VIROLOGY 236, 76–84 (1997)
ARTICLE NO. VY978732

Concurrent Suppression of Virus Replication and Rescue of Movement-Defective Virus in Transgenic Plants Expressing the Coat Protein of Potato Virus X

Charles Spillane,*[,1] Jeanmarie Verchot,†[,1] Tony A. Kavanagh,* and David C. Baulcombe†[,2]

*Department of Genetics, Trinity College, Dublin 2, Ireland; and †The Sainsbury Laboratory, Norwich Research Park, Colney Lane, Norwich NR4 7UH, United Kingdom

Received May 22, 1997; returned to author for revision June 20, 1997; accepted July 14, 1997

A line of transgenic tobacco expressing the coat protein (CP) of potato virus X (PVX) was resistant against a broad spectrum of PVX strains. Inoculation of leaves and protoplasts with PVX expressing the jellyfish green fluorescent protein reporter gene revealed that this resistance mechanism suppressed PVX replication in the initially infected cell and systemic spread of the virus. Cell-to-cell movement was also slower in the resistant plants. The resistance at the level of replication was effective against wild-type PVX and also against movement-defective isolates with a frameshift mutation or deletion in the CP ORF. However, the cell-to-cell movement defect of the mutant viruses was rescued on the resistant plants. Based on these results it is proposed that the primary resistance mechanism is at the level of replication.

INTRODUCTION

Virus resistance in plants containing viral CP transgenes may be mediated by the protein or the RNA product of the transgene (Baulcombe, 1996). Protein-mediated resistance relies on the ability of transgenic CP to interfere with functions of the viral CP that are essential for the infection cycle. If the CP molecule has multiple functions, the resistance may operate at several levels. For example, the tobacco mosaic virus (TMV) CP produced in transgenic tobacco caused resistance against TMV by suppression of virion disassembly and viral vascular transport (Register and Beachy, 1988; Osbourn *et al.,* 1989; Wisniewski *et al.,* 1990; Reimann-Philipp and Beachy, 1993). Similarly CP-mediated resistance against alfalfa mosaic virus (AlMV) operated at the level of virion disassembly and, in some lines, at the level of viral RNA replication (Taschner *et al.,* 1994).

RNA-mediated virus resistance, as in transgenic plants containing the CP coding sequence of tobacco etch virus (TEV) or potato virus Y (PVY), involves degradation of viral RNA species with a nucleotide sequence that is similar to that of the transgene (Van der Vlugt *et al.,* 1992; Lindbo *et al.,* 1993; Smith *et al.,* 1994; Goodwin *et al.,* 1996). Although the first described examples of this RNA-mediated resistance involved CP transgenes providing potyviral resistance, it is now recognized that the same mechanism can be targeted against parts of the viral genome other than the CP open reading frame (ORF) (Swaney *et al.,* 1995) and against many types of virus, including potexviruses (Mueller *et al.,* 1995; Pang *et al.,* 1996; Prins *et al.,* 1996; Sijen *et al.,* 1996). We refer to this RNA-mediated process as homology-dependent resistance.

From earlier studies it is known that a transgene based on the CP ORF of PVX confers resistance to PVX (Hemenway *et al.,* 1988). This resistance is effective against RNA or virion inocula, indicating that PVX is suppressed at a later stage in the infection cycle than virion disassembly. However, it was not known whether this resistance was mediated by protein or RNA.

In this report we describe an analysis that was designed to characterize the mechanism of PVX resistance in tobacco plants carrying PVX CP transgenes. Our findings suggest that the resistance was due primarily to inhibition of PVX RNA replication in the inoculated cell. Surprisingly, CP defects in mutant PVX could be complemented in part by inoculation to these PVX-resistant plants. We propose that these activities of the transgenic CP are related to the roles of the CP in the normal PVX infection cycle.

MATERIALS AND METHODS

Plasmids

Two binary plasmid constructs were prepared for *Agrobacterium*-mediated transformation of tobacco leaf discs. The plasmid pCPΔ44 contains the CP ORF and 3′ untranslated region of the PVX genome (nucleotides 5606–6436) inserted between the cauliflower mosaic virus (CaMV) 35S promoter and the 260-bp polyadenylation

[1] These authors contributed equally to the work described in this paper.

[2] To whom correspondence and reprint requests should be addressed.

0042-6822/97 $25.00

signal of the nopaline synthase terminator of pROK2 (Jefferson *et al.,* 1987). A four-step cloning procedure was followed to introduce the PVX sequence into the binary vector. First, a partial PVX cDNA clone (pPVXF) containing the 3′ half of the PVX genome (Kavanagh *et al.,* 1992) was linearized with *Apa*I restriction enzyme and treated with nuclease *Bal*31. Second, a *Bal*31–*Sst*I fragment of approximately 700 bp (corresponding in size to the region from the 5′ end of the CP ORF to the 3′ end of the PVX genome) was isolated and ligated to *Eco*RV–*Sst*I-linearized pUBS19 (a derivative of pUC19) (Murphy and Kavanagh, 1988) to produce the plasmid pUBSΔ44 in which the PVX cDNA extended 44 bp 5′ of the coat protein coding region. The PVX cDNA was excised from pUBS19 by digestion with *Cla*I and *Sst*I and ligated to the *Cla*I–*Sst*I-linearized pICI9H (Marsh *et al.,* 1984) to generate an intermediate plasmid. Finally the PVX cDNA was isolated from the intermediate plasmid by digestion with *Bam*HI and *Sst*I and inserted into pROK2 cut with the same enzymes.

The binary vector pCPΔ3 contains nucleotides 5643–6133 of the PVX genome inserted into the pROK2 vector. The PVX infectious clone pTXS (Kavanagh *et al.,* 1992) was digested with *Taq*I enzyme and a fragment corresponding to nucleotides 5643–6133 was ligated to an *Acc*I linearized pUC19 plasmid creating the plasmid pPVXTaq. An *Xba*I–*Stu*I fragment derived from pPVXTaq was ligated to an *Xba*I–*Stu*I-linearized pCPΔ44 creating pCPΔ3.

