

Transgenic accumulation of two plant virus coat proteins on a single self-processing polypeptide

Jose F. Marcost† and Roger N. Beachy

Department of Cell Biology, The Scripps Research Institute, Division of Plant Biology – BCC 206, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

An expression cassette based on the highly specific tobacco etch potyvirus (TEV) nuclear inclusion (NIa) proteinase has been developed to produce multiple proteins through the translation of a single self-processing polypeptide. Gene constructs encoding TEV NIa, the tobacco mosaic tobamovirus (TMV) coat protein (CP) and the soybean mosaic potyvirus (SMV) CP were used to develop transgenic tobacco plants. Proper processing of the multifunctional polypeptide was demonstrated, leading to accumulation of separate proteins *in planta*. Moreover, the viral genes expressed in this way were biologically

active and conferred pathogen-derived protection to TMV, TEV and potato potyvirus Y (PVY). Transgenic plants were also derived from gene constructs in which the NIa cleavage site was mutated, resulting in the accumulation of the non-processed polyprotein, as predicted. Although transgenic proteins accumulated in low amounts in all the plant lines analysed, accumulation of the mutant non-processed protein form was greatly increased in plants following infection with TEV, but not TMV, apparently as a consequence of protein stabilization.

Introduction

Pathogen-derived resistance (PDR) (Sanford & Johnston, 1985) against plant viruses has been demonstrated through genetic transformation of plants with genes that encode either wild-type or modified viral proteins (Fitchen & Beachy, 1993; Wilson, 1993). In many cases, the accumulation of transgenic viral proteins correlates with the level of resistance achieved, and protection has been proposed to involve protein interference with specific virus processes (Reimann-Philipp & Beachy, 1993). There are examples, however, in which transgenic proteins do not accumulate, or they do so to low levels, yet significant levels of protection are observed. In some of these examples, non-translatable gene transcripts confer protection (De Haan *et al.*, 1992; Lindbo *et al.*, 1992*a, b*; Van der Vlugt *et al.*, 1992), in some cases as a result of post-transcriptional transgene silencing (Lindbo *et al.*, 1993; Mueller *et al.*, 1995; Pang *et al.*, 1996).

There are certain limitations to PDR, including the limited breadth and level of protection that is achieved via a single

transgene. One option to improve PDR is to co-express multiple viral genes in high, equimolar amounts. The rationale behind this approach is that several specific genes would protect a plant against multiple viruses, or that several genes would interfere with different stages in the infection of a single virus, thus improving the degree of protection. In an effort to accumulate multiple proteins in transgenic plants we developed an expression cassette based on the nuclear inclusion (NIa) proteinase from tobacco etch potyvirus (TEV) (Marcos & Beachy, 1994). The NIa protein is one of three proteinases in TEV that are responsible for processing the viral polyprotein (Carrington & Dougherty, 1987; Riechmann *et al.*, 1992), by cleaving at conserved heptapeptide sequences located in the viral polyprotein (Carrington & Dougherty, 1988; Dougherty *et al.*, 1988). NIa is also capable of proper cleavage of a portion of the TEV polyprotein in transgenic plants (Restrepo-Hartwig & Carrington, 1992). The TEV NIa-based cassette PRO1 (Marcos & Beachy, 1994) included the 49 kDa NIa coding sequence flanked by target heptapeptide sequences and cloning sites for in-frame insertion of two open reading frames (ORFs) (Fig. 1*a*). The cassette was designed to allow translation of a polyprotein that is self-processed, resulting in the accumulation of multiple proteins.

It was previously shown that transgenic expression of the coat protein (CP) of tobacco mosaic tobamovirus (TMV)

Author for correspondence: Roger N. Beachy.

Fax + 1 619 784 2994. e-mail beachy@scripps.edu

† **Present address:** Departamento de Patología Vegetal, CEBAS (CSIC), Apartado Correos 4195, E-30080 Murcia, Spain.

