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# *In vitro* characterization of a cassette to accumulate multiple proteins through synthesis of a self-processing polypeptide

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## Abstract

The strategy for processing the polyprotein encoded by plant potyviruses has been mimicked by constructing an expression cassette based on the nuclear inclusion (Nla) proteinase from tobacco etch virus (TEV). This cassette (pPR01), includes the TEV Nla coding region flanked on each side by its heptapeptide cleavage sequence and cloning sites for the in frame insertion of two different open reading frames. pPR01 allows the synthesis, under the control of a single transcriptional promoter, of two proteins in equimolar amounts as part of a polyprotein which is cleaved into individual mature products by the TEV protease. In *in vitro* reactions the cassette functioned as expected when several different protein-coding sequences were used. The potential uses of pPR01 are discussed.

## Introduction

In recent years, development of plant transformation techniques and strategies for enhancing and controlling gene expression have broadened the practical applications of plant biotechnology. However, the potential of these techniques must deal with the problems encountered when more than one transgene is expressed *in planta*.

Current approaches to express more than one gene in transgenic plants require the use of multiple promoters, which in itself presents problems related to levels of expression from each promoter [16]. For example, the relative levels of expression in potato plants of two genes encoding two viral coat proteins (CP), which were introduced via a single Ti-derived transformation vector, were different in different plant lines [15]. An alternative approach, i.e., re-transforming plants with a second gene, may induce gene-silencing effects [11, 17]. Sexual crossing of different transgenic lines may enhance or inhibit gene expression depending on gene copy number and the nature of gene insertion [13]. Therefore, the relative levels of gene expression can not be predicted but rather are a consequence of experimental variability.

Our laboratory is interested in alternative mechanisms to express multiple genes in a single transgenic line as a way to improve pathogen derived protection against plant viruses. In this context, we have developed a system which allow equimolar accumulation of several proteins under the control of a single transcriptional promoter, thereby avoiding the problems outlined above.

Several plant and animal viruses encode pro-

teinases that cleave the viral polypeptides yielding mature proteins. Plant potyvirus genomes are expressed through the translation of a single polypeptide which is processed to release individual viral proteins [20]. Three viral proteinase activities have been implicated in this processing [7, 24]. One of these, corresponding to the nuclear inclusion (Nla) protein, has been widely studied in the case of tobacco etch potyvirus (TEV) [2, 4], and is responsible for several processing events of the large viral polypeptide. Nla from TEV exerts this function through the recognition and cleavage of a specific heptapeptide [Glu-X-X-Tyr-X-Gln/Gly (or Ser)] [5, 8]. Nla releases itself from the polyprotein in an autoproteolytic reaction [3], and is active both *in cis* (processing polypeptides in which it is included) and *in trans* (cleaving in a different polypeptide). The *cis* protease activity has been assayed with different TEV polyproteins produced *in vitro* which contained Nla and either naturally occurring or mutated versions of the cleavage sequence [2, 4]. Protease activity *in trans* has been studied using as substrates TEV polyproteins that were labeled *in vitro* and incubated with Nla either extracted from infected plants [4, 5, 8, 9] or synthesized in *in vitro* translation reactions [2, 4, 18].

We have taken advantage of this well characterized proteinase activity to develop an expression cassette based on the TEV-Nla protein. This cassette allows the synthesis of two proteins in equimolar amounts as part of a polyprotein that is cleaved into individual mature proteins by the Nla proteolytic activity. The *in vitro* characterization of this expression system demonstrated that it functions as expected when different proteins are used.

## Materials and methods

### *Cloning strategies*

Recombinant DNA manipulation and *Escherichia coli* formation were carried out according to existing protocols [22]. The DNA inserts used for the assembly of the different constructs were obtained by the polymerase chain reaction (PCR, Perkin Elmer Cetus). The expression cassette pPRO1 (see Results and Fig. 1) was assembled in pBluescript II KS (+) (Stratagene) under the transcriptional control of a T7 promoter. Nla and 5′-non-translated (5-NTR) sequences from TEV were obtained by PCR using as DNA template a full-length TEV cDNA clone (kindly provided by Dr J. Carrington, Texas A&M University). Oligonucleotide primers for amplification of Nla were TEVNIA.N and TEVNIA.C (see Table 1 for the sequences of all primers used in this work). These two primers amplified the Nla open reading frame

