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Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA

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Transgenic tobacco plants engineered to express either the potato virus X (PVX) coat protein (CP+) or the antisense coat protein transcript (CP-antisense) were protected from infection by PVX, as indicated by reduced lesion numbers on inoculated leaves, delay or absence of systemic symptom development and reduction in virus accumulation in both inoculated and systemic leaves. The extent of protection observed in CP+ plants primarily depended upon the level of expression of the coat protein. Plants expressing antisense RNA were protected only at low inoculum concentrations. The extent of this protection was even lower than that observed in plants expressing low levels of CP. In contrast to previous reports for plants expressing tobacco mosaic virus or alfalfa mosaic virus CP, inoculation of plants expressing high levels of PVX CP with PVX RNA did not overcome the protection. Specifically, lesion numbers on inoculated leaves and PVX levels on inoculated and systemtic leaves of the CP+ plants were reduced to a similar extent in both virus and RNA inoculated plants. Although these results do not rule out that CP-mediated protection involves inhibition of uncoating of the challenge virus, they suggest that PVX CP (or its RNA) can moderate early events in RNA infection by a different mechanism.

Key words: cross-protection/potato virus X/transgenic plants/coat protein/antisense RNA

Introduction

The practice of cross-protection has been used for many years to reduce crop losses due to viral infection (Fulton, 1986). In general, plants infected with one strain of virus exhibited reduced or delayed symptom development in response to infection with a second strain. More recently, Powell Abel *et al.* (1986) have demonstrated that tobacco plants genetically engineered to express tobacco mosaic virus (TMV) coat protein (CP) were protected against subsequent TMV infection and that this protection was similar in several respects to classical cross-protection. They observed that symptom development in plants expressing the CP gene was delayed and that the extent of the delay was dependent on inoculum concentration. In addition, a significant percentage of these plants did not develop symptoms during the experiment. Nelson *et al.* (1987) further reported that protected tobacco plants exhibited reduced lesion numbers on inoculated leaves and reduced virus accumulation in both inoculated and systemic leaves. Subsequently, genetically-engineered cross-protection was successfully extended to confer protection against alfalfa mosaic virus (AlMV), which differs from TMV in virion structure, genome organization, gene expression and the requirement for CP during initial stages of infection (Tumer *et al.*, 1987; Loesch-Fries *et al.*, 1987; Van Dun *et al.*, 1987). Transgenic tobacco plants expressing the AlMV CP gene also exhibited a delay in symptom development on inoculated and systemic leaves, a reduction in the numbers of lesions on inoculated leaves and a dependence upon inoculum concentration.

Protection on the inoculated leaves of TMV and AlMV transgenic plants was largely overcome by inoculation with viral RNA, suggesting that the presence of the CP on virus particles in the challenge inoculum was necessary for maximum protection (Nelson *et al.*, 1987; Loesch-Fries *et al.*, 1987; Van Dun *et al.*, 1987). Classical cross-protection studies by Sherwood and Fulton (1982) and Dodds *et al.* (1985) also showed that protected plants were much more susceptible to infection with RNA than with virions. It was postulated that the viral CP from the first virus may be inhibiting the uncoating of the challenge virus (Sherwood and Fulton, 1982). However, Zinnen and Fulton (1986) observed that plants initially infected with sun hemp mosaic virus (SHMV) were protected completely against superinfection by both virus and viral RNA of a necrotizing strain of SHMV, suggesting that mechanisms for protection may vary with different viruses.

To investigate the role of CP in cross-protection, we have studied a potexvirus, potato virus X (PVX), for which classical cross-protection studies have been reported (Salaman, 1933; Matthews, 1949). PVX is a flexuous, rod-shaped virus with a single-stranded, positive sense RNA genome of ~6.4 kb, which is capped and polyadenylated (Sonenberg *et al.*, 1978; Morozov *et al.*, 1981). Only the CP gene has been definitively mapped thus far; it is located within the 3′ terminal 900 nt of the genome (Morozov *et al.*, 1983). Translation of this genomic RNA *in vitro* yields a series of large polypeptides of approximately 150–180 kd, but a 25 kd polypeptide corresponding to the CP is not observed (Wodnar-Filipowicz *et al.*, 1980; Bendena and Mackie, 1986). The CP is derived from a subgenomic RNA generated *in vivo* (Dolja *et al.*, 1987). Although PVX and TMV are similar in their genome size and 3′ location of the CP gene, they differ significantly in several ways. In contrast to PVX, the 3′ end of TMV RNA is not polyadenylated, but does have a 3′ non-coding tRNA-like structure that can be charged with histidine (Francki *et al.*, 1985). Although both viruses are rod-shaped and have helical substructures, TMV is a rigid rod of ~300 nm × 18 nm and PVX is a flexuous rod of approximately 515 nm × 13 nm. Assembly of TMV proceeds primarily in the 3′→5′ direction, from an origin of assembly (OAS) sequence located 925 nt up-

stream of the 3′ terminus (Francki *et al.*, 1985). Disassembly of TMV *in vitro* (Wilson, 1984) and *in vivo* (Shaw *et al.*, 1986) appears to proceed 5′→3′ in a co-translational manner. In contrast, assembly of a potexvirus, papaya mosaic virus (PMV) has been shown to proceed 5′→3′ from an OAS located within the 5′ 45 nt of the PMV RNA (Abou Haidar and Erickson, 1985). Disassembly of PMV was observed to occur in a 3′ to 5′ direction *in vitro* at alkaline pH (Lok and Abou Haidar, 1981).