The PVX.GFP and the PVX.GFP.ΔCP plasmids are clones of PVX cDNA with the GFP gene inserted adjacent to a duplicated coat protein promoter and were described previously (Baulcombe *et al.,* 1995). The PVX.GFP.ΔCP construct lacks most of the coat protein gene up to nucleotide 6302 near the 3′ end of the gene. The PVX.GFP.FS plasmid contains a frameshift mutation near the 5′ end of the coat protein ORF. This construct was prepared by restriction of PVX.GFP with *Nhe*I (nucleotide position 5663 in the PVX genome). The Klenow fragment of DNA pol I was used to fill in single strand ends of the restriction site and the plasmid was recircularized by ligation. The presence of the frameshift mutation was confirmed by sequence analysis.

Transformation and regeneration of tobacco

Binary plasmids were mobilized from *Escherichia coli* MC1022 into *Agrobacterium tumefaciens* strain LB4404 by triparental mating using *E. coli* HB101 containing pRK2013 as the helper plasmid (Bevan, 1984). Transconjugant *A. tumefaciens* were selected on kanamycin (50 μg/ml) and streptomycin (500 μg/ml) and used to transform *Nicotiana tabacum* cv. Samsun NN leaf discs (Horsch *et al.,* 1985). Transformed shoots were regenerated in MS medium. F1 and F2 progeny seed of lines CPΔ44 and CPΔ3, derived from transformation experiments using plasmids pCP3Δ44 and pCP3Δ3, respectively, were collected for these studies.

In vitro transcription and inoculation of tobacco

Infectious transcripts of PVX plasmids or the TMV cDNA clone pTB2 (Donson *et al.,* 1991) were prepared using T7 polymerase and m7G(ppp)G cap analogue, as described previously (Chapman *et al.,* 1992b). For some experiments a crude sap inoculum containing PVX virions was prepared from systemically infected *N. tabacum* or *N. clevelandii* by homogenization of tissue in cold 50 m*M* borate buffer (pH 8.2). Extracts were filtered through miracloth or spun at low speed and the supernatant was used directly as a virion inoculum. Inocula of RNA were prepared by grinding fresh tissue in RNA homogenization buffer (Baulcombe *et al.*, 1984) and by repeated extractions with phenol:chloroform. RNA was recovered by ethanol precipitation and resuspended in water for use in plant inoculations. All plants were mechanically inoculated in the presence of carborundum. The titer of the various inocula was assayed by inoculation of *Chenopodium amaranticolor* which is a local lesion host of PVX.

Inoculation of protoplasts and Northern analysis

Protoplasts were prepared as described previously (Chapman *et al.,* 1992b). Infectious transcripts were prepared from 5 μg plasmid DNA in a 50-μl reaction and 10 μl was used for each separate inoculation. Electroporation of plasmid transcripts was carried out as described previously (Chapman *et al.,* 1992b). Protoplasts were incubated in the dark for 24 hr after inoculation and RNA was extracted for Northern analysis in RNA homogenization buffer, as described above. Of total RNA, 1.5 μg was loaded per lane of a 0.9% w/v agarose formaldehyde gel. Electrophoresis and Northern analysis was conducted according to Sambrook *et al.* (1989). The membrane was probed with a ^{32}P-labeled transcript of pTB2-CP in which the PVX CP ORF was substituted for the TMV CP ORF (Bendahmane *et al.,* 1995). This probe hybridized to both TMV and PVX RNAs.

ELISA

The level of PVX in infected plants was determined by indirect ELISA using a monoclonal antibody (MAC58) specific for the coat protein of PVX (Torrance *et al.,* 1986). Four tobacco leaf discs were homogenized in 1 ml of ELISA extraction buffer (PBX, 0.05% Tween 20, 2% polyvinylpyrrolidone) and 5- or 100-μl samples were used for this analysis. Plates were washed three times with PBST (PBX, 0.05% Tween 20) between additions of antibody solutions or plant sap extracts. Plant sap samples and purified PVX virion standards were added to microtiter plates precoated with anti-PVX rabbit polyclonal antibod-

ies (10 mg/ml) diluted 1:1000 in carbonate buffer (15 m*M* Na_2CO_3, 35 m*M* $NaHCO_3$) and incubated at 4° overnight. Anti-PVX sera, diluted 1:1000 in PBST containing 0.2% BSA was added and the plates were incubated at 30° for 2 hr. Alkaline phosphatase-conjugated MAC58 diluted in PBST containing 0.2% BSA was added, incubated for 2 hr at 33°, and the microtiter plate was rinsed four times in PBST buffer. Substrate (*p*-nitrophenol phosphate in diethanolamine buffer, pH 9.8) was added to the microtiter plate, incubated at room temperature, and the absorbance was read at 405 nm. All solutions were as specified by the manufacturers protocol for the ELISA diagnostic kit (Bioreba A.G.) The lower detection limit by ELISA in this analysis was approximately 0.4 ng PVX/mg fresh tissue weight.

Immunosorbent electron microscopy

Samples of infected leaves were ground in 1:10 w/v of phosphate buffer, pH 7.0. PVX antisera was diluted 1:1000 in phosphate buffer, pH 7.0, and 20-μl drops were used to float carbon-coated gold grids (3.05 mm mesh). Grids were incubated 1 hr at 25–35° and after washing with phosphate buffer were floated on drops of extracted samples, left overnight at 4°, and subsequently washed three times with phosphate buffer and once with water. For electron microscopy the grids were stained with 2% uranyl acetate.