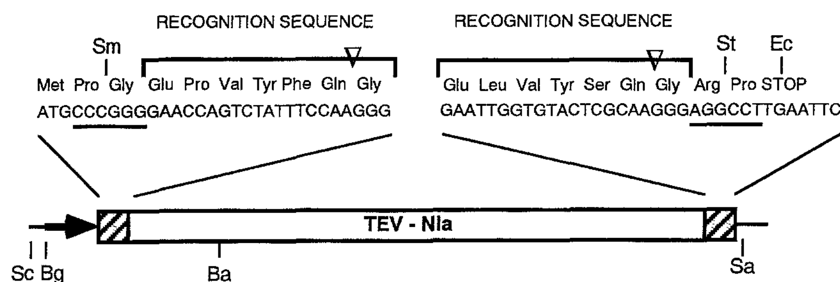


*Fig. 1.* Schematic diagram of the expression cassette pPRO1. The open box represents the Nla open reading frame. The shaded areas enlarged above show (as both nucleotide and amino acid sequence) the heptapeptide recognition sequence for the Nla proteolytic activity at both N- and C-termini of Nla; the engineered *Sma* I and *Stu* I cloning sites (underlined) for the in frame introduction of different genes; and start ATG and stop TGA codons. The Nla processing site between Gln and Gly is indicated as an open arrowhead. The sequence of the TEV 5′ untranslated region is also indicated with a black arrow upstream of Nla. Relevant unique restriction enzyme sites are indicated: Ba (*Bam* HI), Bg (*Bgl* II), Ec (*Eco* RI), Sa (*Sal* I), Sc (*Sac* I), Sm (*Sma* I), and St (*Stu* I).

*Table 1.* Sequences of the oligonucleotide primers used.[1]

| Primer | Sequence |
|---|---|
| TEVNIA.N | 5′-GCTCTAGA CCCGGG GAACCAGTCTATTTCCAAGGG-3′ |
| TEVNIA.C | 5′-GCGAATTCAAGGCCT CCCTTGCGAGTACACCAATTCA-3′ |
| TEVNTR.5 | 5′-GCCGAGCTC AGATCT AAATAACAAATCTCAACACAACA-3′ |
| TEVNTR.3 | 5′-TCCCCCGGG CATGGCTATCGTTCGTAAATGG-3′ |
| TEVNIA.N2[2] | 5′-TGGCCCGGG GAACCAGTCTATTTCCA**T**GGG-3′ |
| TEVNIA.C3[2] | 5′-GCGAATTCAAGGCCT CCCA**T**GGGAGTACACCAATTCA-3′ |
| TMVCP.51 | 5′-AAAGGCCT TCTTACAGTATCACTACTCC-3′ |
| TMVCP.31 | 5′-AGGCCCGGG AGTTGCAGGACCAGAGGTCC-3′ |
| SMVCP.N1 | 5′-AAAGGCCT TCAGGCAAGGAGAAGG-3′ |
| SMVCP.C2 | 5′-AGGCCCGGG CTGCGGTGGGCCCATGC-3′ |
| GUS.N2 | 5′-AAAGGCCT GTAGAAACCCCAACCCG-3′ |
| GUS.C1 | 5′-CGGAATTC TCATTGTTTGCCTCCCTGCTG-3′ |

[1] Nucleotides annealing to the target genes are underlined with a single line. Nucleotides corresponding to restriction enzymes sites located at the 5′ end of the oligonucleotides are doubly underlined.

[2] Nucleotides changed in TEVNIA.N2 and TEVNIA.C3, when compared with TEVNIA.N and TEVNIA.C respectively, are written in bold characters. These changes introduced an *Nco* I site.

plus the sequences encoding the two specific heptapeptide cleavage sequences located at each end of Nla in the TEV genome. In addition, the primers contained either *Xba* I and *Sma* I (TEVNIA.N) or *Stu* I and *Eco* RI (TEVNIA.C) sites. The PCR product was directionally cloned into pBluescript using *Xba* I and *Eco* RI to yield pBCNla. Oligonucleotide primers for amplification of the 5-NTR of TEV were TEVNTR.5 and TEVNTR.3. In this case the primers contained either *Sac* I and *Bgl* II (TEVNTR.5) or *Sma* I (TEVNTR.3) sites. The final step in the assembly of pPRO1 was a *Sac* I-*Sma* I directional insertion of the TEV 5-NTR into pBCNla. Mutagenesis within the heptapeptides encoding the protease cleavage sequences was accomplished with primers TEVNIA.N2 and TEVNIA.C3, which contained either one or two nucleotide changes (when compared to TEVNIA.N and TEVNIA.C, respectively) that mutated the glutamine located at position −1 (relative to the cleavage site) to histidine (introducing at the same time an *Nco* I site which was used to recover the recombinant clones).