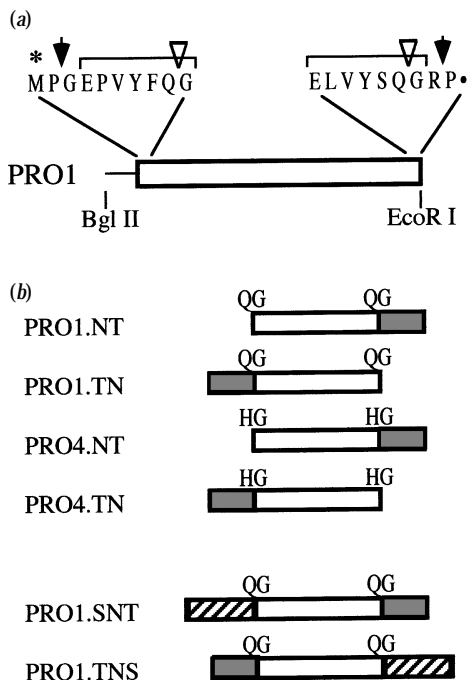


Fig. 1. (a) Schematic diagram of the Nla-based expression cassette PRO1. The TEV Nla protein sequence and TEV 5' untranslated region are shown as an open box and a line, respectively. The single-letter amino acid code at the N and C termini includes the heptapeptides recognized by the Nla proteinase (indicated by a bracket with an open arrowhead at the cleavage site). The cloning sites for insertion of foreign proteins (black arrowhead), the methionine start codon (asterisk) and the stop codon (dot) are indicated. The positions of relevant *Bgl*II and *Eco*RI restriction enzyme sites are also indicated. (b) Schematic diagram of the constructs used for plant transformation. The designation for each construct, which corresponds to the prefix of the plant line names, is indicated at the left. Open boxes represent the TEV Nla ORF, shadowed boxes the TMV CP ORF and hatched boxes the SMV CP ORF, respectively. Underlined letters are used in the names of the plant lines to show the order of the ORFs in the construct. At the boundaries between Nla and CPs are indicated the sequences for either wild-type (QG, in PRO1 constructs) or mutated (HG, in PRO4 constructs) cleavage sites for the Nla proteinase.

confers CP-mediated protection against TMV (Powell-Abel *et al.*, 1986). Likewise, the CP gene from soybean mosaic potyvirus (SMV), a non-pathogen on tobacco, protected tobacco plants against the heterologous potyviruses TEV and potato virus Y (PVY) (Stark & Beachy, 1989). As a first step to determine whether the Nla cassette leads to the accumulation of several functional CPs, TMV CP, alone or in conjunction with SMV CP, was cloned into PRO1, and the resulting gene constructs were introduced into *Nicotiana tabacum* in transformation experiments. Gene constructs were also made in which cleavage by the proteinase was abolished, to determine the effects of such modification on protein accumulation and protection.

Methods

Plant material and plant transformation. Plant line 748 is a transgenic *N. tabacum* cv. Xanthi NN line that expresses the TMV CP under the control of the cauliflower mosaic caulimovirus (CaMV) 35S

transcriptional promoter (Nelson *et al.*, 1987). Plant line 1052 is a transgenic *N. tabacum* cv. Xanthi nn line expressing the SMV CP under the control of the CaMV 35S promoter (Stark & Beachy, 1989).

All the pPRO1-derived plasmids used in this work (Fig. 1b) have been described previously (Marcos & Beachy, 1994). pPRO1-derived plasmids were digested with *Bgl*II and *Eco*RI, and the inserts containing the ORF were purified and ligated into the polylinker region of the plasmid pMON316, digested with the same two restriction enzymes. In this position, the coding sequence is placed between the CaMV 35S promoter and the nopaline synthase 3' untranslated region (Sanders *et al.*, 1987). Leaf disks of *N. tabacum* cv. Xanthi NN were transformed through *Agrobacterium tumefaciens*-mediated gene transfer as described previously (Horsch *et al.*, 1985). The resulting plants were screened for gene insertion by PCR, nopaline accumulation (Otten & Schilperoort, 1978), and accumulation of TMV CP-containing peptides by Western blot analysis.

Plants were grown under greenhouse conditions, and 4–5-week-old plants were inoculated and held in the greenhouse or transferred to a growth-room under artificial light (14 h light/10 h dark) at 25–30 °C.

Protein and RNA analysis. Leaf tissue was frozen in liquid nitrogen and stored at –80 °C. Tissue was ground to a fine powder in liquid nitrogen and proteins were extracted in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 6 M urea, 1% (w/v) SDS and 10% (v/v) β -mercaptoethanol. Extracts were boiled, centrifuged to remove debris, and loaded onto polyacrylamide gels containing SDS and subjected to electrophoresis as described (Laemmli, 1970). Proteins from equivalent amounts of leaf tissue were loaded in each gel lane. Proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell) and probed with either a polyclonal antiserum raised against TMV, a polyclonal antiserum raised against TEV Nla (kindly provided by J. C. Carrington, Texas A&M University, USA), or a polyclonal antiserum raised against the core of the CP from johnsongrass mosaic potyvirus (kindly provided by D. D. Shukla, CSIRO, Melbourne, Australia), which recognizes most potyviral CPs. Antibody binding was detected by immunoreaction with anti-rabbit IgG coupled to horseradish peroxidase (Pierce) and subsequent enhanced chemiluminescence (Amersham).