RESULTS

CP transgenes confer broad spectrum resistance against PVX

The tobacco cultivar Samsun NN was transformed with either of two Ti plasmid constructs containing cDNA of PVX isolate UK3 (PVX_{UK3}) fused to the 35S promoter of CaMV (Fig. 1A). The cDNA in the CPΔ3 construct extends from 7 bases upstream of the PVX CP ORF to the 3′ end of the nontranslated region in the viral genome. The CPΔ44 construct differs only in that there are 44 bases 5′ of the CP ORF. Immunoblot analyses of extracts from transformed tobacco lines confirmed that in these high CP expressing lines selected for further analyses, the transgenic PVX coat protein was approximately 0.1% of the total soluble protein (C.S., unpublished data).

F1 progeny of five CPΔ3 lines and four CPΔ44 lines were screened for resistance to PVX by mechanical inoculation with PVX_{UK3}. All of these lines showed some PVX resistance that was manifest as either an absence or a delay in symptom development and a reduced PVX accumulation in the upper leaves (Table 1). The most resistant line, the progeny of plant CPΔ3#4, was selected for further analyses of the resistance mechanism.

The effect of PVX inoculum strength on the resistance mediated in these transgenic plants was assayed using

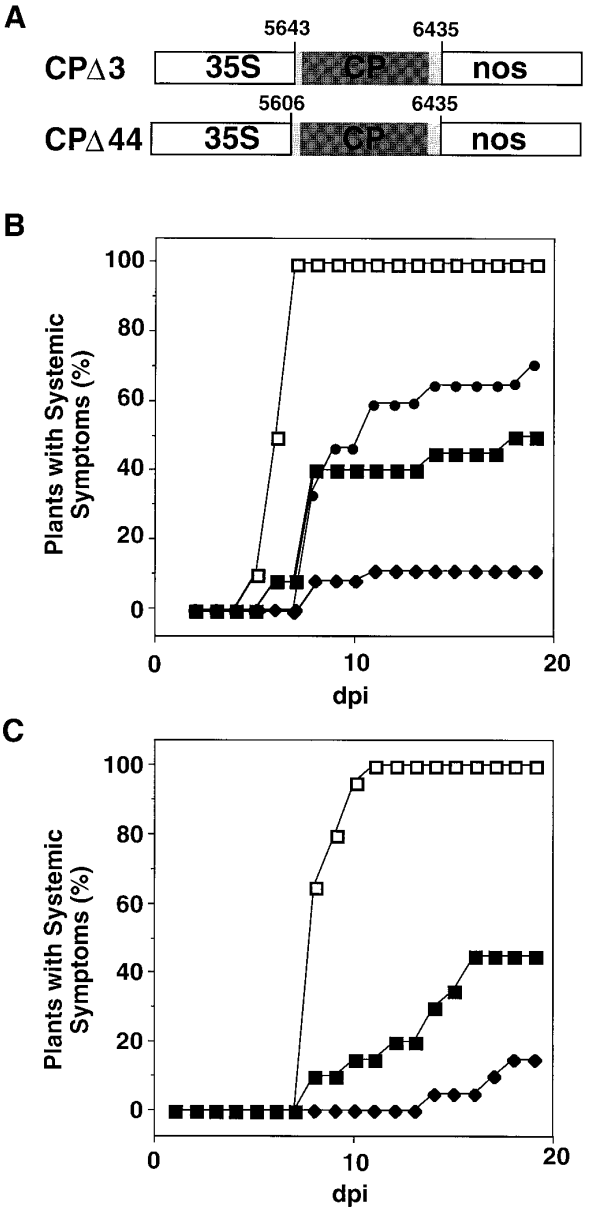


FIG. 1. PVX resistance in line CPΔ3#4 (F1 progeny). Transgene constructs shown in (A) comprised the 35S promoter of CaMV (35S), the CP ORF (positions 5650–6363) and adjacent regions of the PVX genome (with the 5′ and 3′ untranslated regions shaded lighter than the translated region) and the nopaline synthase terminator (nos). The numbers above the diagrams represent the 5′ and 3′ positions of the PVX genome that were present in the CPΔ3 or CPΔ44 constructs. These constructs were transformed into tobacco. (B) and (C) show PVX resistance tests of the F1 progeny of CPΔ3#4. Plants of nontransformed (NT) tobacco (□) or line CPΔ3#4 (●, ■, ♦) were inoculated with crude sap extracts of plants infected either with PVX_{UK3} (B) or PVX_{CP4} (C). Symptoms on the noninoculated leaves were monitored daily. 15 (B) or 20 (C) plants were each inoculated with the sap samples either undiluted (●) or diluted to 10^{-2} (■) or 10^{-3} (□, ♦) in buffer. The titers of the PVX_{UK3} inocula diluted 10^{-2} and 10^{-3} were equivalent to 150 and 26 lfu, respectively, on local lesion host *Chenopodium amaranticolor*. The titers of the PVX_{CP4} inocula diluted 10^{-2} and 10^{-3} were equivalent to 103 and 37 lfu, respectively.

TABLE 1

Susceptibility of F1 Progeny of Line CPΔ3 and CPΔ44 to PVX_{UK3} Infection

Plant line[a]	Number of plants with systemic symptoms[b] 11 dpi	17 dpi
CPΔ3#3	0	10
CPΔ3#4	0	2[c]
CPΔ3#8	4	11
CPΔ3#13	7	13[c]
CPΔ3#16	3	12
CPΔ44#7	5	17[c]
CPΔ44#8	2	15
CPΔ44#20	1	15[c]
CPΔ44#23	13	14
NT	20	20

[a] Nontransformed (NT) or carrying the CPΔ3 or CPΔ44 constructs.