The different open reading frames (ORFs) inserted into pPRO1 included those encoding tobacco mosaic virus (TMV) and soybean mosaic virus (SMV) coat proteins (CP), as well as the *uidA* gene encoding the $\beta$-glucuronidase (GUS) activity from *E. coli*. These ORFs were obtained by PCR using as template cDNAs available in our laboratory; the GUS gene was obtained from Clontech. The primers used for amplification are detailed in Table 1, the prefix indicating the gene to which they were targeted. PCR products corresponding to SMV- and TMV-CP genes were digested with *Stu* I and *Sma* I and inserted either at the *Sma* I or at the *Stu* I sites of pPRO1, depending on the construct. The PCR product corresponding to the *uidA* ORF was digested with *Stu* I and *Eco* RI and inserted at the C terminus of Nla in pPRO1.

### In vitro *transcription and translation*

One $\mu$g of plasmid DNA purified from *E. coli* through Qiaprep mini columns (Qiagen) was first linearized with *Sal* I (which cleaves downstream of pPRO1), and subsequently transcribed *in vitro* with T7 RNA polymerase (Epicentre Technolo-

gies). Size and integrity of transcribed mRNA were confirmed by agarose gel electrophoresis. About 1 $\mu$g of mRNA was used to program *in vitro* translation in 25 $\mu$l volume reactions using a nuclease-treated rabbit reticulocyte lysate system (Promega) according to the manufacturer's protocol. $^{35}$S-Met was used as the labeled amino acid. However, since TMV CP contains no methionine residues, $^{3}$H-Leu was used when this ORF was translated. Proteins translated *in vitro* were analyzed by autoradiography following SDS-PAGE [14].

*Immunoprecipitation assays*

Immunoprecipitation assays were based upon previously described protocols with minor modifications. Briefly, 20 $\mu$l aliquots of *in vitro* translation reactions were diluted to 100 $\mu$l with TBSN (25 mM Tris-HCl pH 7.5, 150 mM NaCl 1% Nonidet P-40) and pre-incubated with protein A Sepharose beads (Sigma) for 15 min on ice. After removing the beads, 1 $\mu$l of an appropriate dilution of a polyclonal antibody raised against TMV CP was added, and the mixture was incubated for 2–4 h at 4 °C with slow shaking. Subsequently, protein A Sepharose beads previously blocked with rabbit reticulocyte lysate were added and the mixture was kept on ice for 15 min with occasional shaking. The Sepharose beads were recovered and washed twice with 0.5 M LiCl, 20 mM Tris-HCl pH 8, once with TBSN, and once with $H_2O$. Finally, beads containing immunoprecipitated labeled proteins were resuspended in SDS-PAGE loading buffer and the proteins were analyzed as described above.

## Results

*pPRO1, a TEV-Nla protease-based expression cassette*

A TEV-Nla-based expression cassette has been constructed to express two different proteins in equimolar amounts. This cassette, named pPRO1 (Fig. 1), was first assembled in pBluescript II KS (+) under the transcriptional control of the T7 promoter. PCR was carried out, using as template a full length TEV cloned cDNA, to obtain an open reading frame encompassing the Nla sequence as well as the target heptapeptides located at its N- and C-termini (TEV nucleotides (nt) 5673 to 6983 as numbered in [1]). The oligonucleotides used for priming the PCR reactions also contained blunt-end restriction sites to allow the in frame insertion of foreign protein sequences (either in a *Sma* I site at the N-terminus or in a *Stu* I site at the C-terminus). ATG start and TGA stop codons were engineered upstream of the *Sma* I site and downstream of the *Stu* I site, respectively. In addition, the 144 nucleotide 5′ untranslated region from TEV RNA, which has been shown to enhance translation *in vitro* and *in vivo* [6], was included upstream of the open reading frame. pPRO1 was sequenced and six changes were found when compared to the previously published TEV sequence. These changes were, according to numbering in [1], GC to CG at nt 5768–5769, A to G at nt 5773, A to G at nt 6235, T to C at nt 6314, and A to G at nt 6961, and were

