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Expression of potyviral polyproteins in transgenic plants reveals three proteolytic activities required for complete processing

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All proteins encoded by the plant potyvirus, tobacco etch virus (TEV), arise by proteolytic processing of a single polyprotein. Two virus-encoded proteinases (NIa and HC-Pro) that catalyze most of the proteolytic events have been characterized previously. The two proteins that are derived from the N-terminal 87 kd region of the viral polyprotein are a 35 kd protein and HC-Pro (52 kd). It is demonstrated in this study that a third proteolytic activity is required to process the junction between these proteins. Proteolysis at the HC-Pro N terminus to separate these proteins occurred poorly, if at all, after *in vitro* synthesis of a 97 kd polyprotein, whereas cleavage of the HC-Pro C terminus occurred efficiently by an autoprocessing mechanism. Synthesis of the same polyprotein in transgenic tobacco plants, however, resulted in complete and accurate proteolysis at both termini of HC-Pro. A point mutation affecting an amino acid residue essential for the proteolytic activity of HC-Pro had no effect on N-terminal processing. Expression in transgenic plants of a construct with a large deletion in the 35 kd protein coding region resulted in partial inhibition of HC-Pro N-terminal cleavage, suggesting that the 35 kd protein may affect the proteolytic event but not in a catalytic role. We speculate that this cleavage event is catalyzed by either a cryptic potyviral proteinase that requires a host factor or subcellular environment for activation, or possibly a host proteinase.

Key words: polyprotein/proteolysis/tobacco etch virus/transgenic plants

Introduction

The potyviruses form a diverse group of plant viruses that cause diseases worldwide (Hollings and Brunt, 1981). This group is characterized genetically as possessing a single-stranded RNA genome of ~10 000 nucleotides (nt) that encodes a single, large translation product (reviewed by Dougherty and Carrington, 1988). This product is a polyprotein precursor that undergoes extensive proteolytic processing to form at least eight mature proteins. Most of these protein cleavage events are catalyzed by two virus-encoded proteinases, NIa and HC-Pro (Carrington and Dougherty, 1987; Hellmann *et al.*, 1988; Carrington *et al.*, 1989a; Garcia *et al.*, 1989).

Using the potyvirus tobacco etch virus (TEV) as a model, NIa (49 kd proteinase) has been shown to catalyze cleavage at five sites, each of which is located in the C-terminal two-thirds of the polyprotein (Figure 1A) (Carrington and Dougherty, 1987; Carrington *et al.*, 1988). These sites are characterized by the conserved heptapeptide sequence Glu-Xaa-Xaa-Tyr-Xaa-Gln-Gly or Ser, where the scissile bond is positioned between the Gln-Gly or Ser dipeptide. Dougherty *et al.* (1988, 1989) have shown that these conserved residues are integral to efficient substrate recognition, and that they may form the basis for differential processing efficiencies at various TEV cleavage sites (Dougherty and Parks, 1989). Allison *et al.* (1986) pointed out that NIa exhibited sequence similarity to the animal picornavirus proteinase 3C, as well as to the plant comovirus 24 kd proteinase.

The second proteinase, HC-Pro, is encoded by sequences near the amino terminus of the polyprotein and functions to process autolytically its C terminus (Carrington *et al.*, 1989a,b). This protein possesses a mol. wt of ~51-58 kd and has been known to exist in potyvirus-infected plants (de Mejia *et al.*, 1985; Thornbury *et al.*, 1985). The primary sequence determinants for substrate recognition by HC-Pro have not been elucidated, although cleavage *in vitro* occurs between Gly763 and Gly764 (Carrington *et al.*, 1989a) (numbering system designates the polyprotein initiator Met as position 1). Based on a systematic genetic analysis, the proteolytic domain of HC-Pro (the C-terminal 20 kd) most closely resembles a cysteine-type proteinase (Oh and Carrington, 1989). HC-Pro also functions as 'helper component', a protein required during aphid-mediated transmission of TEV from plant-to-plant in naturally occurring infections (Thornbury *et al.*, 1985). The domain within the HC-Pro molecule responsible for helper component activity has not been mapped.

Since autolytic cleavage of the C terminus of HC-Pro liberates an ~87 kd product from the remainder of the TEV polyprotein (Figure 1A), the N terminus of HC-Pro must be processed to yield the mature protein. Proteolytic cleavage at the N terminus of TEV HC-Pro has not been reproduced consistently *in vitro* in our laboratory. Faithful processing at all other known cleavage sites within the TEV polyprotein, on the other hand, has been duplicated in cell-free reactions using proteinase isolated from infected plants or proteinase synthesized *in vitro*. This raises the possibility that a third proteinase performs this cleavage event, or that a host factor coupled with NIa or HC-Pro is required. Alternatively, a specific subcellular compartment or condition (pH, for example) may be necessary to permit proper folding of the cleavage site region or to stimulate a viral proteolytic activity that has yet to be identified. To understand the requirements for processing at the HC-Pro N terminus, we have used transgenic plants to express wild-type and mutagenized TEV cDNA encoding HC-Pro and flanking regions. The results indicate that neither HC-Pro nor NIa function to process the HC-Pro N terminus *in vivo*, but that a third proteolytic activity is necessary for this cleavage event.