Total RNA was extracted following a small-scale procedure described previously (Verwoerd *et al.*, 1989). RNA analysis by Northern blot hybridization to 32 P-labelled TMV CP cDNA was done essentially as described by Sambrook *et al.* (1989).

Viruses and virus inoculations. TMV (UI strain) was propagated on *N. tabacum* cv. Xanthi nn, and TEV and PVY were propagated on *N. tabacum* cv. Xanthi NN. TMV was purified as described (Padgett & Beachy, 1993; Tobias *et al.*, 1982). Prior to inoculation with TMV, plants were topped, kept for 1 day in the dark and TMV was diluted in inoculation buffer IB (20 mM potassium phosphate pH 7.0, 1 mM EDTA) and rubbed onto leaves dusted with carborundum. TEV and PVY inocula were prepared by grinding infected plant tissue in IB (40 mg/400 μ l); dilutions of leaf extracts were rubbed onto leaves dusted with carborundum.

Results and Discussion

Viral coat proteins translated as part of Nla-based polyproteins are properly processed *in planta*

Nicotiana tabacum cv. Xanthi NN plants were transformed with genes comprising the CaMV 35S promoter and one of several derivatives of the proteinase cassette. An initial experiment was designed to examine whether Nla-containing

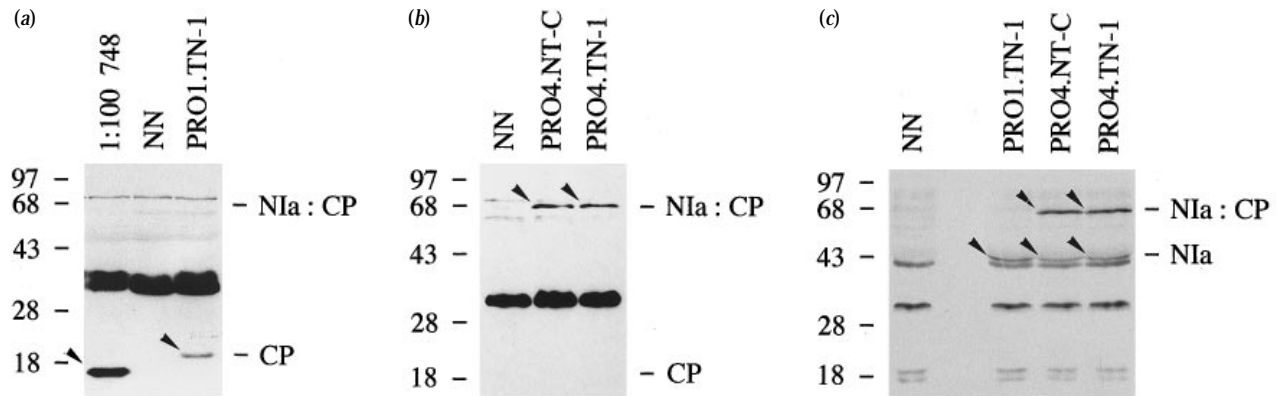


Fig. 2. Western immunoblot analyses of proteins extracted from plant lines PRO1.TN-1, PRO4.NT-C and PRO4.TN-1. The plant lines are indicated above each lane. Protein extract from control line 748 (containing TMV CP; Nelson *et al.*, 1987) was diluted 1:100 with untransformed tobacco extract (NN) prior to loading on the gel. Proteins were immunostained with either anti-TMV (a) and (b), or anti-TEV NIa (c), antisera. The positions of the TEV NIa-TMV CP fusion protein (68 kDa), and the TMV CP (~18.5 kDa) and TEV NIa (49 kDa) cleaved from the polyprotein are indicated on the right. The molecular mass (kDa) and positions of protein markers are indicated on the left. Small arrowheads indicate transgenic proteins.

polyproteins were self-processed properly *in vivo* due to specific recognition and cleavage by NIa. For this purpose, two versions of the cassette were used: PRO1, which contains the natural heptapeptide recognition sequences for the TEV NIa proteinase, and PRO4, in which a glutamine-to-histidine mutation was engineered at the -1 position of both cleavage sites (Fig. 1b); it was previously shown that such mutations would abolish cleavage by NIa (Dougherty *et al.*, 1988). In both cases, the sequence coding for TMV CP was inserted either before (PRO1.TN and PRO4.TN) or after (PRO1.NT and PRO4.NT) the NIa sequence (Fig. 1b). Proper translation and predicted processing of these four constructs had been demonstrated in previous *in vitro* translation experiments, rendering the TMV CP processed in the case of PRO1 constructs and fused to NIa in PRO4 (Marcos & Beachy, 1994).