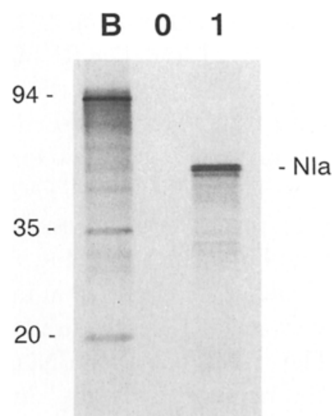


*Fig. 2. In vitro* translation of RNA transcribed from the pPRO1 expression cassette. Translation reactions were programmed with 1 $\mu$g of brome mosaic virus (BMV) RNAs (lane B), with no RNA added (lane 0), and with RNA transcribed *in vitro* from pPRO1 (lane 1). Proteins were synthesized in a nuclease treated rabbit reticulocyte lysate in the presence of $^{35}$S-Met and then analyzed by SDS-PAGE (12.5% polyacrylamide) and autoradiography. The molecular mass (in kDa) and positions of the major proteins translated from BMV RNAs and the position of the 49 kDa TEV Nla protein are indicated.

left unmodified as they did not affect the protease activity of Nla (see below). It should be noted that proteins expressed in pPRO1 will contain additional amino acid residues at both N- and C-termini as a consequence of the Nla target heptapeptide and the cloning strategy used.

Upon *in vitro* transcription and subsequent *in vitro* translation in the presence of $^{35}$S-Met, pPRO1 gave the expected translated peptide of approximately 49 kDa (Fig. 2). Subsequent data demonstrated that this protein corresponded to Nla since it exhibited the proper proteolytic activity when expressed in pPRO1 as part of a polyprotein (see below).

*pPRO1 encodes the Nla protease activity*

To confirm that pPRO1 encodes Nla protease activity, several constructs were engineered in which the CP ORF from a tobacco mosaic virus (TMV) was inserted into the cassette (Fig. 3A). The first two constructs, pPRO1.NT and pPRO1.TN, contained the TMV CP sequence in either of the two possible cloning sites. To demonstrate that processing of the resultant polyprotein was due to recognition and cleavage of the specific heptapeptides by the Nla protease and not to non-specific degradation, two additional controls were designed. First, the C-terminal Nla protease domain was removed with a frameshift mutation at the unique *Bam* HI site, resulting in pPRO1.TΔN. In this construct, processing is not expected despite the presence of the naturally occurring cleavage sequence. Second, the two target heptapeptides were mutated to include a Gln to His change at the −1 position. This mutation at the cleavage site has been previously shown to inhibit the specific processing by Nla [8, 9]. The resulting mutant cassette was named pPRO4 and