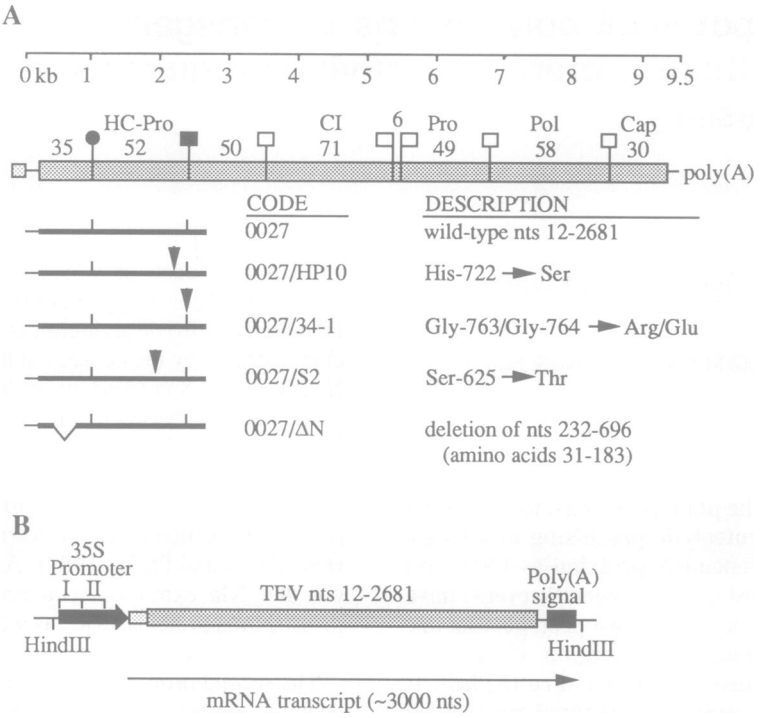

Fig. 1. Tobacco etch virus (TEV) genetic map and sequences used to produce transgenic plants (**A**) The TEV genome is represented underneath the scale bar. The genome-linked protein (VPg) is shown as the stippled box attached to the genomic RNA. The shaded rectangular box represents the open reading frame, while the vertical dashes indicate positions that encode cleavage sites. Vertical lines with white and black boxes represent cleavage sites recognized by the 49 kd proteinase (NIa) and HC-Pro, respectively. The vertical line with the black circle indicates the cleavage site between the 35 kd protein and HC-Pro. Regions expressed in transgenic plants are diagrammed below the genetic map. The vertical arrows indicate the positions of site-directed mutations. (**B**) Each cDNA segment shown in (A) was inserted into pRTL2 (see Materials and methods), and the resulting expression cassette is shown. The sequence that was duplicated in the 35S promoter is shown by the brackets. The 5′ nontranslated region is represented as the small stippled box adjacent to the 35S promoter, whereas the coding sequence is shown as the long shaded box. Each expression cassette was excised with *Hin*dIII, inserted into the binary vector pGA482 (An, 1986), and integrated into the tobacco genome. Abbreviations: CI, cylindrical inclusion protein; Pro, proteinase; Pol, polymerase; Cap, capsid protein.

Results

HC-Pro in virus-infected plants and analysis of anti-HC-Pro serum

Antibodies directed against a segment of HC-Pro synthesized in *Escherichia coli* were produced in rabbits (see Materials and methods). Preimmune and immune sera were tested for reactivity against authentic HC-Pro that was extracted from TEV-infected tobacco plants. A series of total protein extracts from leaves were prepared after 0, 2, 4, 5 and 7 days post-inoculation (p.i.) and analyzed in immunoblot assays. A major protein that co-migrated with the large subunit of RuBP carboxylase (52 kd) was identified using the immune serum after 4 days p.i., and accumulated steadily through day 7 (Figure 2). Since the published mol. wts of HC-Pro from several different potyviruses range between 51 and 58 kd, we conclude that the immunoreactive species identified here represents the TEV HC-Pro. A minor polypeptide of ~30 kd was reactive specifically with immune serum (Figure 2), and may represent a non-specific proteolytic fragment of HC-Pro. Pre-incubation of anti-HC-Pro serum with HC-Pro protein that was used to immunize rabbits resulted in disappearance of both the 52 and 30 kd immunoreactive proteins (data not shown).

