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Internal Cleavage and *trans*-Proteolytic Activities of the VPg-Proteinase (NIa) of Tobacco Etch Potyvirus In Vivo

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The NIa protein of plant potyviruses is a bifunctional protein containing an N-terminal VPg domain and a C-terminal proteinase region. The majority of tobacco etch potyvirus (TEV) NIa molecules are localized to the nucleus of infected cells, although a proportion of NIa is attached covalently as VPg to viral RNA in the cytoplasm. A suboptimal cleavage site that is recognized by the NIa proteinase is located between the two domains. This site was found to be utilized in the VPg-associated, but not the nuclear, pool of NIa. A mutation converting Glu-189 to Leu at the P1 position of the processing site inhibited internal cleavage. Introduction of this mutation into TEV-GUS, an engineered variant of TEV that expresses a reporter protein (β-glucuronidase [GUS]) fused to the N terminus of the helper component-proteinase (HC-Pro), rendered the virus replication defective in tobacco protoplasts. Site-specific reversion of the mutant internal processing site to the wild-type sequence restored virus viability. In addition, the *trans*-processing activity of NIa proteinase was tested in vivo after introduction of an artificial cleavage site between the GUS and HC-Pro sequences in the cytoplasmic GUS/HC-Pro polyprotein encoded by TEV-GUS. The novel site was recognized and processed in plants infected by the engineered virus, indicating the presence of excess NIa processing capacity in the cytoplasm. The potential roles of internal NIa processing in TEV-infected cells are discussed.

The plant potyviruses belong to the 'picornavirus supergroup' of positive-strand RNA viruses (1, 17, 32). The potyviral genome is approximately 10 kb in length and encodes a single polyprotein that is processed by three viral proteinases to yield nine or more mature proteins (38). Two of the proteinases, P1 and helper component-proteinase (HC-Pro), each catalyze cleavage only at their respective C termini (6, 41). The remaining cleavage sites (at least six) are processed by the NIa proteinase (6, 14, 29), a homolog of the picornaviral 3C proteinase. This enzyme possesses a serine-type proteinase fold but contains a nucleophilic Cys residue rather than Ser at the active site (4, 28). In the case of tobacco etch potyvirus (TEV), specificity of NIa is mediated by interaction between a binding site pocket around the active site and a heptapeptide motif, consisting of strictly and structurally conserved residues, between the P6 and P1′ positions of the cleavage site (20). Variation at the conserved and nonconserved positions appears to modulate processing efficiency at different NIa cleavage sites (21, 24). Results of in vitro experiments indicate that processing sites flanking the 6-kDa protein and NIa are cleaved preferentially in *cis*, whereas other sites are processed efficiently in *trans* (5, 7, 8, 27, 39).

The 49-kDa NIa protein is actually a polyprotein consisting of a proteinase domain (27 kDa) near the C terminus and a genome-linked protein domain (VPg; 21 kDa) near the N terminus (8, 25, 33) (Fig. 1A). The VPg domain is attached covalently to the 5′ terminus of viral RNA via a phosphodiester linkage with Tyr-62 (34). The N-terminal domain also contains a nuclear localization signal that directs the majority of NIa molecules to the nucleus of infected cells (11). Although the 49-kDa form is the predominant NIa-related species in TEV-infected plants, at least small amounts of proteolytically processed forms arise by internal cleavage between the proteinase and VPg domains. Within the VPg pool linked to viral RNA, both the full-length NIa and the 21-kDa, N-terminal fragment of NIa have been identified (33). Evidence indicates that NIa catalyzes internal cleavage at a rate much slower than that at other NIa-mediated cleavage sites (25), most likely as a result of a suboptimal context of the cleavage site. While other NIa-mediated cleavage sites in the TEV polyprotein contain conserved Tyr and Gln residues at the P3 and P1 positions, respectively, the internal NIa site contains Thr and Glu. Amino acid substitutions at the P3 and P1 positions of the NIb/capsid protein cleavage site were shown to reduce processing efficiency in vitro (21, 24).