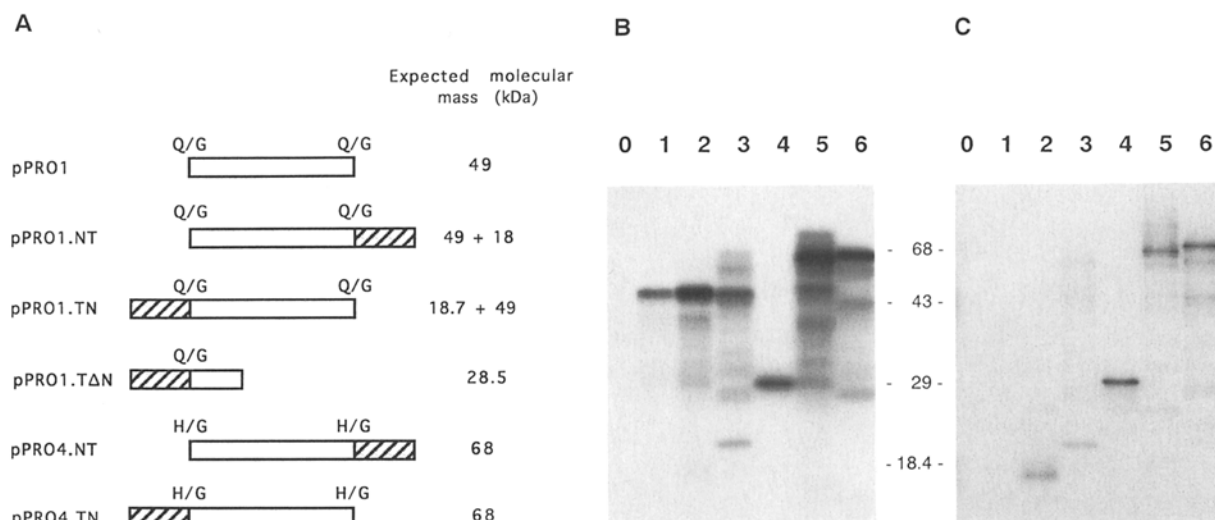


*Fig. 3. In vitro* translation of RNAs transcribed from different pPRO1 derived constructs containing the TMV CP sequence. A. Schematic diagram representing the six different constructs used in this experiment (see text for details). Open boxes represent TEV-Nla sequence. Striped boxes represent TMV CP sequence. The names of the constructs and the expected molecular mass of the translated and processed products are indicated. Q/G indicates the amino acid residues at the cleavage sequence (constructs pPRO1), whereas H/G indicates the His to Gln mutation at −1 position that inhibits processing by Nla (constructs pPRO4). B. *In vitro* translation products obtained from the constructs shown in A. C. Immunoprecipitation analyses using anti-TMV CP antibody of aliquots from the translation samples shown in B. In B and C, proteins were synthesized in a rabbit reticulocyte lysate in the presence of $^{3}$H-Leu and analyzed by SDS-PAGE (15% polyacrylamide) and fluorography. Translation reactions were programmed with no RNA added (lane 0), and with RNA transcribed *in vitro* from pPRO1 (lane 1), pPRO1.NT (lane 2), pPRO1.TN (lane 3), pPRO1.TΔN (lane 4), pPRO4.NT (lane 5), and pPRO4.TN (lane 6). The molecular mass (in kDa) and positions of $^{14}$C-labeled protein markers are indicated.

the corresponding pPRO4.NT and pPRO4.TN were also constructed.

*In vitro* transcription and translation of each construct in the presence of $^{3}$H-leu revealed the expected patterns and sizes of labeled proteins (Fig. 3B). In addition to the 49 kDa protein, a band corresponding to a protein of approximately 18 kDa was detected in pPRO1.NT and pPRO1.TN. 18 kDa is the expected size of TMV CP when expressed in pPRO1 constructs. The CP produced from pPRO1.TN was slightly larger than that produced from pPRO1.NT, in accordance with the numbers of amino acid residues added when the cDNA was cloned at the *Sma* I site versus the *Stu* I site (see Fig. 1). On the other hand, the major proteins resulting from constructs pPRO4.NT and pPRO4.TN migrated at positions corresponding to the size of the precursor polypeptide containing Nla plus TMV CP (68 kDa). Finally, when the protease domain from Nla is absent (pPRO1.TΔN) a single protein of about 28 kDa, corresponding to the truncated protein, was detected.

Immunoprecipitation reactions of the proteins produced *in vitro* using an anti-TMV CP antibody resulted in precipitation of the expected proteins (Fig. 3C). Only those peptides which included TMV CP sequences were selectively immunoprecipitated, whereas the 49 kDa Nla protein was not. Data presented to this point clearly demonstrate that pPRO1 functions as predicted.

Several experiments were carried out to determine whether or not proteolytic processing could occur *in trans*. The labeled peptide that was translated from pPRO1.TΔN was not processed when unlabeled 49 kDa protein translated from pPRO1 was used as source of Nla proteinase (data not shown). This result is in agreement with previously reported data [4].

### *Proteolytic processing of two different proteins introduced in pPRO1*

pPRO1 was further tested with the introduction of coding sequences for two different proteins at the same time. ORFs encoding coat proteins from viruses belonging to different groups, SMV (a potyvirus) and TMV, were inserted in the two possible positions (Fig. 4A, constructs pPRO1.SNT and pPRO1.TNS). The constructs gave the predicted patterns of labeled proteins *in vitro* (Fig. 4B), resulting in the accumulation of proteins with the expected sizes of the Nla (49 kDa), SMV CP (around 30 kDa) and TMV CP (around 18 kDa). As expected, the coat protein cDNAs inserted at the *Sma* I site of pPRO1 gave slightly larger mature proteins than those inserted at the *Stu* I site. Moreover, the more rapidly migrating proteins (predicted to be the TMV CP) comigrated with proteins recovered following immunoprecipitation with anti-TMV CP antibody (see above).