A total of 15 independent R0 transformed plant lines was produced: three containing the PRO1.NT construct, six containing PRO1.TN, two containing PRO4.NT and four containing PRO4.TN. Western blot analyses indicated that transgenic proteins accumulated to unexpectedly low levels (see below). The poor accumulation of proteins was reflected in the failure to detect TMV CP in several plant lines. Eight out of ten lines transformed with constructs in which the CP was translated upstream of the NIa (i.e. PRO1.TN and PRO4.TN) were identified as containing TMV CP. TMV CP was detected in only one plant line, out of five transformants recovered, in which the CP was translated downstream of the proteinase (line PRO4.NT-C, encoding a mutated non-processed polypeptide, see below). R1 seeds were obtained from selected lines to study further the accumulation and processing of transgenic proteins.

Fig. 2 shows Western blot analyses of three representative plant lines transformed with three different constructs, and

illustrates the results obtained in all the lines. TMV CP expressed as part of construct PRO1.TN had an apparent molecular size larger than that of wild-type CP (Fig. 2a). The difference in size (~1 kDa) is presumably due to the nine amino acid residues which are predicted to remain joined to the CP after cleavage of the polyprotein by NIa (Fig. 1a), and parallels previous *in vitro* data (Marcos & Beachy, 1994). Plant lines that contain gene construct PRO1.TN accumulated TMV CP and TEV NIa and did not accumulate detectable levels of the 68 kDa fusion protein (Fig. 2a, c), indicating that proteolytic processing was complete when the heptapeptide recognition site for the NIa proteinase was present. Auto-proteolysis was NIa specific, since it was largely abolished by a mutation in the heptapeptide that is recognized and cleaved by NIa; as predicted, the 68 kDa uncleaved fusion protein accumulated in those lines that contained the mutant PRO4 constructs and it was detected following immunoreaction with either anti-TMV or anti-NIa serum (Fig. 2b, c). However, the detection in PRO4 lines of NIa (in lower amounts than the predicted 68 kDa band observed in the same lanes) (Fig. 2c) and also of CP (in experiments in which the blots were exposed for longer time) [see below, Fig. 5; line PRO4.NT-C at 0 days post-inoculation (p.i.)] suggest that cleavage occurred to some extent in these plants.

Transgenic plants were also produced in which the CP genes from TMV and SMV were cloned into PRO1 in either of the two possible orientations to obtain constructs PRO1.SNT and PRO1.TNS (Fig. 1b). The *in vitro* processing of the polypeptides encoded by these constructs was described previously (Marcos & Beachy, 1994). Ten tobacco R1 lines transformed with each construct were analysed. TMV CP was detected in seven lines, all of which contained construct PRO1.TNS, and exhibited, as described above, a molecular size

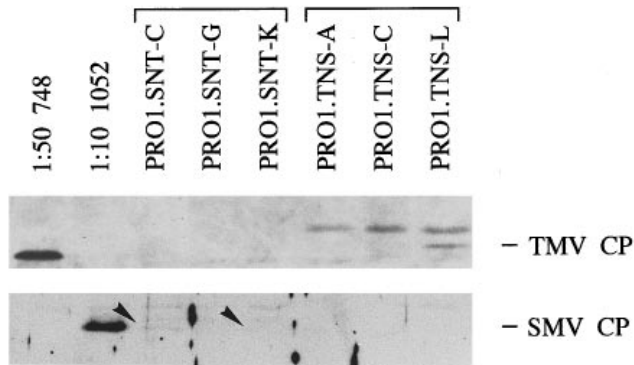


Fig. 3. Western immunoblot analyses of proteins extracted from plant lines transformed with constructs PRO1.SNT and PRO1.TNS. The plant lines are indicated above each lane. Protein extracts from control lines 748 (containing TMV CP; Nelson *et al.*, 1987) and 1052 (containing SMV CP; Stark & Beachy, 1989) were diluted fiftyfold and tenfold, respectively, with extract from untransformed tobacco plants. Proteins were immunostained with either anti-TMV (top panel) or anti-potyvirus CP (bottom panel) antisera. The migrations of the corresponding CPs are indicated on the right. Small arrowheads indicate the faint SMV CP bands (detectable SMV CP was observed in PRO1.SNT-K in the original autoradiograph). No transgenic protein was detected in transformed line PRO1.SNT-G.