[b] Twenty F1 plants inoculated. The titer of the inoculum was the equivalent of 57 local lesion forming units (1fu) on *Chenopodium amaranticolor.*

[c] ELISA of upper noninoculated leaves at 14 dpi revealed that the asymptomatic leaves had significantly less virus than in the symptomatic leaves of nontransgenic plants (Spillane, unpublished data). In the most extreme example, CPΔ3#4, there was no detectable PVX in the upper asymptomatic leaves.

three concentrations of PVX_{UK3} (Fig. 1B) that produced systemic symptoms on nontransformed plants by 5–6 days postinoculation (dpi). In the F1 progeny of CPΔ3#4 inoculated with an undiluted inoculum of PVX_{UK3}, the initial symptoms were delayed until 7 dpi or later. Only 70% of these plants displayed systemic symptoms by 20 dpi. With increasing inoculum dilutions of 10^{-2} and 10^{-3} (Fig. 1B), the proportion of CPΔ3#4 plants with symptoms on the upper leaves at 20 dpi was reduced to 50 or 10%, respectively (Fig. 1B). These data demonstrate that the resistance in line CPΔ3#4, like the CP-mediated resistance against TMV (Powell *et al.,* 1986), is more readily overcome by high titer inocula.

To test the strain specificity of the PVX resistance, the F1 progeny of line CPΔ3#4 were inoculated with various isolates of the serologically distinct subgroups of PVX from Europe and South America (Torrance *et al.,* 1986). These subgroups represent the known extremes of PVX CP sequence variation: the CP ORFs of isolates in the South American subgroup are 78% similar to the CP ORF of PVX_{UK3} at the nucleic acid level and 86% similar at the protein level (Santa Cruz and Baulcombe, 1995). At the nucleotide level the isolates in the European subgroup are all more than 95% similar to PVX_{UK3} in the CP ORF (Santa Cruz and Baulcombe, 1995). After inoculation with RNA or virions of a South American isolate, PVX_{CP4}, the symptoms of PVX infection developed more slowly and in a smaller proportion of the CPΔ3#4 plants than on nontransformed control plants (Fig. 1C, Table 2). The CPΔ3#4 plants were also resistant to other European and South American subgroup isolates of PVX (Table 2). These data demonstrate that the PVX resistance in line CPΔ3#4 is effective against a broad spectrum of PVX isolates and confirms the earlier report (Hemenway *et al.,* 1988) that the CP-mediated resistance against PVX is effective against both virion and RNA inocula.

Resistance in line CPΔ3#4 blocks initial infection and spread of PVX

To characterize the mechanism of PVX resistance in line CPΔ3#4, a PVX vector construct (PVX.GFP) was inoculated to plants from the F2 population. Expression of the jellyfish green fluorescent protein (GFP) from this construct was used as a visual marker to identify the sites of initial virus infection and to monitor cell-to-cell and vascular transport of the virus (Baulcombe *et al.,* 1995).

The pattern of GFP accumulation in the plants of CPΔ3#4 inoculated with PVX.GFP revealed that the PVX resistance mechanism operates at three levels. First, there was suppression of the initial infection. This level of resistance was indicated in three replicate experiments in which PVX.GFP was inoculated to nontrans-

TABLE 2

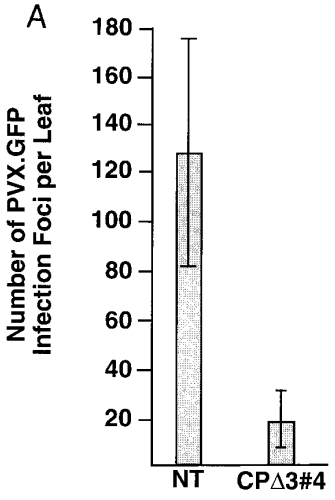
Resistance of Line CPΔ3#4 (F1 generation) against Different Strains of PVX

Plant line[a]	PVX strain[b]	Infected plants[c]
NT	PVX_{UK3}	10/10
CPΔ3#4	PVX_{UK3}	3/10
NT	PVX_{CP4} (RNA)	20/20
CPΔ3#4	PVX_{CP4} (RNA)	3/20
NT	PVX_{DY}	10/10
CPΔ3#4	PVX_{DY}	0/10
NT	PVX_{XS}	10/10
CPΔ3#4	PVX_{XS}	3/10
NT	PVX_{HB}	10/10
CPΔ3#4	PVX_{HB}	1/10
NT	PVX_{P5}	10/10
CPΔ3#4	PVX_{p5}	1/10
NT	PVX_{S6}	20/20
CPΔ3#4	PVX_{S6}	3/20

[a] The plant lines were nontransformed tobacco (NT) or the CPΔ3#4 transgenic line.

[b] The titers of the RNA inocula were standardized at the equivalent of 91–121 1fu on *Chenopodium amaranticolor* except when the inoculum was PVX_{S6} for which the titer was equivalent to 250 1fu. The inocula were all sap (virion) extracts of infected plants, except for PVX_{CP4}, which was an RNA inoculum. PVX_{CP4}, PVX_{HB}, and PVX_{DY} are South American subgroup strains; the other isolates are all of the European subgroup.

[c] Number of infected plants/number of plants inoculated. Nontransformed (NT) and transgenic plants of line CPΔ3#4 were maintained for 15 dpi and the presence of PVX in the upper leaves of infected plants was determined by symptoms.



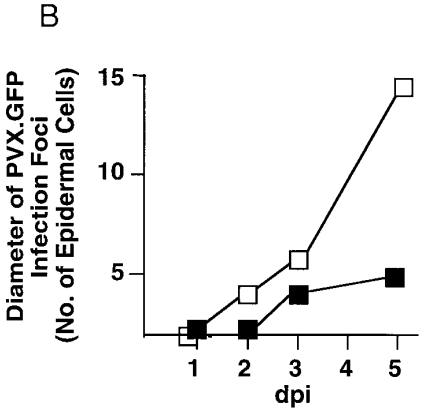


FIG. 2. PVX.GFP inoculated to F2 progeny line CPΔ3#4. (A) The average number of GFP infection foci per leaf of nontransgenic (NT) and transgenic tobacco (line CPΔ3#4) after inoculation with PVX.GFP. Error bars refer to standard errors of 5 replicate samples. Similar data were reproduced in two other experiments. (B) The diameters of infection foci (number of epidermal cells) measured during the first five dpi with PVX.GFP RNA on NT (□) or line CPΔ3#4 (■). Each value represents the average of ten measurements. Similar data were generated from four replicate experiments and with virion as well as RNA inocula.

genic and transgenic plants. In each experiment at 5–7 dpi there were, on average, five- to sixfold fewer sites of infection on the transgenic plants than on nontransgenic plants ($P < 0.05$) (Fig. 2A). The second level of resistance, revealed by the rate of expansion of PVX.GFP foci in inoculated leaves, was suppression of cell-to-cell movement. In nontransgenic plants, PVX.GFP had spread across an average of 13.4 cells by 5 dpi, whereas in line CPΔ3#4 the virus moved two- to threefold slower and was confined to significantly smaller ($P < 0.005$) regions of 5.0 cells in diameter (Fig. 2B).