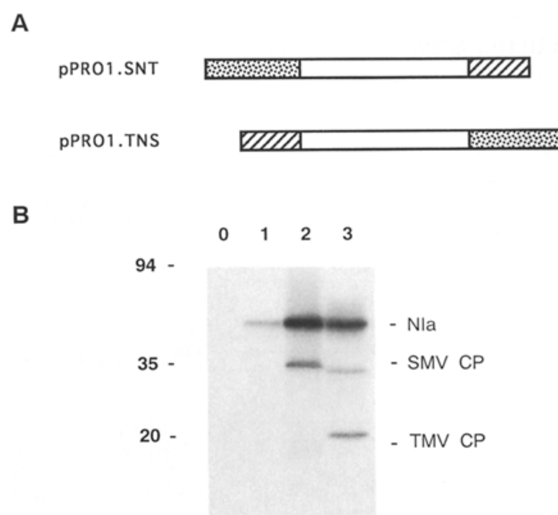


*Fig. 4. In vitro* translation of RNAs transcribed from pPRO1 constructs which contained TMV CP and SMV CP coding sequences. A. Schematic diagram representing the constructs used in this experiment (pPRO1.SNT and pPRO1.TNS). The open box represents TEV-Nla sequence. Striped and dotted boxes represent TMV CP and SMV CP sequences, respectively. B. *In vitro* translation products obtained from pPRO1.SNT and pPRO1.TNS. Translation reactions were programmed with no RNA added (lane 0), and with RNA transcribed *in vitro* from pPRO1 (lane 1), pPRO1.SNT (lane 2), and pPRO1.TNS (lane 3). Proteins were synthesized in a rabbit reticulocyte lysate in the presence of $^{3}$H-Leu and then analyzed by SDS-PAGE (15% polyacrylamide) and fluorography. The molecular mass (in kDa) and positions of the major proteins translated from BMV RNAs, and the positions of the TEV Nla, SMV CP and TMV CP are indicated.

Another construct consisted of the SMV CP positioned at the *Sma* I site of pPRO1 and the open reading frame encoding the $\beta$-glucuronidase activity (GUS) at the *Stu* I site (Fig. 5A, pPRO1.SNG). Following *in vitro* translation in the presence of $^{35}$S-Met the expected profile of mature proteins was generated (Fig. 5B). The polypeptide synthesized upon translation of this construct has a predicted size of about 149 kDa, and is the largest that has been tested with the pPRO1 expression cassette. In this particular case, a high-molecular weight band corresponding to a polypeptide of ca. 110 kDa was present in relatively low amounts. This protein probably corresponds to a fusion of the Nla and GUS peptides, implying that processing was not complete.

A time course of an *in vitro* translation reaction programmed with construct pPRO1.SNG showed the predicted increase in the accumulation of the expected proteins with time (Fig. 5C). Even at short incubation times (15 min) no 149 kDa precursor could be detected, indicating efficient co-translational processing. However, pulse chase experiments with this construct did not demonstrate significant post-translational processing of the small amounts of 110 kDa polypeptide (data not shown).

## Discussion

To achieve the goal of developing a system to facilitate the expression of two different proteins in equimolar amounts, we have exploited the proteolytic processing strategy of the TEV Nla protease. There were several reasons for choosing this protein. First, it is a highly specific proteinase whose cleavage sequence has been well characterized [5, 8, 9, 10]. Second, Nla was active