Given that only a small percentage of TEV NIa molecules are cleaved internally in infected cells, what is the role of the internal cleavage site? One hypothesis states that internal cleavage liberates the C-terminal proteinase domain from the nuclear localization signal, thereby generating a cytoplasmic pool of proteinase necessary for cleavage in *trans* or for additional functions yet to be identified. Alternatively, putative additional functions may be selectively activated or deactivated by separation of the two domains. Here, we examine the nuclear and VPg-associated pools of NIa and NIa internal cleavage products, the requirement for internal NIa processing during TEV infection in protoplasts, and the activity of NIa proteinase at a novel site in a modified viral polyprotein in vivo.

MATERIALS AND METHODS

Plants and virus. An aphid-transmissible strain of TEV was obtained from William G. Dougherty (Oregon State University). *Nicotiana tabacum* cv. 'Xanthi nc' was used for virus infectivity assays, virus purification, nuclear inclusion body purification, and protoplast isolation.

Purification of TEV, TEV RNA, and nuclear inclusion bodies. Virions and TEV nuclear inclusion bodies were isolated from infected plants as described by Dougherty and Hiebert (22, 23). Viral RNA was isolated by disruption of virus

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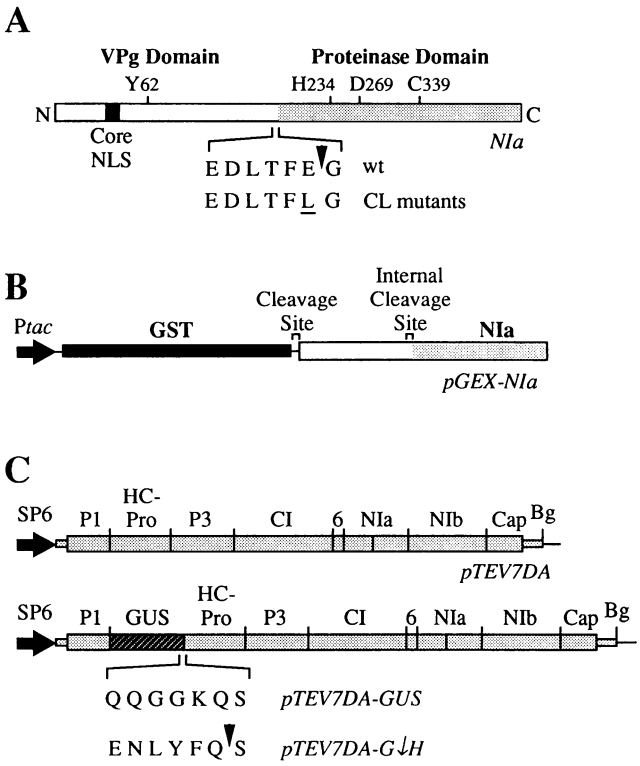

FIG. 1. Diagrammatic representation of NIa and selected recombinant plasmids. (A) Structure of NIa. The core nuclear localization signal (NLS) (black box), VPg domain (open box), and proteinase domain (shaded box) are indicated. Tyrosine (Y) at position 62 is the site of covalent attachment to RNA (34). Catalytic residues (H234, D269, and C339) essential for NIa proteolytic activity (26) are shown. The heptapeptide amino acid sequences around the internal cleavage site in wild-type (wt) and mutagenized (CL) constructs are given below the map, with an arrow indicating the site of processing. (B) Relevant portions of the *E. coli* expression plasmid pGEX-NIa, including the *tac* promoter, the GST and NIa sequences, and NIa-mediated cleavage sites. The cleavage site between GST and NIa sequences is the site naturally found at the NIa N terminus. (C) Relevant portions of three plasmids from which infectious TEV RNAs were derived. Sequences coding for polyprotein cleavage sites are indicated by vertical dashes. The junction sequences between GUS and HC-Pro encoded by pTEV7DA-GUS and pTEV7DA-G ↓ H are shown below the maps. The pTEV7DA-G ↓ H junction sequence constitutes an NIa-mediated cleavage site. Abbreviations: CI, cylindrical inclusion protein; Cap, capsid protein; Bg, *Bgl*II restriction site.

particles in 1% sodium dodecyl sulfate (SDS)–0.1 M ammonium carbonate (pH 9.0)–1 mM EDTA at room temperature, extraction twice with buffer-saturated phenol, extraction twice with water-saturated ether, and precipitation with ethanol. The RNA was resuspended in deionized sterile water. In experiments to analyze the VPg composition, 10 μg of viral RNA was digested with 10 U of RNase T_1 or RNase A for 30 min at 37°C. The products were subjected to immunoblot analysis using anti-NIa serum and ^{125}I-protein A (New England Nuclear) as detailed previously (12). Nuclear inclusion preparations, which were also treated with RNase A under the conditions described above, were analyzed in parallel with the VPg oligonucleotides.