larger than that of the wild-type CP (Fig. 3; lines PRO1.TNS-A, PRO1.TNS-C and PRO1.TNS-L are shown as examples). In some plant lines, an additional immunoreactive band migrating as wild-type TMV CP was detected (Fig. 3, line PRO1.TNS-L); it is predicted that the amino acids that contain the NIa recognition sequence will be located at the surface of the CP (Namba *et al.*, 1989), and may be susceptible to the activity of cellular proteases. SMV CP was much more difficult to detect and only two plant lines, both of which were transformed with construct PRO1.SNT, showed a very faint band of antibody-antigen reaction (Fig. 3, lines PRO1.SNT-C and PRO1.SNT-K).

In conclusion, data presented confirm that heterologous proteins are properly translated and processed *in planta*, as a result of the expression of chimaeric transgenes derived from PRO1 (Figs 2 and 3). No precursor polyprotein was detected in plant lines that harboured PRO1-derived constructs, indicating that processing was highly efficient.

Transgenic proteins translated as part of PRO1 cassettes accumulate to low levels

Low levels of transgenic proteins accumulated in all the plant lines analysed. TMV CP accumulation was more than 100-fold lower than in plant line 748 (Fig. 2a). Line 748 harbours the TMV CP sequence under control of the CaMV 35S promoter and is highly resistant to TMV infection (Nelson *et al.*, 1987). The accumulation of transgenic proteins in the lines described here was less than 0.001% (w/w) of total protein; this level is considerably lower than that expected from a gene whose expression is driven by the CaMV 35S promoter. Northern blot analyses revealed that the steady-

state levels of transgenic mRNA in these plants were within the range found for other transgenes driven by the 35S promoter (not shown), and it was therefore concluded that mRNA levels did not account for the low accumulation of proteins. Previous work has shown that TMV CP accumulates to good levels when expressed as a transgene under the control of the CaMV 35S promoter (Powell-Abel *et al.*, 1986; Nelson *et al.*, 1987). In the present study, we characterized up to 35 plant lines transformed with TMV CP- and NIa-containing constructs and failed to identify a single line with high levels of TMV CP. We therefore concluded that low protein accumulation is likely due to the NIa sequence.

It is also notable that many more lines accumulated the transgenic protein when gene constructs were oriented such that the CP ORF was positioned at the N terminus rather than at the C terminus of NIa; 15 out of 20 transformed lines versus 1 out of 15, respectively, in the case of TMV CP. Restrepo-Hartwig *et al.* (1990) fused the β -glucuronidase gene (*uidA*) to the TEV NIa gene for studies of nuclear targeting, and found that the enzyme activity expressed was higher when the gene was placed N-terminal relative to NIa. These observations suggest that C-terminal proteins from PRO1 constructs may accumulate (at least transiently) but at levels below the limit of detection. The data in Fig. 3 illustrate that protein sequences translated at the N terminus of the NIa were easier to detect (i.e. accumulated to higher levels) than those translated at the C terminus. Our conclusion is that the desired equimolar yields of proteins were not achieved with the PRO1 cassette. One possible explanation for the reduced accumulation of C-terminal proteins could be that the highly effective nuclear targeting of NIa (Restrepo-Hartwig *et al.*, 1990) directs a fraction of the chimaeric polyprotein to the nucleus while causing premature termination of translation. Other considerations, such as the relative stability of different proteins, should also be taken into account. We are currently attempting to elucidate the basis for the low levels of accumulation of proteins when expressed through the NIa-based cassette, with the hope of improving its utility.