The third level of resistance led to suppression of long distance movement through the vascular system: in none of the more than fifty F2 CPΔ3#4 plants tested was there long distance movement of PVX.GFP within 16 dpi, as indicated by the symptoms or distribution of GFP detected by UV illumination. All of the nontransformed plants were systemically infected with PVX.GFP within that period. This suppression of long distance movement was more complete than in the experiments that employed the F1 progeny of CPΔ3#4 (Fig. 1 and Tables 1 and 2). We attribute this higher resistance to the higher transgene copy number and more uniform segregation of the transgenes in the F2 generation. The F2 plants were also uniformly resistant against nonrecombinant (wild-type) PVX (CS and DCB, data not shown).

Suppression of PVX accumulation in protoplasts of line CPΔ3#4

PVX.GFP was inoculated to protoplasts to determine whether the PVX resistance in the F2 generation of line

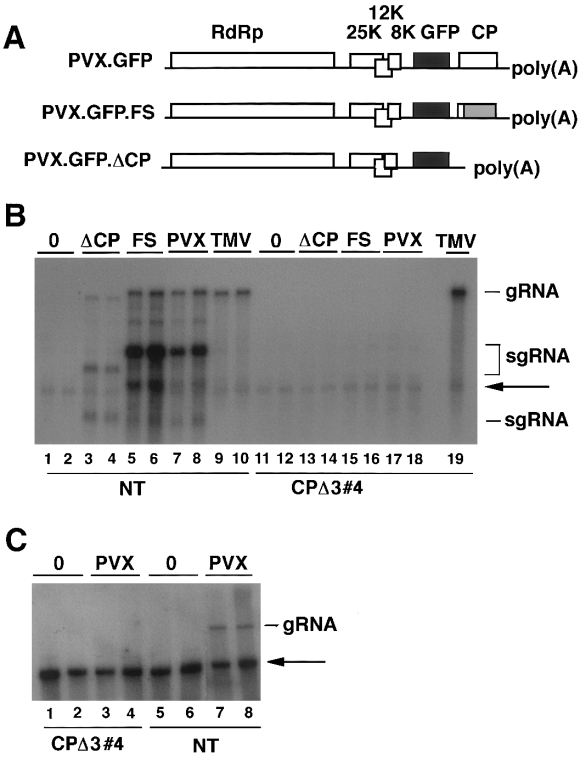


FIG. 3. PVX resistance in protoplasts of line CPΔ3#4. PVX.GFP cDNA constructs (A) were transcribed *in vitro* to produce infectious RNA. Each ORF in the PVX cDNA is indicated as a boxed region with the M_r of PVX-encoded proteins indicated (K, kDa). GFP indicates the ORF of the green fluorescent protein and CP the coat protein ORF. The out-of-frame region of the CP ORF in the frameshift mutant PVX.GFP.FS is shown as a shaded region. (B and C) Northern analysis of RNA that was extracted from protoplasts of transgenic (CPΔ3#4) and nontransgenic (NT) tobacco. The cells were either mock inoculated (O) or were inoculated with PVX.GFP (PVX), PVX.GFP.ΔCP (ΔCP), PVX.GFP.FS (FS), or TMV. The probe in (B) detected genomic (gRNA) and subgenomic (sgRNA) species. The probe in (C) detected the negative-strand RNA of PVX. Both probes detected minor RNA species in mock-inoculated samples (→) that are due to nonspecific hybridization with host RNA.