Construction of recombinant plasmids. All numbering of TEV nucleotides and amino acid residues was based on that of Allison et al. (1). Wild-type and mutagenized NIa coding sequences were introduced into a glutathione *S*-transferase (GST) fusion vector, pGEX-2T (Pharmacia). First, the TEV fragment consisting of nucleotides 5412 to 7098 was subcloned into pTL7SN.3 (10), generating pTL7SN.3-5471. The Glu-189 codon (GAA) of NIa at nucleotides 6253 to 6255 was changed to a Leu codon (TTG) by site-directed mutagenesis (31) of pTL7SN.3-5471, forming pTL7SN.3-5471/CL. A substitution of Leu for Glu-189, which occupies the P1 position at the internal NIa cleavage site, was predicted to debilitate substrate activity, as indicated in previous studies (20). The sequence GAATTC, which constitutes an *Eco*RI site, was inserted by site-directed mutagenesis immediately after nucleotides 5673 and 6982 in both pTL7SN.3-5471 and pTL7SN.3-5471/CL. The *Eco*RI-*Eco*RI fragment, coding for the last six amino acid residues of the 6-kDa protein and the complete NIa, was isolated from both plasmids and subcloned into pGEX-2T. The resulting constructs, pGEX-NIa and pGEX-NIa/CL, encoded a GST/NIa polyprotein containing a cleavage site between GST and NIa and either a wild-type or mutagenized internal NIa processing site (Fig. 1B).

The sequence coding for the defective NIa internal cleavage site was transferred to two plasmids, pTEV7DA and pTEV7DA-GUS, each containing a full-length copy of the TEV genome downstream from a bacteriophage SP6 promoter (Fig. 1C). These plasmids were nearly identical to pTEV7D and pTEV7D-GUS.HC described previously (19) except that the plasmids used in the present study contained only one (rather than three) nonviral nucleotide between the promoter and TEV sequence. Transfer of the mutagenized cleavage site sequence first required construction of pTL7SN.3-5485/CL, a plasmid harboring TEV nucleotides 5412 to 8517, including the defective internal cleavage site sequence. The sequence between nucleotides 5917 and 7166 was excised from pTL7SN.3-5485/CL by digestion with *Bam*HI and *Sal*I and inserted between the corresponding *Bam*HI and *Sal*I sites of pTEV7DA and pTEV7DA-GUS, resulting in pTEV7DA/CL and pTEV7DA-G/CL, respectively. Virus derived from infection by transcripts containing the β-glucuronidase (GUS) gene in the TEV genome was termed TEV-GUS.

A reversion mutation, in which the Leu-189 codon (TTG) was changed to the original Glu codon (GAA), was introduced into pTEV7DA-G/CL. First, the wild-type sequence in pTL7SN.3-5485/CL was restored by oligonucleotide-directed mutagenesis. The *Bam*HI-*Sal*I fragment containing TEV nucleotides 5917 to 7167 was then used to replace the corresponding fragment in pTEV7DA-G/CL, generating pTEV7DA-G/CL_r.

The sequence coding for a NIa cleavage site was engineered between the GUS and HC-Pro sequences in pTEV7DA-GUS (Fig. 1C). An oligonucleotide specifying the heptapeptide Glu-Asn-Leu-Tyr-Phe-Gln-Ser was introduced by site-directed mutagenesis after the GUS sequence in the intermediate vector pTL7SN.3-0027DA-GUS, resulting in pTL7SN.3-0027DA-G ↓ H. This heptapeptide sequence was identical to the NIb/capsid protein cleavage site motif and was shown to confer NIa processing susceptibility in vitro when introduced at foreign sites within heterologous proteins (9, 24). A *Bst*EII-*Bst*EII fragment from pTEV7DA, containing TEV nucleotides 1430 to 9461, was excised and subcloned in place of the corresponding fragment in pTL7SN.3-0027DA-G ↓ H, generating the full-length clone pTEV7DA-G ↓ H.