We analyzed transgenic plants expressing the PVX CP or the corresponding antisense RNA for protection from PVX infection to learn more about the mechanisms involved. Here we report that plants expressing high levels of CP are protected from PVX infection to a greater extent than the plants expressing lower levels of CP or antisense RNA. In contrast to the protection reported in other systems, protection in plants expressing PVX CP is not overcome by inoculation with PVX RNA. Potential mechanisms for protection that are supported by these data are discussed.

Results

Expression of the PVX CP gene or its antisense RNA in trangenic plants

A PVX cDNA was inserted in both sense and antisense orientations between the cauliflower mosaic virus (CaMV) 35S promoter and the pea rbcS E9 3′ end into the expression vector pMON9818 (Cuozzo *et al.*, 1988). The resulting sense and antisense constructs, pMON9809 and pMON9810, were transferred into tobacco plants by *Agrobacterium tumefaciens* mediated transformation of leaf disks (Horsch *et al.*, 1985). Several regenerated plants initially were analyzed for expression of the corresponding 1.2 kb transcripts, which should include 35 nt from the CaMV 35S leader, 844 nt of the cDNA clone, and ~280 nt due to the 3′ end and polyadenylation. Transcript levels in two transgenic plants that were found to express the highest levels of the sense (CP+) and antisense (CP-antisense) RNAs are demonstrated in Figure 1A. Scanning densitometry of this autoradiogram indicated that transcript levels are ~3-fold higher in the CP+ plant, 6665, than in the CP-antisense plant, 7017. One possible reason for this difference is that the poly(T) tail at the 5′ end of the antisense construct alters the transcription of this sequence or that the resulting transcript is less stable. In general, the corresponding transcript levels compared among other transgenic plants were similar to these, or were 2- to 5-fold lower.

The transgenic plants were further analyzed for expression of PVX CP. As expected, the plants expressing antisense PVX CP transcript did not produce PVX CP (data not shown). The plants expressing sense PVX CP transcript produced a 25 kd protein that cross-reacted with antibody to PVX and co-migrated with authentic PVX CP, as indicated in Figure 1B. By comparison with purified PVX, 40 μg of total leaf extract from transgenic plant 6665 contained ~40 ng of PVX CP. In general, the range of CP expression observed by Western analysis of transgenic plants was estimated to be 0.02−0.1% of the total protein.

Transgenic plants are protected from PVX infection

Experiments to determine whether transgenic plants were susceptible to PVX infection generally included progeny of four transformed plant lines: two that express similar levels of CP, 6665 and 6859, one that expresses antisense CP RNA, 7017 and a vector control, 6820. These plants were self-fertilized and progeny were screened for the presence of nopaline synthase activity; the expression of this gene usually segregates with expression of the CP gene. Segregation ratios were 3:1 (CP+:CP−) for these lines, indicating that they each contain a single active copy of the PVX CP cDNA. In the initial experiment, 8−10 nopaline-positive progeny were inoculated with either 0.5 or 5.0 μg/ml of PVX. Symptom development on both inoculated and systemic leaves was monitored visually each day, up to 18 days post-inoculation. In addition, disks from the inoculated and second systemic leaves of each plant were sampled at various timepoints (see Materials and methods) in order to quantitate the extent of virus replication and spread. This method allowed us to determine the time course of PVX multiplication and spread, and to detect differences between control and transgenic plants. In addition, the endogenous levels of CP in transgenic plants were not sufficient to interfere with quantitation of virus accumulation. The inoculated leaf samples from the two CP+ lines, 6665 and 6859, contained significantly lower concentrations of PVX than did the

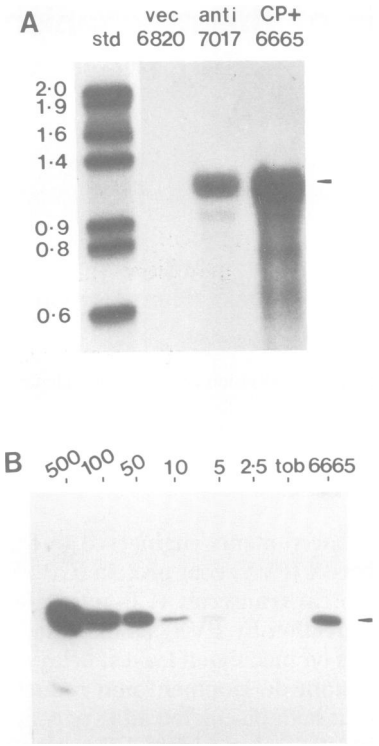

Fig. 1. Analysis of PVX CP and CP transcript levels in transgenic tobacco plants. (**A**) Total RNA isolated from transgenic tobacco leaves was detected by Northern blotting. RNA samples (40 μg/lane) were electrophoresed on a 1.4% formaldehyde/agarose gel, which was blotted to nitrocellulose and then probed with a PVX CP cDNA fragment labeled by nick-translation. The autoradiogram depicts RNA contained in a plant transformed with the vector only (6820), a plant transformed with the antisense orientation of the CP gene (7017) and a plant transformed with the sense orientation of the CP gene (6665). The first lane contains λDNA digested with *Hin*dIII and *Eco*RI. (**B**) Protein extracted from transgenic tobacco leaves was separated on a 12.5% SDS−polyacrylamide gel and CP was identified by immunoblotting. The first six lanes contain decreasing concentrations from 500 to 2.5 ng of purified PVX. The seventh and eighth lanes contain 40 μg of total protein extracted from non-transformed tobacco (tob) and transgenic plant 6665 respectively. The arrow at left denotes the position of the 25 kd PVX CP.