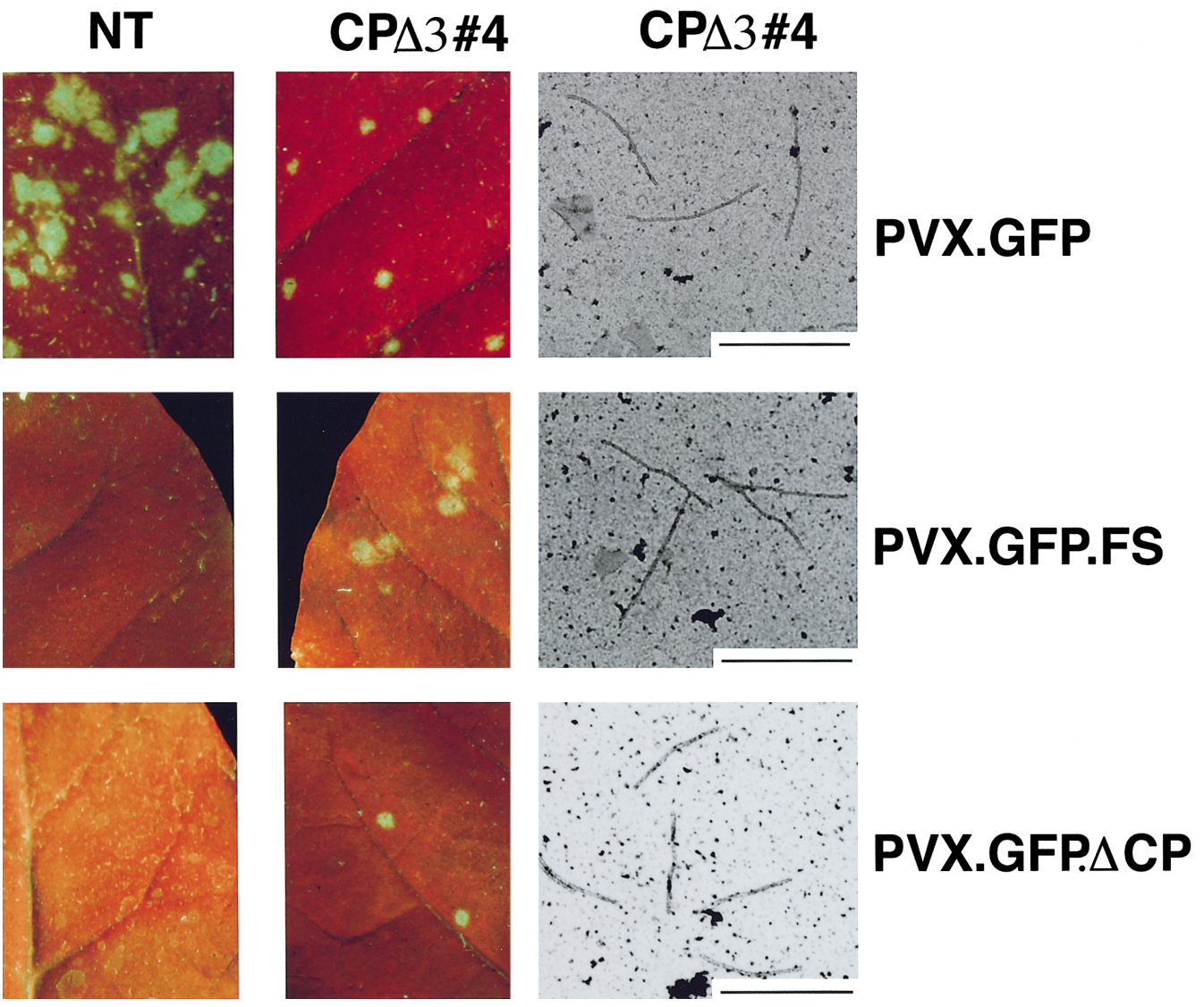


FIG. 4. GFP infection foci on leaves of nontransgenic (NT) or CPΔ3#4 leaves inoculated with PVX.GFP (7 dpi), PVX.GFP.FS (14 dpi), or PVX.GFP.ΔCP (14 dpi). The leaves were photographed under UV light. The right hand panels show immunosorbent electron microscopy of virus particles isolated from the infection foci. The scale bar in these electron micrographs is 0.5 μm.

CPΔ3#4 was due to suppression of virus accumulation within initially infected cells. Northern analysis of protoplast RNA extracted at 24 hr postinoculation revealed that the positive strand RNA of PVX.GFP accumulated in nontransgenic protoplasts (Fig. 3B, lanes 7 and 8). However, in the protoplasts of line CPΔ3#4, PVX.GFP RNA failed to accumulate to detectable levels (Fig. 3B, lanes 17 and 18). There was also suppression of PVX.GFP negative strand RNA accumulation (Fig. 3C, lanes 3 and 4 compared with lanes 7 and 8), indicating that PVX replication in CPΔ3#4 protoplasts was inhibited. In contrast, TMV RNA accumulated to similar or slightly higher levels in the protoplasts of line CPΔ3#4 (Fig. 3B, lanes 9, 10, and 19). From this control it can be ruled out that the low accumulation of PVX.GFP was an artefact due to the condition of the protoplasts of line CPΔ3#4.

The protoplasts of line CPΔ3#4 were also inoculated with two mutant viruses, PVX.GFP.FS and PVX.GFP.ΔCP (Fig. 3A). The PVX.GFP.FS mutant has a frameshift mutation at the beginning of the coat protein ORF while most of the CP ORF is deleted in PVX.GFP.ΔCP (Baulcombe *et al.,* 1995). The mutant viral RNAs accumulated at substantially lower levels in the CPΔ3#4 protoplasts than in protoplasts of a nontransgenic plant (Fig. 3B, lanes 3–6 compared with lanes 13–16). Thus, the CP mutations had no effect on the resistance observed in protoplasts of line CPΔ3#4. Since neither of these mutant viruses would produce CP, this result shows that resistance does not require the CP of the inoculated virus. Similarly, be-

cause PVX.GFP.ΔCP was strongly suppressed in protoplasts of CPΔ3#4, it is concluded that the resistance does not require sequence similarity between the CP ORF of the inoculated virus and the transgene.

PVX CP produced in line CPΔ3#4 complements a CP defect in mutant PVX

Neither the PVX.GFP.ΔCP nor the PVX.GFP.FS mutants can move out of the inoculated cells of nontransgenic tobacco (Baulcombe *et al.,* 1995). However, after inoculation to line CPΔ3#4, these mutant constructs produced expanding GFP infection foci (Fig. 4). The rate of expansion was similar to that of the foci produced when PVX.GFP was inoculated to line CPΔ3#4 (Fig. 4 and data not shown). Using immunosorbent electron microscopy, PVX-like particles were identified in leaf extracts of line CPΔ3#4 inoculated with PVX.GFP.ΔCP or the PVX.GFP.FS (Fig. 4). We could rule out that the mobile, encapsidated virus was a product of recombination between the transgene RNA and the mutant virus because RT-PCR analysis revealed only the mutant PVX RNAs in extracts of line CPΔ3#4 (data not shown). Therefore, we conclude that the transgenic CP in line CPΔ3#4 complemented the CP mutations in the inoculated PVX. However, there was no long distance movement of the mutant isolates in any of the inoculated plants of line CPΔ3#4.

