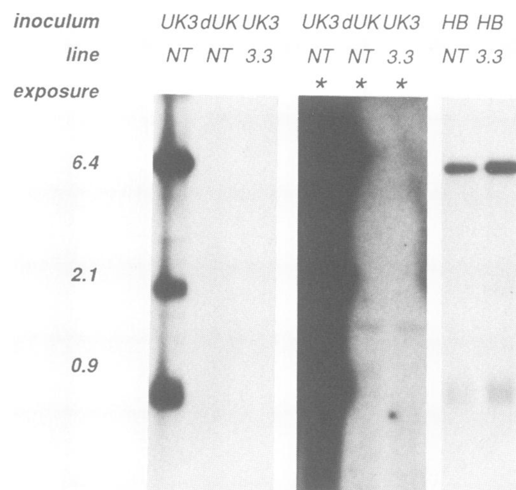

Fig. 7. Northern analysis of PVX RNA in protoplasts of the 3.3 transformed plants. RNA samples were also prepared from protoplasts of NT and T (line 3.3) plants at 24 h post-inoculation with a saturating level of RNA of either PVX_{UK3} (UK3), PVX_{HB} (HB) or of transcripts of pTXSΔ*Bgl*II (dUK), as a control for residual inoculum. This construct is a non-infectious derivative of PVX_{UK3} deleted within the 166 kDa protein between sites for *Bgl*II at positions 966 and 2695. The RNA for infection was produced by *in vitro* transcription. Aliquots of RNA from 30 000 protoplasts were analysed by Northern blotting with ^{32}P-labelled RNA probes specific for the 2 kb at the 3′ end of either the PVX_{UK3} or PVX_{HB} genomes. The autoradiographs were all exposed for <2 h except for the protoplast samples identified by (★) which were exposed for 14 h. The major RNA species detected by this probe were the genomic RNA and two 3′ co-terminal mRNAs of 2.1 kb and 0.9 kb which are labelled by size. The sub-genomic RNAs are most prominent in the protoplast samples inoculated with PVX_{UK3}. The long exposure of the protoplast RNA samples showed faint non-specific hybridization to host RNAs between 1 and 2 kb in length.

Discussion

The major finding reported in this paper is the extreme degree of resistance to PVX associated with transgenic expression of a mutant form of the gene for the 166 kDa protein. The absence or very low level of accumulation of PVX RNA in inoculated leaves and in protoplasts of the transformed plant indicates that the primary effect was at the level of virus replication. In the 3.3 line, PVX accumulation was not detected in protoplasts given 5 μg/ml PVX RNA, which is close to the level required to produce maximal infection of non-transformed protoplasts, although there was some virus accumulation in inoculated leaves given a much higher level of inoculum (>100 μg/ml PVX RNA). Presumably the very concentrated inoculum allowed the virus to overcome, to a limited extent, the inhibiting effects of expressing the mutant forms of the 166 kDa protein, but was below that required for the virus to spread beyond the inoculated leaf.

The recent report of resistance in tobacco expressing an unmodified form of the PVX gene encoding the 166 kDa protein (Braun and Hemenway, 1992) prompts the question as to whether mutation of the gene is necessary for the resistance phenotype. Our data with the lines expressing the GAD and the GED mutations indicate that even the type of mutation affects the degree of resistance. This suggestion is reinforced by the report of a similar differential in the effects of ADD and other mutations of the replicase subunit encoded by Qβ phage when expressed in transformed *E.coli*: the cells expressing the ADD form were the most resistant to infection by Qβ phage (Inokuchi and Hirashima, 1987). We offer two explanations to reconcile the apparent difference of the resistance effects of the ADD or GED and GAD mutations with the resistance from the unmodified gene for the 166 kDa protein (Braun and Hemenway, 1992). One possibility is that there are two types of replicase-mediated resistance: resistance through ectopic and unregulated expression of the wild type gene or a dominant negative effect of certain mutant forms of the gene. Alternatively, there may be only a single mechanism based on the effect of a dominant negative mutant and, as suggested by Braun and Hemenway (1992), the unmodified gene expressed in their transgenic lines may not have had full wild type replicase activity. This suggestion was prompted by the low specific infectivity (0.2% of PVX RNA) of the *in vitro* transcripts of the cDNA which was the source of the gene expressed in the transgenic plants. The plasmid pTXS used in the present work produced transcripts which were 4% as infectious as PVX RNA and, in a derivative of this plasmid in which the length of the 3′ poly(A) tract was increased from 25 to 80 nucleotides, the specific infectivity was 80% of PVX RNA (Chapman, 1992). It remains possible, therefore, that partial or total inactivation of the 166 kDa protein is necessary for resistance in transgenic plants, although the results reported here suggest that the nature of the inactivating mutation may be important. It will be necessary to construct and test transgenic plants expressing the unmodified gene for the PVX 166 kDa protein from our highly infectious cDNA clone in order to determine the importance of mutations in the gene for the 166 kDa protein.