Processing of HC-Pro in vitro and in transgenic plants

Numerous reports have appeared showing that translation of several potyviral genomic RNAs or synthetic transcripts in the rabbit reticulocyte lysate system results in accumulation of a high mol. wt polyprotein precursor containing HC-Pro, without subsequent proteolytic conversion to mature-sized HC-Pro (e.g. Hiebert *et al.*, 1984; Carrington *et al.*, 1989a). As shown previously and in Figure 3, translation of transcripts from pTL-0027 (with a coding capacity for a 97 kd protein representing the N terminus of the TEV polyprotein—Figure 1A) yielded proteolytic products of 10 and 87 kd. The latter consists of HC-Pro (52 kd) linked to the protein derived from the extreme N terminus of the viral polyprotein; even though we have not detected the N-terminal protein *in vivo* and therefore cannot assign an accurate mol. wt, we will refer to it as the '35 kd protein' (i.e. 87 minus 52 = 35). Although the proteolytic domain of HC-Pro efficiently self-processed the polyprotein to remove the 10 kd protein fragment flanking its C terminus, the peptide bond linking the 35 kd protein and HC-Pro remained stable. Addition of active NIa proteinase to this polyprotein failed to induce proteolytic cleavage (data not shown). Translation of pTL-0027/HP10 transcripts, which code for the 97 kd polyprotein containing a proteinase-inactivating mutation, resulted in accumulation of the 97 kd polyprotein. Several minor products also were detected after translation of both transcript preparations, and we suspect they represent prematurely terminated polypeptides rather than proteolytic fragments. If any were the result of proteolysis at the HC-Pro N terminus, unique proteins of 52 and 62 kd would have been detected among the translation

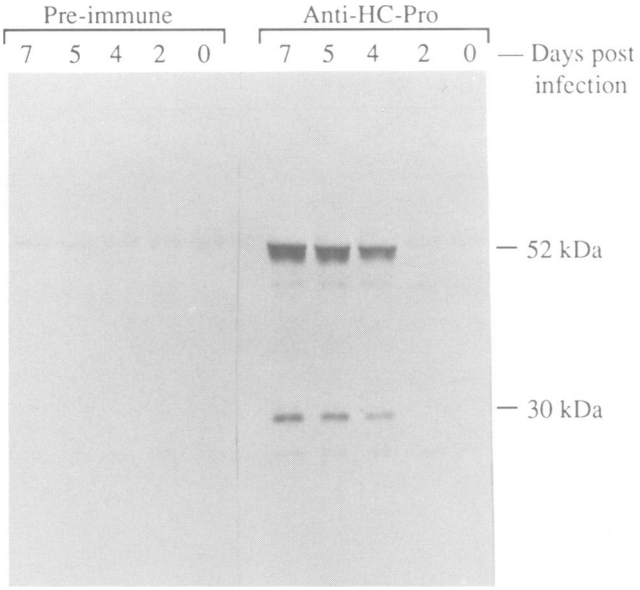

Fig. 2. Immunoblot analysis of extracts from TEV-infected plants using anti-HC-Pro serum. Total SDS-soluble protein extracts from tobacco plants that had been infected 0, 2, 4, 5 or 7 days previously were prepared, subjected to SDS–gel electrophoresis, transferred to nitrocellulose membranes, and probed with anti-HC-Pro or pre-immune sera and alkaline phosphatase-linked, goat anti-rabbit IgG.

products of pTL-0027 and pTL-0027/HP10 transcripts respectively. It thus appears that neither HC-Pro nor NIa function *in vitro* at this site.

To test the hypothesis that cleavage at the N terminus of HC-Pro is stimulated by conditions or factors *in vivo*, the TEV cDNA fragment used to construct pTL-0027 was introduced into the plant expression vector pRTL2 (forming pRTL2-0027) and integrated into the tobacco genome by the leaf disk transformation procedure. Polyprotein synthesis and accurate N- and C-terminal processing would be predicted to yield an HC-Pro molecule of 52 kd, whereas partial cleavage at only one terminus would render products of 87 and/or 62 kd.

RNA extracts were prepared from individual transformed plants and screened by Northern blot hybridization. The 35S-promoter/TEV sequence/poly(A) transcriptional unit should have generated an mRNA of ~3000 nt (Figure 1B). An RNA species of approximately this size was detected in extracts from plant U-6B transformed with pRTL2-0027 sequences, but not in plants transformed using the binary vector pGA482 (Figure 4A). Several plants have been transformed with pGA482 sequences and will heretofore be referred to as 'control plants'. Southern blot analysis using total DNA extracts from plant U-6B indicated that the TEV sequences were integrated intact (data not shown).

Total SDS-soluble protein extracts were prepared from control and U-6B plants, and were analyzed in immunoblot experiments using anti-HC-Pro serum and [^{125}I]protein A. Autoradiography revealed accumulation of a 52 kd protein in extracts from plant U-6B, but not in control plant extracts (Figure 4B). The immunoreactive protein migrated through SDS–polyacrylamide gels with the same mobility as authentic HC-Pro, suggesting that the polyprotein synthesized in the transgenic plant was accurately and completely processed. The minor 30 kd protein that is often associated

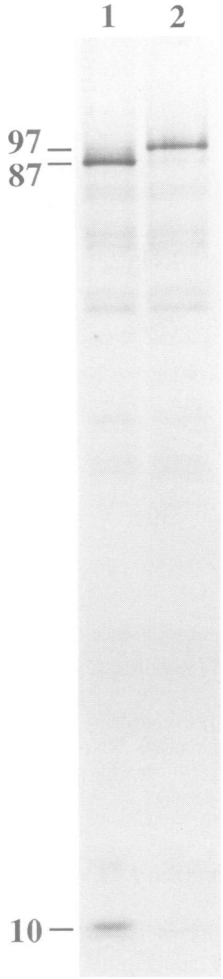

Fig. 3. Cell-free synthesis and processing of HC-Pro-containing polyproteins in the rabbit reticulocyte lysate system. Transcripts were synthesized from *Bam*HI-linearized pTL-0027 or pTL-0027/HP10 and translated *in vitro* in the presence of [^{35}S]methionine. pTL-0027/HP10 is identical to pTL-0027 (Carrington *et al.*, 1989a) except that it contains a proteinase-inactivating mutation that affects HC-Pro. Radiolabeled products were analyzed by SDS–PAGE and autoradiography. **Lane 1**, translation products of pTL-0027 transcripts; **lane 2**, translation products of pTL-0027/HP10 transcripts. The sizes (in kd) of the radiolabeled proteins are indicated at the left.