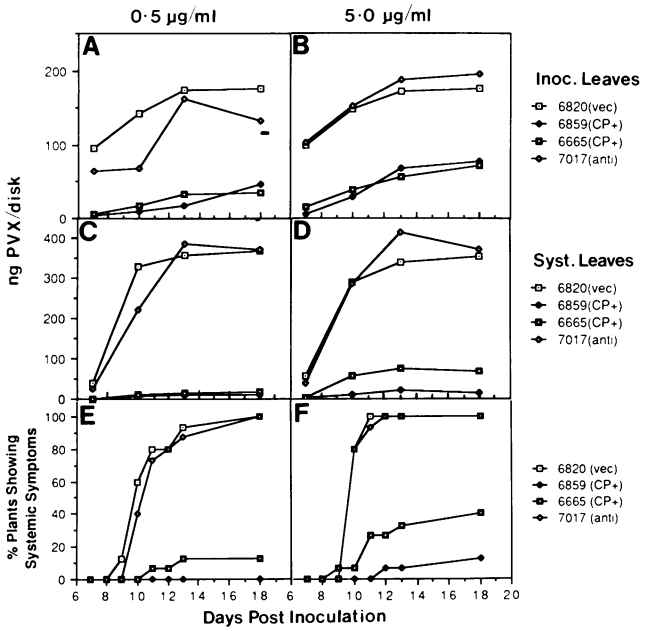

Fig. 2. Virus accumulation in transgenic plants after inoculation with PVX. Nopaline-positive progeny (8−10) from two plant lines expressing PVX CP (CP+), 6859 and 6665, one line expressing antisense CP RNA (CP-anti) 7017, and a vector control (vector), 6820, were inoculated with 0.5 (A,C,E) and 5.0 (B,D,F) μg/ml PVX. Samples were collected from inoculated (A,B) and 2nd systemic leaves (C,D) at 7, 10, 13, and 18 days post-inoculation, and virus levels were quantitated by ELISA. All plants were visually scored for onset of systemic symptom development (E,F).

vector control plants at all timepoints when the inoculum concentration was 0.5 μg/ml (Figure 2A). In contrast, the corresponding levels in the CP-antisense plants were generally lower up to 10 days post-inoculation, but similar to controls at later time points. The difference in PVX levels between control plants and transgenic CP+ plants was more pronounced in leaf samples from second systemic leaves (Figure 2C). The PVX levels in systemic leaves of CP-antisense plants were not significantly lower than those in controls.

Similar results were obtained when the inoculum concentration was increased to 5.0 μg/ml (Figure 2B,D). Progeny from the two CP+ lines still were significantly protected, although PVX levels in inoculated leaves were approximately 2-fold higher by day 18 than at the lower inoculum concentration. PVX levels in second systemic leaves of the 6859 plants were identical at 18 days after inoculation with either concentration; corresponding levels in 6665 plants increased ~3-fold after inoculation with the higher concentration. Plants expressing antisense RNA were not significantly protected on inoculated or systemic leaves after inoculation with 5 μg/ml of virus. In general, PVX levels detected in control plants were similar at both inoculum concentrations. Similar PVX levels were also observed in control plants when inoculum concentrations of 5 and 20 μg/ml were used (unpublished data). This type of response is typical of PVX infection in a systemic host (P.E.Thomas, unpublished data). Although the numbers of lesions initially induced are proportional to the virus inoculum concentration, the virus ultimately spreads throughout the inoculated leaves and the rest of the plant to similar levels. Thus, since all of the control plants exhibited distinct symptoms by 7 days, quantitation of virus levels before 7 days post-inoculation may be

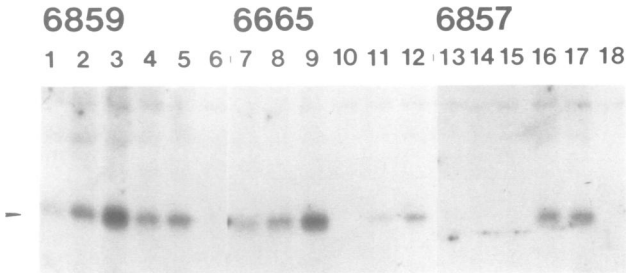

Fig. 3. Comparison of CP levels in progeny plants from three transgenic plant lines. Protein was extracted from transgenic tobacco leaves and CP was detected by Western immunoblotting. Six nopaline-positive progeny were analyzed from each of three transgenic lines: 6869 (lanes 1−6), 6665 (lanes 7−12) and 6857 (lanes 13−18). Each lane contains 15 μg of total leaf protein.

necessary to detect differences in the controls that are dependent on inoculum concentration.

The visual observations of symptom development were consistent with the quantitative data. All of the control plants had developed lesions on inoculated leaves by 18 days post-inoculation at 0.5 μg/ml inoculum, while only 15−20% of the progeny from both of the CP+ plants lines had lesions. At the higher inoculum concentration, only 40−50% of the CP+ plants had lesions. Furthermore, there were relatively few lesions on the CP+ plants that did develop lesions compared with numbers on the control plants. The time course of symptom development on the inoculated and systemic leaves of all plants paralleled the time course of PVX accumulation. Specifically, both symptoms and virus levels on inoculated and second systemic leaves increased between 7 and 13 days and then leveled off.