The question of whether wild type replicase sequences can bring about resistance to virus infection in transgenic plants extends beyond the PVX work, as it has not yet been ruled out from the other examples of replicase-mediated resistance that mutation is necessary to obtain the resistance. The gene for the TMV 54 kDa protein giving TMV resistance in transgenic tobacco (Golemboski *et al.*, 1990) and in transiently transformed tobacco protoplasts (Carr *et al.*, 1992) was modified by addition of five polylinker-derived codons at the 3′ end or, alternatively, could be considered as a deleted form of the 183 kDa protein of which it is the C-terminal domain. The PEBV gene for the 54 kDa homologue giving PEBV resistance in *N.benthamiana* was also mutated in a PCR step used in transfer of the gene to the expression

cassette (MacFarlane and Davis, 1992). In addition, there is a report of resistance to cucumber mosaic virus (CMV) in plants expressing a deleted and truncated replicase component encoded by RNA-2 of CMV (Anderson *et al.*, 1992). An attractive corollary of the hypothesis that inactivation of the replicase gene is necessary for replicase-mediated resistance is an explanation for the lack of resistance in plants expressing functional subunits of the putative replicases of brome mosaic and alfalfa mosaic viruses (Taschner *et al.*, 1991; Mori *et al.*, 1992).

It should be stressed that we cannot, with the available data, state whether or not the resistance was RNA- or protein-mediated. The observation of resistance with the ADD rather than with GAD or GED mutations, indicates a protein-mediated effect as RNA-mediated resistance is less likely to be affected by the few nucleotide differences in a 4 kb RNA whereas a protein-mediated effect may be sensitive to single amino acid differences in domains affecting the overall structure of the protein. In addition, we cannot rule out the possibility that, if protein-mediated, the resistance results from low level expression of truncated forms of the 166 kDa protein present in extracts of the transgene plants (Figure 3).

The interpretation of the effect of mutations in the transgenically expressed replicases of plant viruses is complicated by the apparent lack of correlation between the level of expression and the degree of resistance in the plants expressing the ADD mutant of the 166 kDa protein. The two lines which were fully susceptible (3.2 and 3.8) to PVX both expressed the transgene at a higher level than the resistant line 3.3. There was a similar lack of correlation between the level of expression and the degree of virus resistance in the lines expressing the 5′ portion of the PVX gene for the 166 kDa protein (Braun and Hemenway, 1992), in lines resistant to potyviral expression due to expression of the potyviral coat protein or coat protein RNA (Stark and Beachy, 1989; Lawson *et al.*, 1990; van der Vlugt *et al.*, 1992) and in lines resistant to tomato spotted wilt virus due to expression of the coat protein gene (Gielen *et al.*, 1992). In order to draw firm conclusions about the resistance phenotype of the mutations in the gene for the 166 kDa protein it will be necessary to analyse more transformants expressing each of the mutant and the wild type genes and also to understand why there is no correlation between the gross level of transgene expression and the degree of resistance.

There are various suggestions for this lack of correlation. One possibility, that the resistance is a somaclonal artefact and not directly associated with the transgene is unlikely, as the resistance is specific to the strains of PVX which are similar to the source of the transgene. Other possible explanations are based on the potential for variation in the cell specificity or timing of expression of the transgenes. The analysis of transgenes, transgene expression and resistance in the progeny of 3.1 may shed some light on this problem: this line contains two or more transgenes only one of which is associated with resistance.

In addition to the information derived from the analysis of the resistance mechanism in the transgenic plants expressing viral replicase, transgenic expression may indicate in other ways how these proteins work. For example, the partition of the PVX 166 kDa protein in the P30 fraction of the transgenic plants (Figure 2) indicates that subcellular localization of this protein, possibly in association with membranes (David *et al.*, 1992) requires neither an active protein nor the presence of other viral proteins.

Materials and methods

Mutation of the gene for the 166 kDa protein

The sequence encoding the GDD motif of the 166 kDa protein in PVX_{UK3} was mutated using the PCR (polymerase chain reaction) based procedure of Kammann *et al.* (1989) to generate variants with the sequence indicated in Figure 1. The mutant sequence was substituted between *Bcl*I restriction sites at positions 3507 and 4203 into pPVXML70 (Figure 1) which is a clone of the intact gene for the 166 kDa protein or into pTXS which is a transcription clone of the full length genome of PVX (Kavanagh *et al.*, 1992).