The sequence coding for Gly-Asp-Asp between amino acid positions 347 and 349 in NIb, the RNA-dependent RNA polymerase, was changed by site-directed mutagenesis to a sequence encoding Val-Asn-Asn in pTL7SN-SP. This intermediate vector contained TEV nucleotides from position 7166 to

the 3′ end, including a poly(A) tail and a flanking *Bgl*II site. The TEV sequence from this plasmid was excised with *Sal*I and *Bgl*II and transferred to the corresponding position in pTEV7DA-GUS, forming pTEV7DA-G/VNN.

In vitro transcription and protoplast inoculation. Plasmids used to generate full-length TEV transcripts were purified by cesium chloride gradient centrifugation. Capped transcripts were produced from *Bgl*II-linearized DNA as described elsewhere (19). Transcripts were concentrated approximately fourfold by precipitation on ice in the presence of 2 M lithium chloride (15). Protoplasts were prepared from greenhouse-grown plants and inoculated with transcripts (approximately 10 μg/7.5 × 10^5 protoplasts), using a polyethylene glycol-mediated transfection procedure described previously (10, 35). Protoplasts were cultured at room temperature, and aliquots (2.5 × 10^5 protoplasts) were sampled at 24, 48, and 72 h postinoculation. Cells were lysed and assayed for GUS activity as described previously (10, 30).

Infection of plants. Plants were dusted with carborundum and inoculated mechanically with nondiluted transcripts directly after in vitro transcription. Following the appearance of virus-induced symptoms, leaves were harvested, ground in protein dissociation buffer, and subjected to immunoblot analysis using anti-HC-Pro or anti-GUS serum as described previously (36).

Expression of proteins in *Escherichia coli*. Plasmids pGEX-2T, pGEX-NIa, and pGEX-NIa/CL were propagated in *E. coli* TG1. Induction of GST/NIa polyprotein expression with isopropylthiogalactopyranoside (IPTG), extraction of total proteins, and immunoblot analysis with anti-NIa serum and alkaline phosphatase-linked second antibody have been described elsewhere (13).

RESULTS

The objectives of this study were to examine the composition of VPg-associated and nuclear pools of NIa, to investigate the role of internal cleavage of NIa in vivo, and to test for the presence of NIa proteolytic activity capable of processing an artificial cleavage site in the cytoplasm of infected cells.

Immunoblot analysis of NIa forms in vivo. Although the sizes of NIa-related proteins have been documented previously, it was revealing to compare directly the compositions of nuclear and VPg-associated pools of NIa. Immunoblot analysis using anti-NIa serum was carried out. A single, 49-kDa species was detected in nuclear inclusion body preparations (Fig. 2, lane 3), in total nuclear extracts (data not shown), and in total protein extracts from infected leaves (36). In contrast, three NIa oligonucleotides were detected after incubation of genomic RNA with RNase. RNase A treatment, which cleaved after the first pyrimidine residue (U) at nucleotide position 5, resulted in release of two NIa-related species migrating slightly more slowly than the 49-kDa protein and one migrating just behind the 21-kDa form (Fig. 2, lane 1). The mobility of each form was retarded when VPg oligonucleotides were prepared with RNase T_1, which cleaved after the first guanosine residue at nucleotide position 39 (Fig. 2, lane 2). Three species were also detected after digestion with RNase P_1, which cleaves RNA nonspecifically (data not shown). The basis for two, rather than one, larger NIa forms was not determined. These results indicate that the internal cleavage site is utilized rarely in the NIa pool transported to the nucleus, whereas this site is processed in a significant proportion of genomic RNA-linked NIa.

Effect of mutation of the internal NIa cleavage site in vivo. Both full-length and N-terminal processed forms of TEV NIa

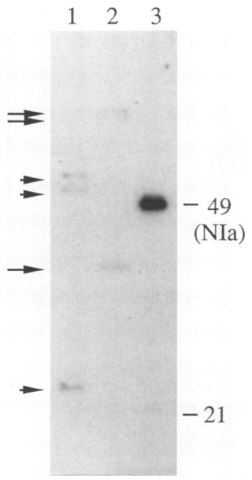

FIG. 2. Immunoblot analysis of NIa-related proteins isolated from TEV-infected plants. Proteins were resolved by electrophoresis in a discontinuous SDS-polyacrylamide gel (12.5%), transferred to a nitrocellulose membrane, and probed with anti-NIa serum and ^{125}I-protein A. The blot was then subjected to autoradiography. Lanes 1 and 2, VPg oligonucleotides prepared by digestion of viral RNA with RNase A (lane 1) or T_1 (lane 2). The short and long arrows indicate the VPg oligonucleotide species derived by treatment with RNases A and T_1, respectively. Lane 3, nuclear inclusion protein treated with RNase A. The sizes of NIa and the N-terminal NIa cleavage product are given in kilodaltons at the right.