Onset of systemic symptom development was delayed by 2 days in CP+ progeny of the 6665 line compared to control plants at the 0.5 μg/ml inoculum concentration (Figure 2E). By 18 days post-inoculation, <10% of these CP+ plants were systemically infected. CP+ progeny of the 6859 line did not develop systemic symptoms by 18 days post-inoculation, while 100% of the controls were systemically infected by this time. The CP-antisense plants did exhibit a 1 day delay in systemic spread compared to the control plants; however, the percentage of CP-antisense plants that developed systemic symptoms was similar to the controls at later time points. In general, plants acquired systemic symptoms earlier at the higher inoculum concentration (Figure 2F). Only 13% of the 6859 plants and 49% of the 6665 plants were systemically infected by day 18 at the 5 μg/ml inoculum concentration. Thus, these data indicate that the progeny of the two CP+ plant lines are significantly protected from PVX infection on both inoculated and systemic leaves at each inoculum concentration tested.

Plants expressing antisense RNA are protected at low inoculum concentrations

The fact that CP+ plants were protected and CP-antisense plants were not in the previous experiment may indicate that CP itself or CP sense transcript, but not antisense RNA, are capable of inhibiting the challenge virus. However, the levels of antisense transcripts, which are lower than the sense transcript levels in regenerated plants and in their progeny (data not shown), may not be sufficient to protect at the inoculum concentrations used previously. Therefore, we inoculated these antisense plants with lower inoculum concentrations

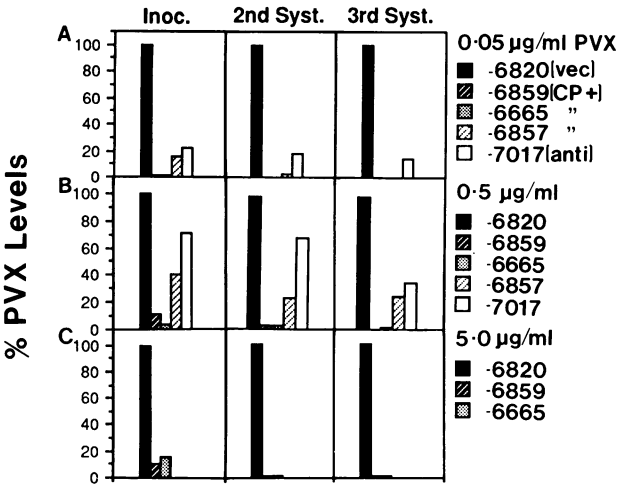

Fig. 4. Accumulation of virus in transgenic plants inoculated with PVX. Progeny (4−8) from three CP+lines, 6859, 6665 and 6857, the CP-antisense line, 7017 and the vector control, 6820, were inoculated with 0.05 µg/ml (**A**) and 0.5 µg/ml PVX (**B**). Progeny from the two best-expressing CP+ lines, 6859 and 6665, and the vector control, 6820, also were inoculated with 5 µg/ml PVX (**C**). At 14 days post-inoculation, both inoculated and systemic leaves were collected and leaf extracts were individually analyzed for PVX levels by ELISA. Values obtained for each plant from a specific line were averaged. These average virus levels of the CP+ and CP-antisense plant lines are represented relative to average levels in the vector control plants. For easy comparison, the value for each line is depicted as a % of the control (100%). Statistical analysis was done to determine if average values in CP+ and CP-antisense plant lines were significantly different from average control values. Only values obtained for Student's *t* test that were less than 0.05 were considered significantly different.

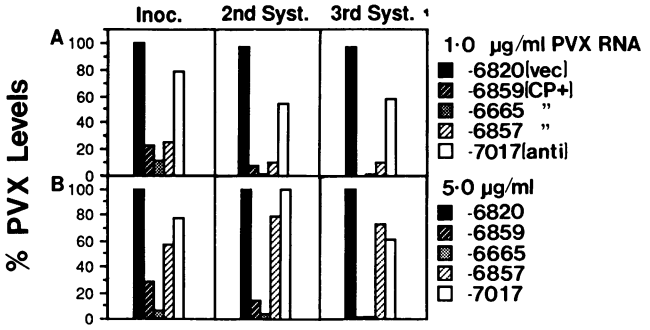

Fig. 5. Accumulation of virus in transgenic plants inoculated with PVX RNA. Progeny (4−8) from three CP+ lines, 6859, 6665 and 6857, the CP-antisense line, 7017 and the vector control, 6820, were inoculated with 1 µg/ml (**A**) and 5 µg/ml (**B**) PVX RNA. PVX levels were analyzed as described in the legend to Fig. 5.

and compared the extent of protection to that observed in plants of a CP+ line, 6857, which express lower levels of CP (and sense transcript) than progeny from the 6859 and 6665 lines. A comparison of the CP levels in nopaline+ progeny plants from these three CP+ lines is illustrated in Figure 3. Although extracts from only two 6857 progeny plants appear to contain CP, the others do contain low levels that are detectable when much larger amounts of total protein are analyzed. In this experiment, only nopaline+ progeny from each of the three CP+ lines, 6665, 6859 and 6857, that expressed detectable levels of CP by Western or ELISA analyses were used. Four to eight progeny from each of the three CP+ lines, the CP-antisense line (7017) and the vector control were inoculated with 0.05, 0.5 and 5 µg/ml PVX. Symptom development was monitored daily, and virus levels were quantitated only at 14 days post-inoculation. This time point was chosen for sampling because the previous experiment indicated that virus accumulation leveled off in the control and transgenic plants by 14 days. Although this is a fairly stringent approach to determine significance of protection, it does eliminate detection of differences that may exist earlier in infection. In addition, intact inoculated and systemic leaves from each plant were sampled instead of the disk sampling used previously to ensure more accurate quantitation of virus levels.