Transcriptions in vitro and inoculation of plants or protoplasts and testing of resistance to PVX

The plasmid pTXS and derivatives containing the modified gene for the 166 kDa protein gene (Figure 1) were linearized and transcribed *in vitro* with m^7GpppG cap analogue, and the transcripts were inoculated into *N.clevelandii* or into protoplasts of *N.tabacum* (cv. Samsun NN) as described by Chapman *et al.* (1992b).

For testing of PVX resistance, the basic procedures of plant inoculation, Northern analysis of RNA and the preparation and analysis of protoplasts have been described previously (Chapman *et al.*, 1992a,b). In the plant experiments the standard inocula were 250 μg/ml total RNA from PVX_{UK3}-infected *N.clevelandii* with a local lesion titre on *C.amaranticolor* approximately equivalent to 1 μg/ml of purified viral RNA. Purified PVX as a source of PVX RNA inoculum was prepared as described by Baulcombe *et al.* (1984). In the protoplast experiments, the inocula for 300 000 protoplasts were 5 μg PVX_{UK3} RNA or the transcripts, prepared as described by Chapman *et al.* (1992b) or 1 μg of transcript of pTHBS which is a transcription clone of the full length cDNA of PVX_{HB} (Kavanagh *et al.*, 1992).

Plant transformation

The mutant version of gene for the 166 kDa protein was first transferred to the *Agrobacterium* transformation vector pRok2, which is essentially the same as pRok1 (Baulcombe *et al.*, 1986), but with the expression cassette in the opposite orientation relative to the T-DNA border sequences and with additional *Sma*I, *Kpn*I and *Sst*I sites in the expression cassette (M.Longstaff and C.Raines, unpublished).

For plant transformation, the binary plasmid vectors were mobilized into *Agrobacterium tumefaciens* (strain LBA4404) using *E.coli* HB101 harbouring pRK2013, as described by Bevan (1984). Transformation of *N.tabacum* (cv. Samsun NN) was carried out using the leaf disc transformation method of Horsch *et al.* (1985). Transformed shoots and roots were selected by propagation on medium containing kanamycin (100 μg/ml) and eventually transferred to a glasshouse for production of mature plants, analysis of expression of the transgene, testing of virus resistance and seed production. The transformed progeny seedlings were identified by assaying for the neomycin phosphotransferase activity (NPT) (McDonnell *et al.*, 1987) encoded by a gene carried on pRok2 adjacent to the expression cassette.

Preparation of antiserum against the 166 kDa protein

The antigen for antiserum production was a fusion protein derived from a *lacZ* fusion plasmid (pEX2) (Stanley and Luzio, 1984), containing a *Pst*I fragment from the 166 kDa coding sequence, between nucleotide positions 1597 and 4455 (the termination codon). The 236 kDa protein encoded by this recombinant plasmid (pPVXML8) was then isolated and purified by polyacrylamide gel electrophoresis, as described by Laemmli (1970) and inoculated into rats. Pre-immune sera and immune sera were collected, the immune sera being taken after repeated booster inoculations.

Protein analysis

Protein samples were extracted in a pestle and mortar in 100 mM Tris−HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 0.4 M sucrose, 10% glycerol, 10 mM β-mercaptoethanol (2 ml/g tissue). The homogenate was centrifuged at 3200 r.p.m. for 10 min in a Sorvall SS-34 rotor and the supernatant was re-centrifuged at 18 000 r.p.m. in the same rotor to produce the 30 000 *g* pellet (P30) and supernatant (S30). Aliquots of each fraction were analysed by electrophoresis in polyacrylamide, as described by Laemmli (1970). Equal amounts of protein were loaded to each lane of the gel.

After electrophoresis, the polyacrylamide gel was soaked in 20 mM Tris,

200 mM glycine for 10 min and proteins were transferred in the same buffer onto a nitrocellulose membrane (Schleicher & Schuell) in a Bio-Rad Transblot apparatus at a setting of 150 mA for 16 h. The membrane was rinsed for 1 h, with shaking in blocking solution [phosphate-buffered saline (PBS), 1% Tween 20, 5% milk powder] and incubated for 2 h with an anti-166 kDa protein serum (M.Longstaff, unpublished) diluted 1/500 in PBS with 0.1% Tween 20, 5% milk powder (antiserum buffer). The filter was then rinsed three times, for 5 min in antiserum buffer and incubated for 90 min with horseradish peroxidase-conjugated anti-rabbit antibody, diluted 1/1000 in antiserum buffer. The filters were washed three times in PBS with 0.1% Tween 20. The immunoreaction was detected using the enhanced chemiluminescence kit (Amersham plc, UK). In the samples shown, the major protein migrated with an apparent M_r of 175 000, based on comparison on the same gel of the migration of myosin (M_r 205 000) and β-galactosidase (M_r 116 000). This is in good agreement with the predicted value of 166 000.

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