with HC-Pro (Figure 2), and that is presumed to be a degradation product, was also detected in the transgenic plant U-6B extract.

To be certain that the HC-Pro-related protein identified in the transgenic plants was not the result of an asymptomatic TEV infection, a progeny analysis was conducted using F1 seed obtained from plant U-6B. Nine out of 15 progeny were positive for HC-Pro using the immunoblot assay (Figure 4B); since TEV is not known to be transmitted through seed, the HC-Pro-encoding genome segment must have been integrated stably and inherited. Additionally, extracts from U-6B, control, and TEV-infected plants were screened by immunoblot assay using anti-HC-Pro, anti-NIa (49 kd proteinase), and anti-NIb (58 kd polymerase) sera. While the TEV-infected plant extracts were positive for the presence of each viral protein, U-6B extracts contained only HC-Pro-related protein (Figure 5).

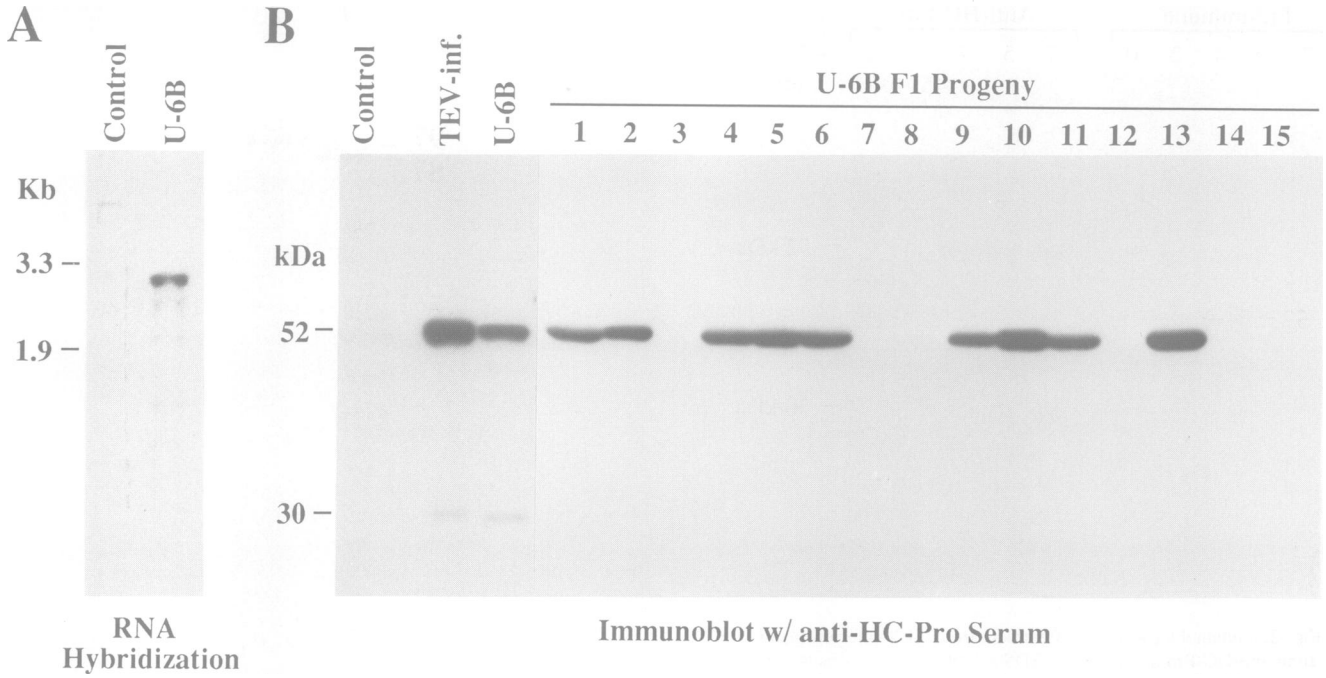

Fig. 4. Expression of HC-Pro-containing TEV sequences in transgenic plants. (**A**) Northern hybridization analysis using total RNA extracts from control and U-6B transgenic plants. RNA was subjected to electrophoresis in 1.5% agarose under denaturing conditions (Carrington and Morris, 1984), transferred to a Genescreen Plus nylon membrane, and hybridized to ^{32}P-labeled cDNA corresponding to genome nucleotides 12–2681 (from pTL-0027). (**B**) Immunoblot analysis, using anti-HC-Pro serum and [^{125}I]protein-A, of protein extracts from control, TEV-infected, and U-6B transgenic plants, and from 15 individual F1 progeny of self-fertilized U-6B.