To facilitate comparison, the data illustrated in Figure 4 represent levels of PVX (ng PVX/mg leaf tissue) in transgenic plants as a percentage relative to levels in control plants (100%). These percentage values were calculated from the actual values averaged from four to eight plants. At the 0.05 µg/ml inoculum concentration, the inoculated leaves of the two CP+ lines, 6665 and 6859, contained only 2% of the virus levels detected in control leaves (Figure 4A). These low levels are similar to endogenous levels of CP in these plants and may not represent accumulation of virus. Protection also was observed in the 6857 plants and CP-antisense plants, which contained 20−30% of control virus levels. Virus accumulation in systemic leaves was inhibited in plants from all three CP+ lines and in the CP-antisense plants. Again, the visual symptoms confirmed these data; protected plants had fewer or no lesions on inoculated leaves and systemic symptom development was delayed or absent in these plants.

Accumulation of virus was somewhat higher in CP+ transgenic plants at the 0.5 than at the 0.05 µg/ml inoculum concentration (Figure 4B), but was still significantly lower than control levels in both inoculated and systemic leaves. The two lines that express higher levels of CP were protected to a greater extent than the 6857 line. As observed previously, the CP-antisense plants were not significantly protected on either inoculated or second systemic leaves at this inoculum concentration. However, virus levels in the third systemic leaves of these plants were significantly lower than in the corresponding control leaves. At the 5 µg/ml inoculum concentration (Figure 4C), progeny from the two CP+ lines 6859 and 6665 exhibited significantly reduced virus levels on both inoculated and sytemic leaves. Progeny of the CP+ 6857 line and the CP-antisense 7017 line were not inoculated at this concentration in this experiment. However, in a previous experiment, antisense plants were not protected on either inoculated or systemic leaves at the 5 µg/ml inoculum concentration (data not shown). In that experiment, the 6857 plants were not significantly protected on inoculated leaves, but were significantly protected from systemic spread. These results indicate that over a 2 log range of inoculum concentration, transgenic CP+ plants are protected. The extent of this protection on inoculated and systemic leaves seems to correlate with average levels of CP in progeny plants. Only at the lowest inoculum concentration, 0.05 µg/ml, was there a significant reduction of virus accumulation in inoculated and systemic leaves of plants expressing antisense RNA. Nevertheless, the fact that protection was observed in plants expressing CP or CP-antisense RNA suggests that different mechanisms may be involved, but that CP-mediated protection may be more effective.

Table I. Comparison of lesion numbers at 5 days post-inoculation with PVX or PVX RNA

Inoculum concentration (μg/ml)	Average number of lesions per leaf[a]			
	Control plants[b]		6665 progeny (CP+)	
	Visual[c]	Stained[d]	Visual	Stained
PVX				
0.5	1 ± 1	2 ± 2	1 ± 1	0
5	14 ± 11	27 ± 16	1 ± 2	2 ± 2
20	35 ± 8	52 ± 29	0	7 ± 5
PVX RNA				
1	3 ± 3	13 ± 8	1 ± 1	1 ± 1
5	11 ± 9	29 ± 16	0	2 ± 2
20	25 ± 15	57 ± 30	1 ± 1	8 ± 5

[a]Plants (6–8) were inoculated on each of two leaves with the indicated concentrations of PVX or PVX RNA. Data shown are means ± SE.
[b]Control plants were non-transformed tobacco.
[c]Chlorotic lesions on inoculated leaves were visually scored.
[d]Lesions on inoculated leaves were stained for starch deposition with IKI solution, and were counted using a dissecting microscope.

Inoculation with RNA does not overcome protection in CP+ plants

The observations that protection in transgenic plants expressing TMV or AlMV CP can largely be overcome by inoculation with viral RNA indirectly support a role for endogenous CP in prevention of uncoating of the challenge virus, at least as an early event after inoculation (Nelson *et al.*, 1987; Loesch-Fries *et al.*, 1987; van Dun *et al.*, 1987). Thus, to investigate the mechanism of PVX protection in transgenic plants, we inoculated CP+ plants or CP-antisense plants with two concentrations of PVX RNA. These concentrations were chosen on the basis of previous infectivity titration experiments, which indicated that 1 and 5 μg/ml RNA inocula were similar to 0.5 and 5 μg/ml virus inocula in terms of lesion numbers on inoculated leaves. We scored symptom development and collected leaves at 14 days to quantitate virus accumulation. After inoculation with 1 μg/ml RNA, virus levels in all three CP+ plant lines were significantly reduced in both inoculated and systemic leaves compared to control plants (Figure 5A). The inoculated leaves of CP+ plants developed fewer lesions than did leaves from control plants, although the lesions were not actually counted in this experiment. In contrast, virus levels in CP-antisense plants were not significantly different from control levels. This is not surprising as these plants were not protected when inoculated with the equivalent 0.5 μg/ml virus inoculum. It remains to be tested whether antisense plants would be protected at lower RNA inoculum concentrations. Virus levels were still significantly reduced in both inoculated and systemic leaves of 6859 and 6665 plants as the RNA inoculum concentration was increased to 5 μg/ml (Figure 5B). However, protection in progeny of the CP+ line (6857) that express lower levels of CP was overcome when RNA inoculum was increased to 5 μg/ml.