are present in the VPg pool. Additionally, both full-length and C-terminal processed forms are fully functional as proteinase (8, 25). It may be questioned, therefore, whether processing at the internal NIa cleavage site is physiologically relevant. To address the importance of processing at this site, the effects of mutations at the NIa internal cleavage were determined. A substitution resulting in conversion of Glu-189 (the P1 position residue of the internal cleavage site) to Leu was made first in pGEX-NIa, a bacterial expression plasmid encoding a GST/NIa polyprotein (Fig. 1B). In cells expressing a wild-type NIa sequence fused to GST, both full-length (49-kDa) and processed (21-kDa) NIa forms accumulated after induction as a result of utilization of the cleavage sites between GST and NIa and between the VPg and proteinase domains within NIa (Fig. 3A, lane 2). For reasons that were not determined, the 27-kDa proteinase domain was not detected consistently in these experiments. In cells expressing the GST/NIa polyprotein containing an altered internal cleavage site, processing occurred at the NIa N terminus to yield the 49-kDa protein but not at the internal, modified site (Fig. 3A, lane 3). This result indicated that the mutation inactivated the internal cleavage site but not the proteolytic activity of NIa.

The Glu-189-to-Leu mutation was transferred to a full-length TEV cDNA clone to generate pTEV7DA/CL. Transcripts were produced by using bacteriophage SP6 polymerase and applied to tobacco plants by mechanical inoculation. Whereas transcripts synthesized from the wild-type plasmid, pTEV7DA, were infectious in 7 of 11 plants, transcripts from pTEV7DA/CL failed to infect any of 10 inoculated plants, as judged by a lack of virus-induced symptoms. Although authentic TEV RNA contains a VPg rather than a cap structure linked to the 5′ terminus, noncapped SP6 transcripts of the TEV genome were noninfectious (unpublished observations). All experiments reported here, therefore, were conducted by using m^7GpppG-capped transcripts.

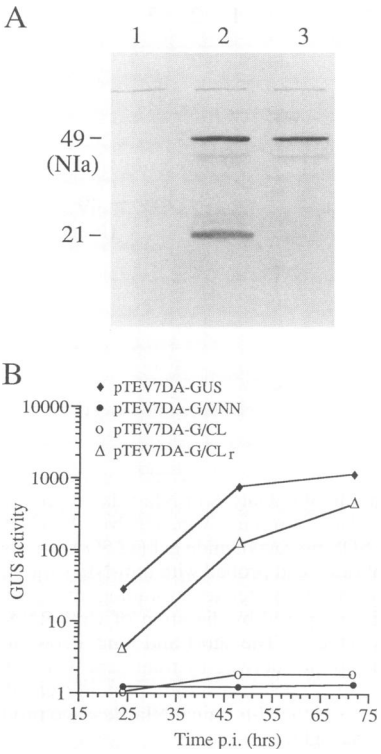

FIG. 3. Mutational analysis of the NIa internal cleavage site. (A) Processing of NIa-containing polyproteins in *E. coli*. Total protein extracts from cultures containing the expression vector pGEX-2T (lane 1), pGEX-NIa (lane 2), or pGEX-NIaCL (lane 3) containing the Glu-189-to-Leu substitution in NIa were subjected to immunoblot analysis using anti-NIa serum and alkaline phosphatase-linked second antibody. The 21-kDa protein in lane 2 resulted from NIa-mediated processing at both the GST/NIa junction and the internal NIa cleavage site. (B) Effect of the internal cleavage site mutation on TEV-GUS amplification in protoplasts. Equivalent concentrations of transcripts generated from the plasmids indicated were introduced into 7.5×10^5 protoplasts by polyethylene glycol-mediated transfection. GUS activity (pmoles of substrate cleaved per minute per 10^5 protoplasts) was measured at 24, 48, and 72 h postinoculation (p.i.).