DISCUSSION

In this description of line CPΔ3#4 we have confirmed that plants carrying PVX CP transgenes are resistant against PVX. In addition, we have shown that the PVX resistance was effective against a broad spectrum of PVX strains (Fig. 1 and Table 1), was expressed in the lines with the highest levels of CP transgene expression (CS and TAK, unpublished data), and could be overcome by concentrated inocula (Fig. 1). The primary level of resistance (i.e., suppression of PVX accumulation in the initially infected cells), was reflected in the fewer initial infection sites on leaves and in the low level of PVX.GFP replication in protoplasts of the resistant plants. There was also suppression of PVX movement in the resistant plants affecting both cell-to-cell and long distance spread.

In principle the resistance mechanism in line CPΔ3#4 could be either protein- or RNA-mediated. However, if the resistance is RNA-based, it must involve the 133 nucleotide residues from the 3′ noncoding region of the PVX genome. This is the only RNA that is common to both the transgene RNA and to the mutant PVX.GFP.ΔCP that was prevented from replicating in the protoplasts of line CPΔ3#4 (Fig. 3). We consider that this small region of RNA is unlikely to confer the strong resistance that we have described here (Fig. 3) based on previous results (Angell and Baulcombe, 1997). These data showed that transgenic lines expressing the 3′ noncoding region of the PVX RNA as part of a defective PVX genome were not resistant against PVX. It is therefore more likely that the resistance in line CPΔ3#4 is protein- rather than RNA-mediated. Consistent with this proposal are the observations (Fig. 1 and Table 2) that the resistance in line CPΔ3#4 is different from the RNA-mediated and homology-dependent virus resistance associated with transgene silencing: it is associated with high level expression of the CP transgene, whereas homology-dependent resistance is associated with low level transgene expression; it is effective against a broader spectrum of PVX strains than homology-dependent resistance; it is overcome by high titer inocula, whereas homology-dependent resistance is effective against high titer inocula.

To explain the resistance and mutant-complementation phenotypes of line CPΔ3#4 we propose, as suggested originally by Hemenway *et al.* (1988), that the transgenic CP interacts with the viral origin of assembly (OAS) located in the 5′ part of the PVX genome (Sit *et al.,* 1994). If PVX moves between cells as virions, as suggested previously (Allison and Shalla, 1974; Chapman *et al.,* 1992a; Oparka *et al.,* 1996), this interaction would rescue the cell-to-cell movement defect of the PVX CP mutants. Encapsidation would also sequester RNA, leaving a reduced pool of positive strand PVX RNA available for replication. In addition, the interaction would block translation of the 5′ ORF of the PVX genome that encodes the PVX RNA polymerase (Huisman *et al.,* 1988). Both the reduced availability of positive strand RNA and the block on RNA polymerase translation would result in low levels of PVX replication and accumulation in initially infected cells and protoplasts.

An attractive feature of this model is that a single interaction, between the CP and the OAS, accounts for the reduced initial infection by PVX and the complementation of movement phenotypes of the CP transgenic line. Furthermore, the detection of PVX particles in extracts of line CPΔ3#4 inoculated with the mutant PVX provides direct evidence that the proposed interaction of the CP and OAS was taking place in these plants (Fig. 4).

The resistance at the level of long distance movement of PVX is outside the scope of this model. One likely explanation invokes suppression of long distance movement as a secondary consequence of the reduced PVX replication in the PVX-resistant line. Alternatively there could be separate resistance mechanisms affecting long distance movement and PVX replication. These alternatives are currently under investigation.

In practical terms the CP-mediated resistance mechanism described here has both advantages and disadvantages relative to other types of pathogen-derived resistance against potexviruses. It has broader spectrum efficacy than the highly strain-specific homology-dependent

resistance in plants with a replicase transgene (Mueller *et al.,* 1995) but a narrower range than the resistance in plants expressing a defective movement protein (Beck *et al.,* 1994). Conversely, the CP-mediated resistance was stronger than movement protein-mediated resistance (Beck *et al.,* 1994) but not as strong as homology-dependent resistance (Mueller *et al.,* 1995). Perhaps the best approach to durable and effective pathogen-derived resistance against potexviruses will be to combine two or more of these different types of engineered resistance.

ACKNOWLEDGMENTS

C.S. and T.A.K. gratefully acknowledge financial support for Forbairt (The Irish Science and Technology Agency), Teagasc (the Irish Agriculture and Food Development Authority), BioResearch Ireland, and the Gatsby Charitable foundation. The Sainsbury Laboratory is supported by the Gatsby Charitable Foundation. We are grateful to Wojciech Kaniewski for providing PVX coat protein antiserum for Western analysis and to Simon Santa Cruz for providing the PVX strains. We also thank Giannina Brigneti and Kim Findlay for their assistance with electron microscopy and Maria Rosa Marano and June Chapman for assistance with tobacco protoplast experiments. Sue Angell, Jim English, and Jordi Garcia Mas are thanked for their comments on the draft script. Work with imported and genetically manipulated viral genes was carried out under license from MAFF [PHF11895A/801 (121)].

REFERENCES

Allison, A. V., and Shalla, T. A. (1974). The ultrastructure of local lesions induced by potato virus X: A sequence of cytological events in the course of infection. *Phytopathology* 64, 784–793.

Angell, S. M., and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *EMBO J.* 16, 3675–3684.

Baulcombe, D. C., Flavell, R. B., Boulton, R. E., and Jellis, G. J. (1984). The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. *Plant Pathol.* 33, 361–370.

Baulcombe, D. C., Chapman, S. N., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. *Plant J.* 7, 1045–1053.

Baulcombe, D. C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* 8, 1833–1844.

Beck, D. L., Van Dolleweerd, C. J., Lough, T. J., Balmori, E., Voot, D. M., Andersen, M. T., O'Brien, I. E. W., and Forster, R. L. S. (1994). Disruption of virus movement confers broad-spectrum resistance against systemic infection by plant viruses with a triple gene block. *Proc. Natl. Acad. Sci. USA* 91, 10310–10314.

Bendahmane, A., Köhm, B. A., Dedi, C., and Baulcombe, D. C. (1995). The coat protein of potato virus X is a strain-specific elicitor of Rx1-mediated virus resistance in potato. *Plant J.* 8, 933–941.