Since the RNA inoculation experiments depicted in Figure 5 were done simultaneously with the virus inoculation experiment depicted in Figure 4, we can directly compare these data. Statistical analysis indicated that virus concentrations in control plants were similar, regardless of concentration or form of inoculum. In addition, the levels of virus in the higher expressing CP+ plants were not significantly different at equivalent virus and RNA inoculum concentrations, but were significantly lower than control levels. Thus, RNA inoculation did not overcome protection in plants expressing high levels of PVX CP. To further analyze this protection in terms of earlier events during infection, we quantitated the numbers of lesions formed on leaves of control plants and progeny of the 6665 line after inoculation with comparable concentrations of virus and viral RNA. Since the chlorotic lesions resulting from PVX infection of tobacco frequently are difficult to count accurately, we also quantitated the lesions by staining the leaves for starch with an IKI solution. This method was previously demonstrated to be useful for detecting virus even when lesions were not visible on the unstained leaves (Holmes, 1931; Helms and McIntyre, 1962; Thomas and Zielinska, 1983).

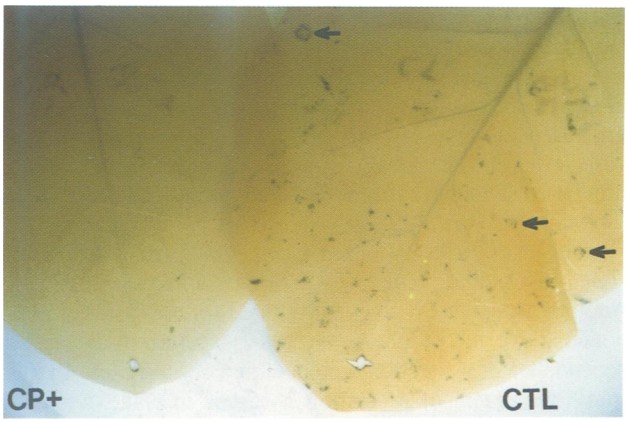

Fig. 6. Comparison of lesion development on inoculated leaves of control and CP+ plants. Five days after inoculation with 20 μg/ml PVX RNA, inoculated leaves of untransformed tobacco (CTL) and 6665 plants (CP+) were stained for starch to locate lesions. The arrows denote lesions in the form of concentric rings.

As indicated in Table I, the numbers of lesions observed on inoculated leaves of control plants, both visually and by staining, were similar at comparable virus and RNA inoculum concentrations. The numbers of lesions on control leaves increased with increasing inoculum concentration, and even greater numbers were detected by staining. The numbers of lesions scored on the CP+ plants were significantly lower than on the controls at all inoculum concentrations. There was no significant difference in lesion numbers on CP+ leaves in response to virus or viral RNA inocula. When chlorotic lesions and/or stained lesions were observed on CP+ leaves, they were generally smaller in diameter than lesions observed on control leaves. A photograph comparing starch lesions on control and CP+ plants after inoculation with PVX RNA is shown in Figure 6. The larger starch lesions in the form of concentric rings were frequently observed on control leaves and correlated well with large chlorotic lesions observed on the corresponding unstained leaves. This pattern of lesion formation also was observed on plants inoculated with virus. These data indicate that transgenic plants expressing high levels of PVX CP are protected to the same extent after both PVX and PVX RNA inoculation.

Discussion

Transgenic tobacco plants that express PVX CP were significantly protected from PVX infection on both inoculated

and systemic leaves. The numbers of lesions observed on inoculated leaves of CP+ plants were reduced or absent compared to controls. In addition, the levels of virus detected in these leaves were significantly lower. Similarly, a delay (or absence) of symptom development and a reduction in virus levels in systemic leaves were observed in CP+ plants. Similar results have been reported previously for TMV (Powell Abel *et al.*, 1986; Nelson *et al.*, 1987), and AlMV (Tumer *et al.*, 1987; Loesch-Fries *et al.*, 1987; Van Dun *et al.*, 1987), and recently for CMV (Cuozzo *et al.*, 1988). Another aspect that has been described for the systems mentioned above and for classical cross-protection studies is the dependence of protection on inoculum concentration. In this study, the extent of protection observed in plants expressing low levels of PVX CP was dependent upon inoculum concentration. Protection on the inoculated leaves of these plants, both in terms of lesion numbers and virus accumulation, was overcome at 5 μg/ml, although systemic protection was still significant. However, the level of protection observed in progeny of the two lines that express higher levels of CP was similar over a 2 log difference in inoculum concentration. Additional experiments will be required to determine whether higher concentrations of PVX will overcome this protection. Loesch-Fries *et al.* (1987) also have reported that AlMV CP-mediated protection (in terms of lesion numbers) is dependent upon levels of CP expression.

One mechanism that has been postulated for both classical protection and CP-mediated protection is prevention of uncoating of the challenge virus (Sherwood and Fulton, 1982). In support of this mechanism, Nelson *et al.* (1987), Loesch-Fries *et al.* (1987) and Van Dun *et al.* (1987) have reported that protection in CP+ plants can be largely overcome by inoculation with corresponding viral RNA, as indicated by lesion numbers on inoculated leaves. Although these data support a role for CP in prevention of uncoating of the challenge virus, they do not rule out the possibility that CP might prevent virus uptake. It is also possible that prevention of uncoating by CP could lead to reduced cell to cell spread of the virus if plant viruses migrate from cell to cell as intact virions. Thus, RNA inoculation would overcome protection initially, but then virus spread would be inhibited. A delay in systemic symptom development and systemic virus accumulation is observed in TMV CP+ plants after inoculation with TMV RNA, even when virus levels in inoculated leaves are comparable to levels in control plants (P.Powell, R.Nelson and R.Beachy, unpublished results).