Pathogen-derived resistance against multiple viruses in plants transformed with NIa-based cassettes

Experiments were conducted to evaluate the suitability of the NIa-based system to confer CP-mediated protection against multiple viruses. Table 1 shows the level of protection in plant lines upon inoculation with TMV. Lines PRO1.TNS-A, PRO1.TNS-C and PRO1.TNS-L, which accumulated detectable levels of TMV CP, were moderately protected. Lower levels of protection were observed in plant lines PRO1.SNT-C and PRO1.SNT-K, although TMV CP could not be detected in any line transformed with construct PRO1.SNT. The level of protection achieved was less than that of line 748, presumably due to the differences in the accumulation of transgenic CP (Fig. 3). An untransformed internal control line (Table 1,

Table 1. Evaluation of coat protein-mediated protection to TMV in transgenic plant lines

	Plant line†	No. of lesions‡
Expt 1 (100 ng/ml)	Non-transgenic	217 ± 30
	748	0 ± 0(*)
	PRO1.ctrl	161 ± 51
	PRO1.SNT-C	91 ± 45(*)
	PRO1.SNT-G	196 ± 57
	PRO1.TNS-A	43 ± 16(*)
	PRO1.TNS-C	39 ± 17(*)
Expt 2 (40 ng/ml)	Non-transgenic	117 ± 29
	PRO1.SNT-K	58 ± 37(*)
	PRO1.TNS-A	16 ± 7(*)
	PRO1.TNS-L	42 ± 19(*)

† Plant line 748 accumulates high levels of TMV CP and is highly resistant to TMV (Nelson *et al.*, 1987). Plant line PRO1.ctrl is a non-transgenic internal control line produced during the plant transformation experiments described in this work. Other plant lines are described in the text.

‡ Leaves were inoculated with TMV at two different concentrations depending on the experiment. Eight plants (one leaf per plant) were inoculated and the average number of necrotic local lesions ± standard deviation is given. Values statistically lower than those shown by the non-transgenic tobacco NN control at 95% confidence (Student's *t*-test) are marked (*).

PRO1.ctrl) was not protected, as predicted. Similar results were obtained in plant lines transformed with PRO1-derived constructs which contain only TMV CP (not shown).

It was previously shown that the SMV CP provides heterologous resistance against TEV and PVY (Stark & Beachy, 1989). When plant lines described here were challenged with TEV as a 1:1000 dilution of infected sap, a moderate delay and a decrease in the number of infected plants were observed in plant lines PRO1.SNT-C, PRO1.TNS-A and PRO1.TNS-L (Fig. 4a), and in PRO1.TNS-C (not shown). Furthermore, plants that became infected exhibited milder symptoms than non-transformed plants. However, all the plants became infected when the inoculum concentration was raised (1:100 dilution of infected sap) (not shown). Plants were also protected against PVY infection (Fig. 4b). On the basis of these studies, it was concluded that these lines exhibited a low degree of protection against the heterologous TEV and PVY, in agreement with previous data (Stark & Beachy, 1989). Control line 1052, which accumulates high levels of SMV CP (Fig. 3), was fully protected against TEV infection at the inoculum concentration used in Fig. 4 (not shown). On the other hand, there was no protection against either TEV or PVY in control line PRO1.ctrl (Fig. 4a, b) and line 748 (not shown).

Certain plant lines transformed with constructs lacking SMV CP sequences exhibited a low degree of protection against TEV (Fig. 4c), but not PVY (not shown). In these