Bevan, M. W. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* 12, 8711–8721.

Chapman, S. N., Hills, G. J., Watts, J., and Baulcombe, D. C. (1992a). Mutational analysis of the coat protein gene in potato virus X: Effects of virion morphology and viral pathogenicity. *Virology* 191, 223–230.

Chapman, S. N., Kavanagh, T. A., and Baulcombe, D. C. (1992b). Potato virus X as a vector for gene expression in plants. *Plant J.* 2, 549–557.

Donson, J., Kearney, C. M., Hilf, M. E., and Dawson, W. O. (1991). Systemic expression of a bacterial gene by a tobacco mosaic virus-based vector. *Proc. Natl. Acad. Sci. USA* 88, 7204–7208.

Goodwin, J., Chapman, K., Parks, T. D., Wernsman, E. A., and Dougherty, W. G. (1996). Genetic and biochemical dissection of transgenic RNA-mediated resistance. *Plant Cell* 8, 95–105.

Hemenway, C. L., Fang, R. X., Kaniewski, W. K., Chua, N. H., and Tumer, N. E. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO J.* 7, 1273–1280.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. (1985). A simple and general method of transferring genes into plants. *Science* 227, 1229–1231.

Huisman, M. J., Linthorst, H. J. M., Bol, J. F., and Cornellissen, B. J. C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *J. Gen. Virol.* 69, 1789–1798.

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907.

Kavanagh, T. A., Goulden, M., Santa Cruz, S., Chapman, S. N., Barker, I., and Baulcombe, D. C. (1992). Molecular analysis of a resistance-breaking strain of potato virus X. *Virology* 189, 609–617.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M., and Dougherty, W. G. (1993). Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. *Plant Cell* 5, 1749–1759.

Marsh, J. L., Erfle, M., and Wykes, E. J. (1984). The pIC plasmid and phage vectors with versatile sites for recombinant selection by insertional inactivation. *Gene* 32, 481–485.

Mueller, E., Gilbert, J. E., Davenport, G., Brigneti, G., and Baulcombe, D. C. (1995). Homology-dependent resistance: Transgenic virus resistance in plants related to homology-dependent gene silencing. *Plant J.* 7, 1001–1013.

Murphy, G., and Kavanagh, T. A. (1988). Speeding up the sequencing of double-stranded DNA. *Nucleic Acids Res.* 16, 5198.

Oparka, K. J., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Santa Cruz, S. (1996). Viral coat protein is targeted to, but does not gate, plasmodesmata during cell-to-cell movement of potato virus X. *Plant J.* 10, 805–813.

Osbourn, J. K., Watts, J. W., Beachy, R. N., and Wilson, T. M. A. (1989). Evidence that nucleocapsid disassembly and a later step in virus replication are inhibited in transgenic tobacco protoplasts expressing TMV coat protein. *Virology* 172, 370–373.

Pang, S. Z., Slightom, J. L., and Gonsalves, D. (1993). Different mechanisms protect transgenic tobacco against tomato spotted wilt and impatiens necrotic spot tospoviruses. *Bio/Technology* 11, 819–824.

Pang, S. Z., Jan, F. J., Carney, K., Stout, J., Tricoli, D. M., Quemada, H. D., and Gonsalves, D. (1996). Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. *Plant J.* 9, 899–909.

Powell, P. A., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* 232, 738–743.

Prins, M., Resende, R. D., Anker, C., Vanschepen, A., Dehaan, P., and Goldbach, R. (1996). Engineered RNA-mediated resistance to tomato spotted wilt virus is sequence-specific. *Mol. Plant–Microbe Interact.* 9, 416–418.

Register III, J. C., and Beachy, R. N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology* 166, 524–532.

Reimann-Philipp, U., and Beachy, R. N. (1993). Coat protein-mediated resistance in transgenic tobacco expressing the tobacco mosaic-virus coat protein from tissue-specific promoters. *Mol. Plant–Microbe Interact.* 6, 323–330.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Santa Cruz, S., and Baulcombe, D. C. (1995). Analysis of potato virus X coat protein genes in relation to resistance conferred by the genes *Nx, Nb* and *Rx1* of potato. *J. Gen. Virol.* 76, 2057–2061.

Sijen, T., Wellink, J., Hiriart, J-B., and Van Kammen, A. (1996). RNA-mediated virus resistance: Role of repeated transgenes and delineation of targeted regions. *Plant Cell* 8, 2277–2294.

Sit, T. L., Leclerc, D., and Abouhaidar, M. G. (1994). The minimal 5′ sequence for in-vitro initiation of papaya mosaic potexvirus assembly. *Virology* 199, 238–242.

Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A., and Dougherty, W. G. (1994). Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation, and fate of nonessential RNAs. *Plant Cell* 6, 1441–1453.

Swaney, S., Powers, H., Goodwin, J., Rosales, L. S., and Dougherty, W. G. (1995). RNA-mediated resistance with nonstructural genes from the tobacco etch virus genome. *Mol. Plant–Microbe Interact.* 8, 1004–1011.

Taschner, P. E. M., Van Marle, G., Brederode, F. T., Tumer, N. E., and Bol, J. F. (1994). Plants transformed with a mutant alfalfa mosaic virus coat protein gene are resistant to the mutant but not to wild-type virus. *Virology* 203, 269–276.

Torrance, L., Larkins, A. P., and Butcher, G. W. (1986). Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. *J. Gen. Virol.* 67, 57–67.

Van der Vlugt, R. A. A., Ruiter, R. K., and Goldbach, R. W. (1992). Evidence for sense RNA-mediated protection to PVY^N in tobacco plants transformed with the viral coat protein cistron. *Plant Mol. Biol.* 20, 631–639.

Wisniewski, L. A., Powell, P. A., Nelson, R. S., and Beachy, R. N. (1990). Local and systemic spread of tobacco mosaic virus in transgenic tomato. *Plant Cell* 2, 559–567.