In contrast, we have found that transgenic plants expressing PVX CP are protected from infection by PVX RNA. At equivalent virus and RNA inoculum concentrations, CP+ plants develop reduced numbers of lesions and some plants do not develop any lesions on inoculated leaves (Table I). Generally, lesions detected on CP+ plants by starch staining were smaller in diameter than lesions detected on control plants (Figure 6). Whether these smaller starch lesions reflect a block in virus multiplication at the site of infection or a block in virus spread remains to be determined. In addition, virus levels in inoculated and systemic leaves are reduced to a similar extent in virus and RNA inoculated CP+ plants at 14 days post-inoculation. These data do not obviate a role for CP in prevention of uncoating, but do suggest that PVX CP and/or its RNA can inhibit the initial events during challenge RNA inoculation by some other mechanism. Although it is possible that endogenous CP RNA could interact with challenge viral RNA, viral replicase or some host factor to deter replication and transport, such a scenario would also appear to be possible for TMV and AlMV; however, RNA inoculation does overcome protection in these systems. Thus,it seems more likely that endogenous PVX CP is somehow interacting with the challenge viral RNA.

Several mechanisms can be proposed in light of the differences between TMV and PVX assembly and disassembly. Most assembly studies of potexviruses have been done with PMV, which is assembled 5′→3′ from an OAS located with the 5′-most 45 nt of the RNA (Abou Haidar and Erickson, 1985). Disassembly of PMV has not been extensively characterized, but does occur 3′→5′ *in vitro* at alkaline pH (Lok and Abou Haidar, 1981). In contrast, assembly of TMV proceeds primarily in the 3′→5′ direction from an origin of assembly (OAS) sequence located 925 nt upstream of the 3′ terminus (Francki *et al.*, 1985). Wilson (1984) has demonstrated that cotranslational disassembly of relaxed TMV virions occurs from 5′→3′ *in vitro*. Similar cotranslational disassembly studies with potexviruses have not been successful, either because the mechanism for disassembly is significantly different or the conditions used to swell TMV virions are not successful with potexviruses (Wilson and Shaw, 1985). Assuming that PVX and PMV assembly/disassembly are similar, it is possible that PVX CP in transgenic plants could bind at or near the OAS/5′ region of the challenge viral RNA and subsequently prevent translation of the replicase and/or interfere with replication. This interaction might result in recoating, at least partially, of the viral RNA. Such mechanisms could account for the observed reduction in lesion numbers and virus accumulation in RNA inoculated PVX CP+ plants. Our observation that the lower expression CP+ plants were not as well protected from RNA inoculation on inoculated and systemic leaves as the plants that express higher levels of CP would also support these mechanisms.

Systemic infection in CP+ plants was delayed in response to RNA inoculation, and the extent of protection observed was similar to that observed in response to virus inoculation. Regardless of the mechanism(s) involved, inhibition of infection on the inoculated leaves could delay spread of the virus throughout the plants. Alternatively the endogenous CP also could be interfering with the cell to cell or systemic spread of the virus by similar or different mechanisms. Although lesion numbers observed on inoculated leaves of control plants were dependent upon the concentration of PVX or PVX RNA inocula, virus levels in inoculated and systemic leaves of these plants were similar at all inoculum concentrations at 14 days post-inoculation. Thus, these data suggest that the reduced PVX levels in systemic leaves of CP+ plants are not a result of fewer lesions on the inoculated leaves.

Although genetically engineered cross protection with viral CP is similar in several respects to classical cross protection, the mechanisms involved may not necessarily be the same. We want to determine to what extent other viral-encoded genes, parts of genes, or transcripts might also confer protection. As there is precedence for antisense inhibition of gene expression in plant systems (Ecker and Davis, 1986; Rothstein *et al.*, 1987), it is possible that the antisense viral RNA might inhibit replication of the challenge virus by RNA−RNA interactions or function to compete for host or viral components. We have demonstrated that transgenic

plants expressing PVX CP antisense transcripts were significantly protected on both inoculated and systemic leaves, but only at the lowest virus inoculum concentration tested. To achieve protection in the antisense plants at higher inoculum concentrations, better levels of expression of endogenous antisense transcript may be needed. In addition, the antisense RNA may not be as inherently effective as CP in detering challenge virus by virtue of the mechanism involved. In support of the latter statement, we observed that the virus levels in the antisense plants were similar to levels in the low expressing CP+ plants only at the lowest inoculum concentration. At a 10-fold higher inoculum concentration, these CP+ plants still were protected from systemic infection whereas the antisense plants were not. Recent results with plants expressing CMV CP antisense transcripts also support this (Cuozzo *et al.*, 1988). In addition, we have analyzed protection in transgenic plants expressing only the 5′ 135 nt of the PVX CP transcript in either the sense or antisense orientation (unpublished data). The levels of these transcripts were ~6-fold lower than the full length sense transcripts. In addition, no polypeptide that reacts with an antibody to PVX can be detected in the plants expressing the truncated sense transcripts. At the inoculum concentrations tested, plants expressing sense or antisense transcripts from this region of the PVX CP did not confer any protection from infection by PVX. These results support a role for the involvement of CP in protection from PVX. In addition, these data demonstrate that, at present, CP-mediated protection is more effective than CP-antisense-mediated protection.