Expression of mutagenized TEV sequences in transgenic plants

The proteolytic activity of HC-Pro *in vitro* serves only to cleave its C terminus (see Figure 3). Given that HC-Pro is the only known proteinase within the N-terminal 97 kd region of the TEV polyprotein, it is conceivable that this enzyme functions to autoprocess both termini *in vivo*, but with the aid of a host factor during cleavage of the HC-Pro N terminus. To test this hypothesis, a point mutation resulting in the conversion of His722 to Ser was introduced into pRTL2-0027, and modified construct (termed pRTL2-0027/HP10) was expressed in transgenic plants F-1D and F-2C. This mutation completely eliminates the C-terminal proteolytic activity of HC-Pro *in vitro* (Figure 3; Oh and Carrington, 1989). In addition, clustered point mutations resulting in conversion of the HC-Pro C-terminal Gly763/Gly764 cleavage site to Arg/Glu was introduced into pRTL2-0027, forming pRTL2-0027/34-1, which was expressed in plants E-6A and E-4D. This mutation renders the C-terminal cleavage site non-functional (Carrington *et al.*, 1989a) but presumably has no effect on the enzymatic active site of HC-Pro.

Immunoblot analysis of SDS-soluble protein extracts from transgenic plants F-1D and F-2C revealed the presence of a ~62 kd HC-Pro-related protein (Figure 6, lanes 3 and 4). This product appeared to possess a mol. wt of ~10 kd greater than that of HC-Pro identified in extracts from TEV-infected tissue (lane 1) and from plant U-6B (lane 2). Since cleavage at the C terminus of HC-Pro was inhibited by the mutation, this product appeared to have resulted from proteolysis of the polyprotein only at the HC-Pro N terminus. This interpretation is supported by analysis of HC-Pro-related protein produced in the pRTL2-0027/34-1-transformed plants, E-6A and E-4D. These plants contained sequences encoding a polyprotein with a defective cleavage site at the C terminus of HC-Pro, and would be predicted to yield a stable product of 62 kd consisting of HC-Pro linked to the small polypeptide flanking its C terminus. An immunoreactive product of ~62 kd, which co-migrated during SDS–PAGE with the protein identified from F-1D and F-2C plants, was detected in extracts from E-6A and E-4D (Figure 6, lanes 7 and 8).

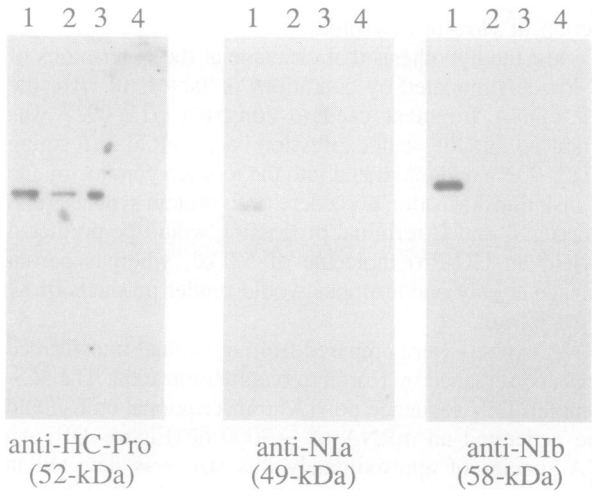

Fig. 5. Immunoblot analysis of TEV-infected, U-6B, and control plants using anti-HC-Pro, anti-NIa, and anti-NIb sera. Duplicate samples of protein extracts were analyzed using the antisera indicated and [^{125}I]protein-A. **Lane 1**, TEV-infected plant extract; **lane 2**, U-6B transgenic plant extract; **lane 3**, U-6B transgenic plant extract prepared ~30 days after the extract shown in lane 2; **lane 4**, control plant extract (transformed using vector pGA482). The mol. wts of HC-Pro, NIa and NIb are indicated at the bottom.