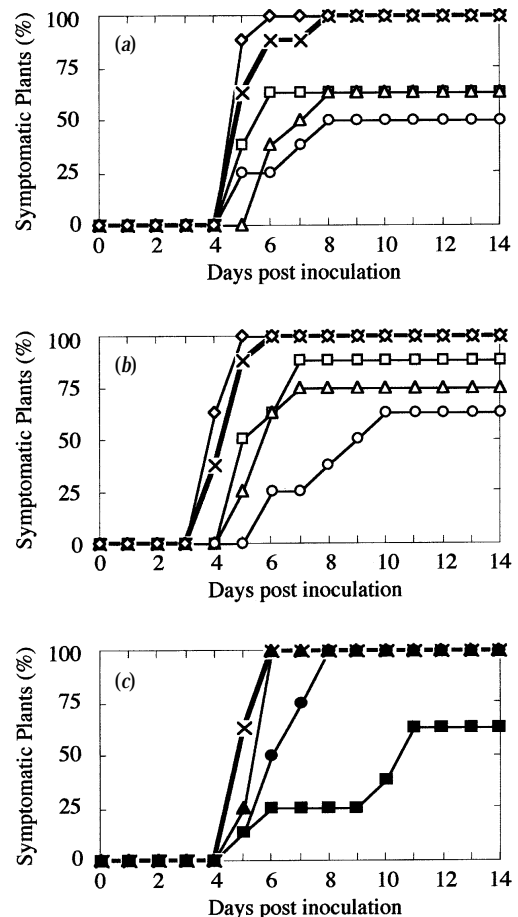


Fig. 4. Protection against potyvirus infection in plant lines transformed with PRO1-derived constructs. (a) Protection against TEV infection in untransformed control line PRO1.ctrl (◇), and plant lines PRO1.SNT-C (□), PRO1.TNS-A (△) and PRO1.TNS-L (○). (b) Protection against PVY infection in the same plant lines [same symbols as in (a)]. (c) Protection against TEV infection in plant lines PRO1.TN-1 (■), PRO4.NT-C (▲) and PRO4.TN-1 (●). In (a), (b) and (c), tobacco NN control is represented by a cross and a thicker line. Eight plants per line were inoculated (1:1000 dilution of sap from infected tobacco leaves) and the percentage of plants showing symptoms (vertical axis) on successive days p.i. (horizontal axis) was scored.

experiments, protection was also observed as a decrease in the accumulation of the TEV CP of the challenging virus (not shown), even in lines PRO4.NT-C and PRO4.TN-1 in which all the plants became infected. From these data, it was concluded that protection against TEV in lines PRO1.SNT and PRO1.TNS is likely due, at least in part, to the expression of the NIa gene. Previous results demonstrated that the NIa proteinase of tobacco vein mottling potyvirus (TVMV) conferred protection against infection by TVMV, although not against other potyviruses (Maiti *et al.*, 1993). Similar results were obtained with the NIa protein of PVY (Vardi *et al.*, 1993). Our work extends these results to a third potyvirus, TEV.

Taken together, these data indicate that viral genes expressed *in planta* as part of the NIa self-processing polypeptides are biologically active in conferring pathogen-derived

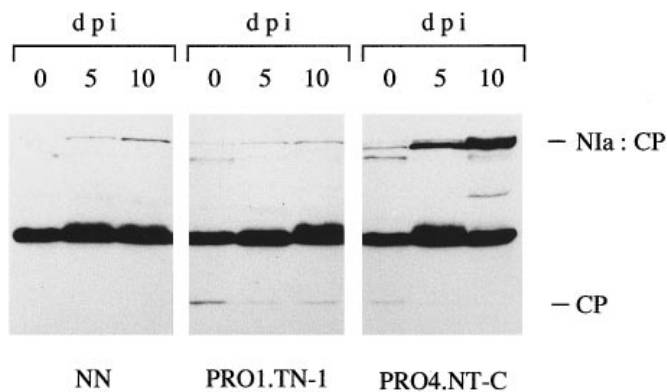


Fig. 5

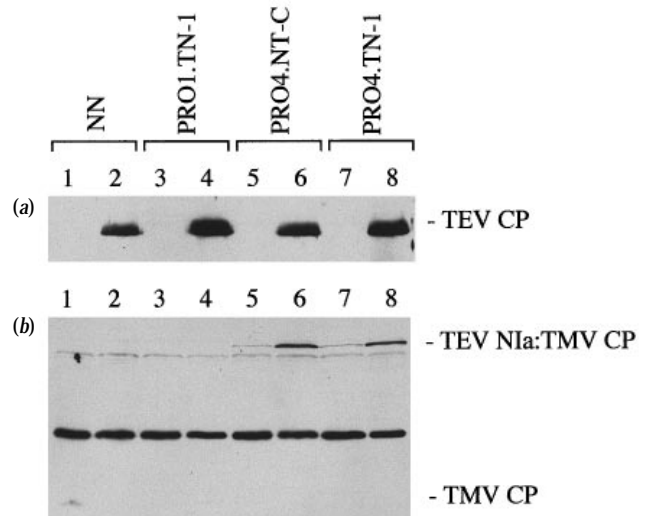


Fig. 6

Fig. 5. Changes in accumulation of transgenic proteins upon TEV infection in plant lines transformed with PRO1-derived constructs. Western immunoblot analysis of protein extracts from TEV-infected systemic leaves of untransformed tobacco NN and plant lines PRO1.TN-1 and PRO4.NT-C, at 0, 5 and 10 days p.i. Plants were infected with a 1:500 dilution of sap from TEV-infected tobacco leaves. Proteins were immunostained with an anti-TMV serum. The migrations of the TEV NIa-TMV CP fusion protein and the TMV CP cleaved from the polyprotein are indicated on the right.