Previous studies of cross-protection with virus infected plants and transgenic plants indicated that the protection observed in both cases was similar in many respects. The work described here extends the applicability of the CP-mediated protection to a flexuous, rod-shaped potexvirus. We demonstrate that plants expressing PVX CP are significantly protected from PVX infection and that it is possible to obtain protection through expression of the CP-antisense RNA. In addition, we show that protection in PVX CP+ plants was not overcome by RNA inoculation, unlike the previous studies with transgenic plants expressing TMV or AlMV CP. This observation suggests that PVX CP or its RNA can inhibit the early events during RNA infection by a mechanism other than inhibition of uncoating. Understanding the mechanisms involved in the CP-mediated protection observed in transgenic plants will augment our knowledge of the initial steps of viral infection, and will facilitate the development of virus-resistant plants.

Materials and methods

Virus and viral RNA purification

PVX used for infection experiments was obtained from Dr P.E.Thomas, USDA, Prosser, Washington, and is a local isolate. The virus was propagated in *Nicotiana tabacum* (cv Samsun) and purified as described by Shepard and Shalla (1972). RNA was isolated from PVX by addition of 100 μg/ml proteinase K, 0.5% SDS, 50 mM Tris−HCl, pH 8.0, and was subsequently purified by phenol/chloroform extractions and ethanol precipitation.

Cloning and plant transformation

A partial PVX CP cDNA clone (p3a) that lacked the 5′ terminal 10 codons was obtained from Dr K.G.Skryabin. This clone was synthesized by oligo(dT) priming of PVX RNA (wild strain) and cloned into the *Pst*I site of pBR322 using dG:dC tailing (Morozov *et al.*, 1983). To recover the 5′ end of the CP gene, a synthetic *Bam*HI−*Pst*I fragment containing 18 bases of authentic 5′ non-coding sdequence and 66 bases corresponding to the first 22 codons was used to replace the smaller *Pst*I−*Pst*I fragment containing the dG:dC tail and codons 11−22. The dG:dC tail and part of the dA:dT at the 3′ end of the gene were removed by *Bal*31 digestion of the larger *Hpa*II−*Pst*I fragment subcloned into pUC18. Then, a *Cla*I site was created at the 3′ end of this subclone by linker addition. Finally, the *Xho*I−*Cla*I (~170 bp) was used to replace the original 3′ end sequence from p3a and the *Pst*I−*Cla*I sequence from pBR322. The final cDNA construct (in pEMBL 12+) contained 18 bp of 5′ non-coding sequence, 714 bp of PVX CP coding sequence (including TAA), 72 bp of 3′ non-coding sequence and 40 bp of dA:dT. The *Bam*HI−*Cla*I fragment from this clone was inserted into *Bgl*II−*Cla*I sites of the expression pMON 9818 (Cuozzo *et al.*, 1988) between the CaMV 35S promoter and the small subunit E9 3′ end. Both the sense and antisense orientations of the PVX CP gene were subcloned into this vector, resulting in pMON9809 and pMON09810, respectively. These plasmids were mated into *A.tumefaciens* strain 3111SE (Rogers *et al.*, 1987); the modified *A.tumefaciens* then was used to transform *N.tabacum* (cv Samsun) leaf disks.

Analysis of transgenic plants

Levels of PVX CP in transgenic tobacco leaves were determined by immunoblotting as described by Tumer *et al.* (1987), except 2-mercaptoethanol was not included in the extraction buffer. Blots were incubated first with rabbit anti PVX antiserum (ATCC) and then [^{125}I]protein-A. PVX CP transcripts were isolated by the LiCl method (Tumer *et al.*, 1986) and analyzed by Northern blotting, using a nick-translated PVX CP cDNA fragment as a probe.

Cross-protection experiments

Progeny from self-fertilized transgenic plants were analyzed for presence of nopaline (Otten and Schilperoort, 1978). Generally, 4−15 nopaline+ progeny (4−6 weeks old) were inoculated on each of two carborundum-dusted leaves with various concentrations of purified virus or viral RNA in 100 mM sodium phosphate buffer, pH 7.0. Plants were subsequently rinsed with H_2O to remove carborundum and were maintained in a growth chamber set for 10 h dark/14 h light (250 μE) period, 22°C and 50% humidity. Symptom development on inoculated and systemic leaves was scored visually each day, generally for 1−21 days post-inoculation. Leaf samples were collected for quantitation of virus levels. Either 1 cm disks were sampled from both inoculated leaves and two systemic leaves of each plant at various time points, or corresponding intact leaves (including midribs) were removed from each plant at 14 days post-inoculation. Leaf samples collected for starch staining were processed according to the method of Thomas and Zielinska (1983).

Quantitation of PVX levels in inoculated plants by ELISA

PVX was extracted from leaf disk samples by grinding in 50 μl of PBS/0.05% Tween/0.2% ovalbumin with a Wheaton overhead stirrer or from whole leaf samples by homogenization with 3 ml of the same buffer per gram of tissue using a Waring Blendor. Levels of PVX in these samples were quantitated by ELISA, as described by Kaniewski and Skotland (1979). Specifically, extracts (5−20 μl) and purified PVX standards were applied to microtiter plants coated with rabbit anti PVX γ-globulin, incubated at 4°C for 16 h, and washed three times with PBS/0.05% Tween. Samples then were reacted with a 1/5000 dilution of alkaline phosphatase/PVX antibody conjugate for 4 h at 37°C. After washing plates three times with PBS/Tween, the *p*-nitrophenylphosphate substrate was added and absorbance at 410 nm was monitored using a Dynatech plate reader. Values were converted to ng PVX per disk (each disk weighs ~0.03 g) or to ng PVX per g tissue for whole leaf samples. Student's *t* test was used to determine whether the PVX levels in transgenic plants were significantly different from levels in control plants.

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