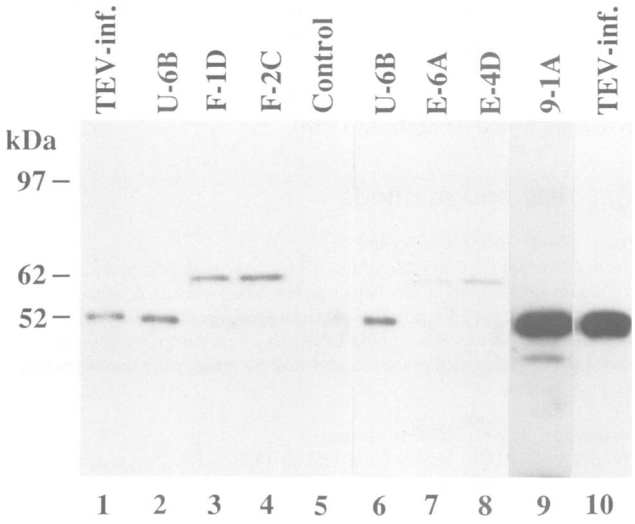

Fig. 6. Effects of point mutations in HC-Pro on polyprotein processing in transgenic plants. Protein extracts from each of the plants indicated were analyzed by immunoblot assay using anti-HC-Pro serum and [^{125}I]protein-A. Plants F-1D and F-2C (**lanes 3** and **4**) were transformed using pRTL2-0027/HP10 sequences, which contained the HC-proteinase-inactivating His722 to Ser mutation. Plants E-6A and E-4D (**lanes 7** and **8**) were transformed using pRTL2-0027/34-1, which contained the Gly763/Gly764 to Arg/Glu C-terminal cleavage site mutation. Plant 9-1A (**lane 9**) contained pRTL2-0027/S2 sequences, which contained the Ser625 to Thr point mutation. The sizes of the proteins detected (52 and 62 kd), as well as the electrophoretic position of the size standard phosphorylase b (97 kd), are shown at the left.

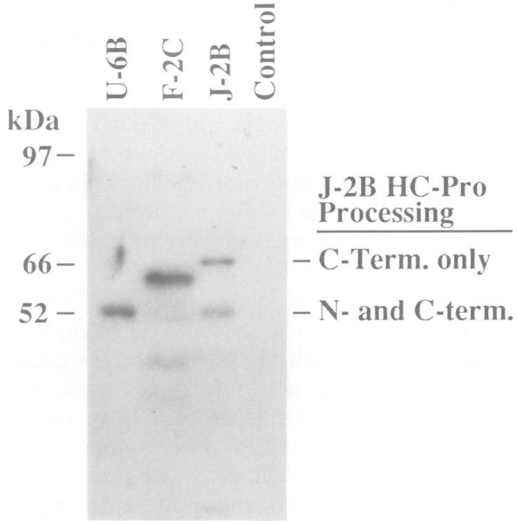

Fig. 7. Effect of deletion of a segment of the 35 kd protein on polyprotein processing in transgenic plants. Protein extracts from each of the plants indicated were analyzed by immunoblot assay using anti-HC-Pro serum and [^{125}I]protein-A. Plants U-6B and F-2C were described in Figures 4–6. Plant J-2B was transformed using pRTL2-0027/ΔN sequences. This construct was produced by deleting coding sequences for ~55% of the 35 kd protein, which is found adjacent to the N terminus of HC-Pro in the TEV polyprotein. The proteolytic products that were expected to result from N- and/or C-terminal processing of HC-Pro are described in the text.

We have noted previously (Carrington *et al.*, 1989b; Oh and Carrington, 1989) that the sequence Gly-Asn-Ser-Gly, found at positions 623–626 in the TEV polyprotein near the center of HC-Pro, resembles the motif surrounding the active-site serine residue of serine-type proteinases (Neurath, 1984). This led to speculation that it may be part of a distinct proteolytic domain that functions to process the N terminus of HC-Pro. To test this hypothesis, a point mutation resulting in the conversion of Ser625 to Thr was incorporated into pRTL2-0027 and integrated in transgenic plant 9-1A. Such a mutation would destroy the activity of a serine-type proteinase. However, this mutation had little effect on N- or C-terminal processing of HC-Pro, since an immunoreactive product of ~52 kd (the size of accurately processed HC-Pro) was detected in extracts from plant 9-1A (Figure 6, lane 9).

Little is known regarding the function of the 35 kd protein located to the N-terminal side of HC-Pro in the viral polyprotein. We have tested the possibility that it functions as the proteinase to process its C terminus (or the HC-Pro N terminus) *in vivo* by expressing a deleted form in transgenic plants. An *Nde*I–*Nde*I DNA fragment, representing the coding sequence for amino acid residues 30–184 of the 35 kd protein, was excised from pRTL2-0027 to form pRTL2-0027/ΔN (Figure 1A), which was used to prepare transgenic plant J-2B. This alteration resulted in deletion of polypeptide sequences totalling ~19 kd. Previous studies have shown that deletions in this protein have no effect on the C-terminal autolytic activity of HC-Pro (Carrington *et al.*, 1989a,b). Therefore, we predicted accumulation of mature-sized HC-Pro if the deletion had no effect on processing, or accumulation of a 68 kd product if proteolysis at the HC-Pro N terminus were impaired. Immunoblot analysis of extracts from plant J-2B using anti-HC-Pro serum revealed accumulation of both predicted proteins (Figure 7). Based on scanning densitometric analysis, 40% of the immunoreactive product was 52 kd (indicating accurate proteolysis at both the N- and C-terminal cleavage sites of HC-Pro), whereas 60% of the product co-electrophoresed with the bovine serum albumen size standard (66 kd).

Discussion

Extensive use has been made previously of *in vitro* transcription and translation systems to reproduce faithfully the events associated with viral polyprotein processing. Using the potyvirus TEV, processing of six of the seven known cleavage sites has been studied after reaction of synthetic radiolabeled substrates with TEV proteinases synthesized *in vitro* or isolated from infected plants. However, requirements for cleavage at the junction between the 35 kd protein and HC-Pro (i.e. the N terminus of HC-Pro) have not been elucidated. We have analyzed this proteolytic event using transgenic plants that express this region of the TEV genome. Unlike the products obtained after synthesis in the rabbit reticulocyte lysate system, expression of a polyprotein containing the 35 kd protein and HC-Pro in transgenic plants resulted in apparently accurate and complete cleavage at both the N and C termini of HC-Pro.