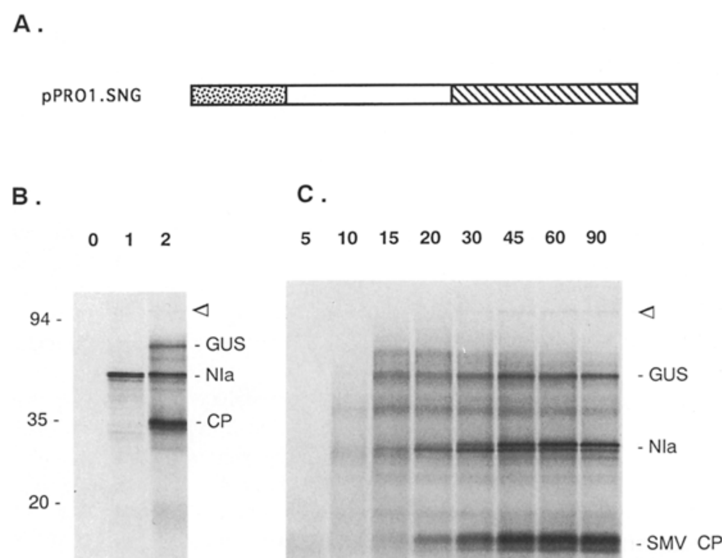


*Fig. 5. In vitro* translation of RNAs transcribed from a pPRO1 construct which contained SMV CP and $\beta$-glucuronidase coding sequences. A. Schematic diagram representing the construct used in this experiment (pPRO1.SNG). The open box represents TEV-Nla sequences. Dotted and striped boxes represent SMV CP and $\beta$-glucuronidase sequences, respectively. B. *In vitro* translation products obtained from pPRO1.SNG. Translation reactions were programmed with no RNA added (lane 0), and with RNA transcribed *in vitro* either from pPRO1 (lane 1) or pPRO1.SNG (lane 2). Proteins were synthesized in a rabbit reticulocyte lysate in the presence of $^{35}$S-Met and then analyzed by 12.5% SDS-PAGE and autoradiography. C. Time course of translation programmed with RNA transcribed from pPRO1.SNG (as in B, lane 2). Samples were withdrawn at the times (in minutes) indicated at the top of each lane and analyzed by SDS-PAGE (10% polyacrylamide) and autoradiography. In A the molecular mass (in kDa) and position of the proteins translated from BMV RNAs is indicated. In A and B the position of the TEV NIa, GUS, and SMV CP proteins are also indicated. An open arrowhead indicates the position of a 110 kDa polypeptide present in small amounts.

*in vitro* when the cleavage sequences were inserted either into several locations in TEV polyproteins [5, 10] or into non-viral proteins [21]. Finally, Nla cleaves its substrate heptapeptide properly *in vivo* when expressed as a transgene in plants [19].

Characterization of the expression cassette pPRO1 *in vitro* demonstrated that Nla functions as expected with different protein sequences, including others not presented in this work. In most of the constructs, cleavage was so effective that non-processed precursors could not be detected. In only two cases (an example is shown with pPRO1.SNG) were minimal amounts of non-cleaved precursors detected, indicating a lack of complete processing. This result does not, however, preclude the future utility of this approach *in vivo*.

There are several potential applications of this Nla-based expression cassette in addition to those involving transgenic plants. For example, transient expression assays, in which pPRO1 expression is driven either by conventional transcription promoters or by plant viral vectors, could be developed. Another option is to use the cassette in prokaryotic systems since Nla proteases from different potyvirus have been shown to be active when expressed in bacterial cells [12, 23].

The number of foreign proteins translated as part of a Nla-containing polyprotein is not, theoretically, limited to two and other versions of pPRO1 could allow more than two proteins to be translated and processed. It should be noted, however, that foreign proteins expressed in pPRO1 will have additional amino acids at both $NH_2$ and COOH termini, which may affect the biological activity of some proteins.

Several practical applications of pPRO1 which imply its expression in plants as a transgene are currently being tested in our laboratory. First, we hope to engineer coat protein mediated resistance (CPMR) against viruses that belong to different groups by inserting two different coat proteins into pPRO1. In initial experiments, constructs containing Nla and the CP of TMV (Fig. 3A) were introduced in *Nicotiana tabacum* via *Agrobacterium tumefaciens* transformation. Preliminary data indicate that TMV CP expressed *in vivo* as part of pPRO1 confers CPMR (data not shown). Second, constructs that include CP sequences with the gene for $\beta$-glucuronidase will enable us to use GUS activity as a probe for the levels of expression of the CP. This approach will be useful for studying those examples in which there is poor correlation between the levels of CP accumulation and the degree of resistance, providing additional important data on the molecular mechanism(s) of CPMR in these cases.

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