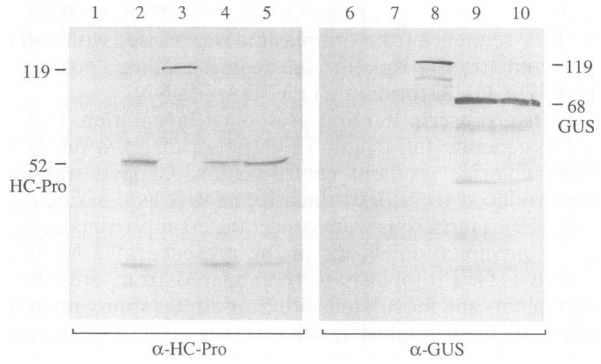

FIG. 4. Immunoblot analysis of HC-Pro- and GUS-related proteins encoded by TEV-GUS derivatives from pTEV7DA-GUS and pTEV7DA-G ↓ H in infected plants. Duplicate immunoblots were prepared with total protein extracts from systemically infected leaves of plants inoculated with wild-type TEV-HAT (lanes 2 and 7) or with transcripts from pTEV7DA-GUS (lanes 3 and 8) or pTEV7DA-G ↓ H (lanes 4, 5, 9, and 10). Two pTEV7DA-G ↓ H transcript-inoculated plants were analyzed. Extracts from mock-inoculated plants were applied to lanes 1 and 6. The blots were probed with anti-HC-Pro (left) or anti-GUS (right) serum. Molecular sizes of GUS/HC-Pro (119 kDa), GUS (68 kDa), and HC-Pro (52 kDa) are indicated.

The internal cleavage site mutation was transferred to the GUS-containing, full-length cDNA clone to produce pTEV7DA-G/CL. In addition, a site-directed reversion mutation, in which the mutant Leu codon was changed to the wild-type Glu codon, was generated and subcloned into pTEV7DA-G/CL, yielding pTEV7DA-G/CL_r. Protoplasts were inoculated with transcripts from these plasmids, from nonmutagenized pTEV7DA-GUS, and from pTEV7DA-G/VNN, and GUS activity over time was used as a surrogate marker for TEV RNA amplification. Transcripts from pTEV7DA-G/VNN encoded a defective RNA-dependent RNA polymerase (NIb) containing a substitution of Val-Asn-Asn for the highly conserved Gly-347–Asp-348–Asp-349 motif. This nonreplicating mutant served to indicate GUS activity levels resulting from translation of input transcripts rather than newly synthesized RNAs.

Transcripts from pTEV7DA-G/CL failed to induce significant levels of GUS activity and were comparable to the replication-defective transcripts from pTEV7DA-G/VNN (Fig. 3B). The nonmutagenized transcripts from pTEV7DA-GUS, as well as transcripts from the cleavage site reversion mutant pTEV7DA-G/CL_r, stimulated GUS activity that increased over the 72-h incubation period. The inability of transcripts containing the cleavage site defect to amplify and the acquisition of amplification activity after restoration of the wild-type sequence suggest that NIa internal processing is required for TEV viability.

***trans* processing at an artificial NIa cleavage site in vivo.** The need for TEV NIa proteolytic activity in the cytoplasm, superimposed on the transport of most NIa molecules to the nucleus, is somewhat paradoxical. Is proteolysis by NIa a regulated, localized process in which all *cis* and *trans* reactions occur in an ordered complex prior to nuclear translocation, or is there a freely diffusible proteinase pool that catalyzes cleavage at the *trans*-processing sites? Such a proteinase pool would be expected to recognize and cleave a processing site introduced at a novel position in the polyprotein within the cytoplasm. To test the ability of NIa to recognize an introduced site, an artificial processing site (Glu-Asn-Leu-Tyr-Phe-Gln-Ser, within which cleavage occurs after Gln residue) was engineered between the GUS and HC-Pro sequences in the recombinant virus derived from pTEV7DA-GUS (Fig. 1C). The introduced cleavage site was identical to that at the junction between NIb and capsid proteins. Given that P1 and HC-Pro autoproteolytically function to separate the N-terminal proteins from the NIa-containing polyprotein segment, cleavage of the GUS/HC-Pro polyprotein should occur only if NIa encounters by chance the introduced site (i.e., in *trans*). This reaction should occur only in the cytoplasm, as HC-Pro, GUS, and GUS/HC-Pro fusion proteins accumulate in this subcellular compartment (11, 16, 36; unpublished observations).