We present evidence in this paper supporting the hypothesis that a third proteolytic activity is required for complete processing of the TEV polyprotein. Whereas most proteolytic cleavage sites in the TEV polyprotein are processed by either NIa or HC-Pro, cleavage at the N terminus of HC-Pro requires a distinct proteinase. Since the HC-Pro N terminus was cleaved in transgenic plant U-6B which lacked the coding sequence for proteinase NIa, clearly this enzyme has no role in affecting this proteolytic step.

Similarly, since the HC-Pro N terminus was efficiently processed in transgenic plants F-1D and F-2C which lacked a functional catalytic domain within HC-Pro (due to a point mutation), the proteolytic activity of HC-Pro plays no part in promoting N-terminal cleavage. This site must, therefore, be processed by a third proteolytic activity of either viral or host origin. If a third potyviral proteinase is involved, there may also be a 'host factor' required to activate or potentiate the enzyme since its activity in the reticulocyte lysate system is extremely low or non-existent.

Contrary to previous speculation (Oh and Carrington, 1989), the HC-Pro amino acid sequence (Gly-Asn-Ser-Gly, positions 623–626), which resembles a highly conserved region surrounding the active-site Ser of serine-type proteinases (Neurath, 1984), appears to play no role in proteolysis to generate the HC-Pro N terminus. Substitution of Ser625 with Thr had no effect on this proteolytic event in transgenic plants. Since no other proteolytic role can be ascribed to this domain, the sequence similarity with the serine type proteinase active site may be fortuitous.

By expression of a deletion construct in transgenic plants, we tested the hypothesis that the 35 kd protein adjacent to HC-Pro in the TEV polyprotein functions as the third proteolytic enzyme. In such a case, the 35 kd protein would perform a function similar to the foot-and-mouth disease (picorna) virus L polypeptide, which autolytically releases from the N terminus of the viral polyprotein (Strebel and Beck, 1986). Deletion of a 19 kd internal segment from the 35 kd protein resulted in a polyprotein that was partially defective for HC-Pro N-terminal processing. We suggest that the partial proteolytic inhibition was not due to disruption of a putative proteolytic domain within the 35 kd protein, since a deletion of this magnitude most likely would have eliminated activity completely. It is possible that the phenotype observed was due to perturbation of a polyprotein structural feature. Although the deleted segment of the protein resided ~100 amino acid residues away from the cleavage site, 'long-range' effects of deletions and mutations have been well documented in other viral polyprotein systems (Nicklin *et al.*, 1987; Ypma-Wong *et al.*, 1988). The intermediate processing phenotype may also be explained by inhibition of transport of the polyprotein to a subcellular compartment containing the required proteinase, or to a compartment that possesses the necessary physical, chemical or cofactor requirements to complement a viral proteolytic activity.

If the third proteinase is encoded by the TEV genome, it must reside near the N terminus of HC-Pro or the extreme C terminus of the 35 kd protein. Conserved sequence motifs that characterize the four major classes of proteinases (Barrett, 1986) are absent from these regions. This may indicate the involvement of a novel proteolytic activity encoded within these sequences. Alternatively, we cannot exclude the possibility of a host proteinase that functions at the 35 kd protein–HC-Pro boundary. The existence of a host enzyme would explain the apparent inefficiency of proteolysis at this site *in vitro*. Experiments designed to detect a specific proteinase activity in membrane-bound and soluble protein extracts from infected and non-infected plants have been inconclusive (data not shown). Since plant tissue is often a rich source of diverse proteinases as well as proteinase inhibitors, identification of a specific proteolytic enzyme used by TEV would require extensive biochemical fractionation. The possibility of a host proteinase involvement must be viewed reservedly, however, since no other members of the 'picornavirus-like superfamily' (picornaviruses, comoviruses) recruit host enzymes for polyprotein processing (Krausslich and Wimmer, 1988).

Materials and methods

Virus, plants, bacterial strains

The highly aphid-transmissible strain of TEV was used. *Nicotiana tabacum* cv. Xanthi nc was used as host for virus infection, whereas *N.tabacum* cv. Havana 425 was used for production of transgenic plants. *Escherichia coli* strain HB101 was used for cloning DNA, and *Agrobacterium tumefacians* strain LBA4404 was used as the recipient host for binary plant transformation vectors.