Fig. 6. Accumulation of proteins upon TEV infection in plant lines transformed with PRO1-derived constructs. Untransformed tobacco plants (NN) and plant lines PRO1.TN-1, PRO4.NT-C and PRO4.TN-1 were either mock (lanes 1, 3, 5 and 7) or TEV inoculated (lanes 2, 4, 6 and 8) (1:500 dilution of sap from TEV-infected leaves) and systemic tissues were harvested at 10 days p.i. for analysis. (a) Western blot analysis of protein extracts immunostained with an antiserum raised against the core domain of potyviral CPs. (b) Western blot analysis of protein extracts immunostained with an anti-TMV antiserum. Migrations of TEV NIa-TMV CP fusion protein and TMV CP are indicated on the right.

protection, despite the low level of accumulation of the proteins encoded by the transgene. In the present study, we have demonstrated the feasibility of applying this approach to express multiple biologically active genes. Von Bodman *et al.* (1995) used a system based on TMV NIa to produce functional enzymatic activities of two different proteins, although data on accumulation and processing of transgenic proteins were not presented.

Increased accumulation of transgenic proteins upon infection with TEV

During experiments in which plant lines PRO1.TN-1, PRO4.NT-C and PRO4.TN-1 were inoculated with TEV, systemic leaves were harvested at different times after inoculation and analysed using anti-TMV serum for the accumulation of transgenic proteins. These assays revealed an increase in the accumulation of mutant NIa-CP fusion protein following TEV infection of lines PRO4.NT-C (Fig. 5) and PRO4.TN-1 (see below), but not of line PRO1.TN-1 in which all the polyprotein was cleaved (Fig. 5). Moreover, the amount of TMV CP in line PRO1.TN-1 was not significantly changed following infection by TEV. The increased amount of uncleaved protein was greatest in plants that became infected and

accumulated TEV, indicating that increased accumulation was a consequence of infection with TEV.

To eliminate the possibility that increased accumulation of the fusion protein was a consequence of plant age rather than TEV infection, plants were either mock- or TEV-inoculated and tissue was analysed at 10 days p.i. for the presence of TEV CP and transgenic TMV CP (Fig. 6). In these experiments, a high inoculum concentration was used in order to overcome protection and thus better study the increase of transgenic protein effect. The increase in the amount of fusion proteins, but not of processed TMV CP, was confirmed to be dependent on TEV infection (Fig. 6*a, b*). Increased accumulation of the fusion protein could result from either an increase in levels of mRNA of the transgene or an increase in protein stability. The fact that the change in the level of TMV CP-related proteins was observed in plant lines that accumulated derivatives of the PRO4, but not PRO1, cassette supports the second possibility since both constructs have the same regulatory elements and they only differ in three nucleotides at the processing sites (Marcos & Beachy, 1994). Northern blot analysis was carried out to confirm that steady-state levels of transgene-specific mRNAs were the same in mock- or TEV-inoculated plants (not shown). It was concluded that the increased accumulation of transgenic polyprotein following infection with TEV was most

likely a consequence of increased protein stability. Moreover, increased accumulation of NIa-TMV CP fusion protein in PRO4 lines was specific to TEV infection and it did not occur when plants were infected with either PVY or TMV (not shown).

In many cases of PDR, it is not possible to study the accumulation of transgenic protein(s) following challenge by virus because the transgene(s) encodes protein(s) that cannot be readily distinguished from those produced by the challenging virus. Farinelli *et al.* (1992) used strain-specific antibodies and reported that accumulation of transgenic CP from PVY^N increased upon inoculation of the plants with PVY^O. In our study, the distinct molecular size of the NIa-TMV CP fusion protein allowed us to distinguish between transgenic products and those resulting from virus infection. The increase in the accumulation of transgenic fusion protein due to stabilization may imply that, in the absence of virus infection, transgenic proteins do not accumulate well due to protein instability. We suggest that the fusion protein is stabilized because of its interaction with product(s) of TEV infection. An attractive hypothesis is that transgenic proteins containing TEV NIa are included in the nuclear inclusions formed by the NIa and NIB replicase following virus infection (Riechmann *et al.*, 1992).

Lindbo *et al.* (1993) reported a marked decrease in the steady-state levels of transgene mRNA upon virus inoculation as a consequence of an RNA-mediated co-suppression-like mechanism. The differences between the changes of the accumulation of transgenic products reported by Lindbo *et al.* (1993) and those described here emphasize the complex nature of the interactions between viral transgene products and the viruses from which they were derived. Where possible, future studies should address the changes in the accumulation of transgenic viral proteins after virus challenge to increase our understanding and develop more effective strategies for PDR.

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