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

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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., 1992a, 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 protease 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 heptapeptide sequences and cloning sites for in-frame insertion of two open reading frames (ORFs) (Fig. 1a). 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)
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 potyvirus 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 BglII and EcoRI, 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 32P-labelled TMV CP cDNA was done essentially as described by Sambrook et al. (1989).

**Viruses and virus inoculations.** TMV (U1 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/400μl); 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
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 pro- teolytic 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
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 Nla-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 Nla 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 Nla; 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 Nla gene for studies of nuclear targeting, and found that the enzyme activity expressed was higher when the gene was placed N-terminal relative to Nla. 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 Nla 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 Nla (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 Nla-based cassette, with the hope of improving its utility.

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

Experiments were conducted to evaluate the suitability of the Nla-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

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

† 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 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-I 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 NLa gene. Previous results demonstrated that the NLa 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 NLa 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 NLa self-processing polypeptides are biologically active in conferring pathogen-derived...
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 TVMV Nla 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 Nla–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. 6a, 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 Nla–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 PVYN increased upon inoculation of the plants with PVYO. In our study, the distinct molecular size of the Nla–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 Nla are included in the nuclear inclusions formed by the Nla and Nlb 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 in 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.

We are grateful to C. Fux for plant transformation and S. Leitner for growth and maintenance of plant material. We acknowledge the gift of polyclonal antiserum against the central core of potyviral coat proteins from Dr D. D. Shukla (CSIRO, Australia). We acknowledge the gift of tobacco plants related to homology-dependent gene silencing. The authors wish to acknowledge the suggestions made during the review process of this manuscript. J.F.M. was recipient of a postdoctoral fellowship from the Ministry of Science (Spain). Other support was provided by RO1-AI 27161 from the National Institutes of Health (USA).

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Powell-Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G.,


Received 12 September 1996; Accepted 14 March 1997