Production of anti-HC-Pro serum

Complementary DNA representing TEV nt 1429–2181, encoding a 28 kd fragment from the middle of HC-Pro, was inserted into the non-fusion vector pKK233-2 (Amann and Brosius, 1985) to form pKK1422. *E.coli* cultures (500 ml) containing pKK1422 were induced by the addition of 2 mM IPTG. After 3 h, the cells were harvested and lysed at room temperature for 25 min in 25 ml lysis buffer (20 mM Tris–HCl, pH 8.2, 8% sucrose, 100 mM KCl, 5 mM EDTA, 0.1% Nonidet P-40 and 2.5 mg/ml lysozyme). The viscosity of the lysate was reduced by the addition of 10 mM $MgCl_2$ and 10 μg/ml DNase I. The insoluble fraction containing the HC-Pro fragment was collected by sedimentation at 6000 r.p.m. for 10 min in a Sorval SS34 rotor. The pellet was resuspended in lysis buffer (15 ml) and incubated at room temperature for 15 min. The insoluble fraction was collected by centrifugation and washed 5 times in lysis buffer (minus lysozyme). This procedure yielded several mg of highly purified protein that was nearly free of detectable *E.coli* proteins. Protein (500 μg) was emulsified in Freund's complete (for initial immunization) or incomplete (for subsequent boosters) adjuvant, and injected into New Zealand White Rabbits as described (Dougherty and Hiebert, 1980). Serum was collected at several intervals and used without further purification.

Similar procedures were used to produce anti-NIa (49 kd proteinase) and NIb (58 kd polymerase) serum, and will be described more fully elsewhere.

Protein, RNA extraction and analysis

Total SDS-soluble protein was extracted from plant leaf tissue by grinding in 10 vol of protein dissociation buffer (Laemmli, 1971) using a Kontes ground glass pestle in a 1.5 ml microcentrifuge. The samples were then heated for 3 min at 100°C, and the insoluble material was removed by centrifugation. Protein extracts were analyzed for the presence of HC-Pro or HC-Pro containing polyproteins by SDS–PAGE and Western blot analysis (Towbin *et al.*, 1979). After transfer to nitrocellulose, the proteins were reacted with anti-HC-Pro serum (at 1:1000 dilution) followed by [^{125}I]protein A (170 ng, 58 μCi/μg). Blots were subjected to autoradiography using Kodak X-omat AR5 film.

Isolation of total RNA from leaf tissue and Northern blot analysis was as described previously (Carrington and Morris, 1984). ^{32}P-Labeled probes were generated by the random priming method (Feinberg and Vogelstein, 1983) using a DNA fragment corresponding to TEV nt 205–2681.

Construction of recombinant plasmids for plant transformation

Five TEV cDNA constructs were assembled for integration and expression in transgenic tobacco plants (Figure 1a). Each was generated in two steps: (i) insertion of cDNA into vector pRTL2 [which contains the cauliflower mosaic virus 35S promoter and poly(A) signal, see below]; and (ii) excision of the 35S promoter/TEV cDNA/35S poly(A) signal expression cassette with *Hin*dIII and insertion into the binary vector pGA482 (An, 1986). Vector pRTL2 was derived from pRT101 (Topfer *et al.*, 1987) by creating a tandem duplication in the 35S promoter region between −90 and −418 relative to the transcriptional start site, and inserting TEV cDNA corresponding to nt 12–204 immediately after the start site (Figure 1b). This region of the TEV genome contains the 5′ non-translated region and the initial coding sequence for 20 amino acid residues.

A *Sac*I–*Hpa*I restriction fragment representing TEV nt 205–2681 was inserted into pRTL2 to create pRTL2-0027; coupled with the sequence already in the vector, the resulting plasmid contains TEV cDNA, representing nt 12–2681 which encodes a ~97 kd polypeptide. A point mutation resulting in the conversion of His722 to Ser was transferred from pTL7S-2027/HP10 to pRTL2-0027, creating pRTL2-0027/HP10. This mutation completely abolishes HC-Pro proteolytic activity *in vitro* (Oh and Carrington, 1989)

A clustered point mutation resulting in the conversion of the dipeptide Gly763/Gly764 to Arg/Glu was subcloned from pTL-1527/34-1 to pRTL2-0027, creating pRTL2-0027/34-1. The 34-1 mutation destroys the cleavage site recognized by HC-Pro at its C terminus (Carrington *et al.*, 1989a). pRTL2-0027/HP2 was produced by introducing a point mutation (Taylor *et al.*, 1985) resulting in the conversion of Ser625 (TCT codon) to Thr (ACT) in pRTL2-0027. pRTL2-0027/ΔN was generated by deletion of an *Nde*I–*Nde*I fragment, representing TEV nt 232–696, from pRTL2-0027. This alteration maintains the reading frame but results in deletion of amino acid residues 31–185 from the 35 kd protein encoded near the 5′ terminus of the genome (Figure 1A).

The expression cassette was excised from each of these plasmids with *Hin*dIII and inserted into pGA482. The resulting plasmids were mobilized into *A.tumefaciens* strain LBA4404 by the tiparental mating procedure (Ditta *et al.*, 1980). Tobacco cells were transformed and regenerated by the leaf-disk transformation procedure (Horsch *et al.*, 1985) as modified previously (Carrington and Freed, 1990).

In vitro transcription and translation

Plasmid pTL-0027 contains the same TEV cDNA insert as found in pRTL2-0027 (Figure 1A), but harbors an SP6 promoter to facilitate synthesis of RNA transcripts *in vitro*. pTL-0027/HP10 contains the His722 to Ser mutation described above, which inactivates the proteolytic domain of HC-Pro. Methods for transcription and cell-free translation have been described (Carrington and Dougherty, 1987).

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