Plants infected by wild-type TEV produced HC-Pro of 52 kDa, while plants containing virus derived from pTEV7DA-GUS accumulated the 119-kDa GUS/HC-Pro fusion protein (Fig. 4). This product was recognized by anti-HC-Pro and anti-GUS sera (Fig. 4, lanes 3 and 8). Plants infected by virus derived from pTEV7DA-G ↓ H, which encoded the artificial cleavage site between GUS and HC-Pro, accumulated individual 52-kDa HC-Pro and 68-kDa GUS proteins of the proper sizes (Fig. 4, lanes 4, 5, 9, and 10). The proteinase, therefore, was active at the artificial site, suggesting the presence of an

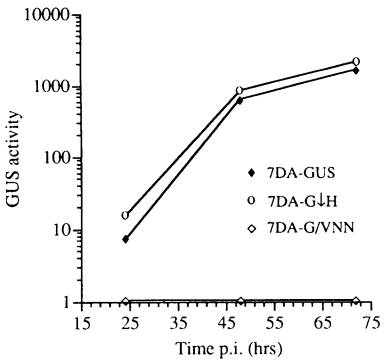

FIG. 5. Amplification of TEV-GUS derived from pTEV7DA-G↓H transcripts in protoplasts. Equivalent concentrations of transcripts generated from the plasmids indicated were introduced into 7.5 × 10^5 protoplasts by polyethylene glycol-mediated transfection. GUS activity (pmoles of substrate cleaved per minute per 10^5 protoplasts) was measured at 24, 48, and 72 h postinoculation (p.i.).

NIa activity that may be diffusible in the cytoplasm. Although we cannot formally exclude the possibility that cleavage occurred by an autoproteolytic mechanism resulting from an interaction between the cleavage site and NIa within a precisely folded polyprotein, the presence of this site at an artificial position within the polyprotein argues against this idea.

To determine the relative replicative abilities of pTEV7DA-GUS and pTEV7DA-G↓H-derived viruses, protoplasts were inoculated with transcripts from each plasmid and GUS activity was assayed over 72 h. Viruses derived from pTEV7DA-GUS and pTEV7DA-G↓H induced comparable levels of GUS activity over time (Fig. 5), indicating that the introduced cleavage site had little or no effect on virus viability. Essentially no GUS activity was detected in protoplasts inoculated with pTEV7DA-G/VNN transcripts (Fig. 5).

DISCUSSION

Internal cleavage of NIa. The internal cleavage site between the VPg and proteinase domains is processed in a subset of TEV NIa molecules in infected plants. A precise determination of the proportion of NIa that undergoes internal processing was not possible, as processed forms of NIa in total protein extracts were difficult to detect because of their low quantities (36). It is clear, however, that the proportion of protein processed internally is substantially higher in VPg-associated NIa than in NIa localized in the nucleus. In fact, analyses of VPg associated with plum pox and tobacco vein mottling potyvirus RNAs revealed only the processed, N-terminal domain of NIa (37, 40). The finding of apparent full-length forms linked to TEV RNA in this and previous studies by others (33) suggests that cleavage is not strictly required for VPg activity during RNA replication. The presence of full-length NIa linked to genomic RNA molecules, which obviously arise as products of RNA replication involving VPg activity, implies that nonprocessed NIa is active as VPg. It is possible that the full-length form (or an NIa-containing polyprotein) is the only active VPg molecule and that internal processing after RNA linkage generates the smaller, N-terminal species. Alternatively, both full-length and processed forms of NIa may be functional as VPg.

Despite the fact that most NIa molecules in TEV-infected plants are not processed between the VPg and proteinase domains, an active internal cleavage site appears to be required for virus infectivity. The mutation affecting Glu-189 in NIa eliminated both internal proteolytic processing in an *E. coli* expression system and virus viability in plants and protoplasts. The lack of infectivity was not due to another fortuitous mutation that escaped detection, as oligonucleotide-directed reversion of the altered codon to the wild-type sequence restored virus activity. At this point, the viral function that requires internal NIa processing is not clear. One possibility is that proteolytic cleavage generates a cytoplasmic form of the NIa proteinase that lacks the N-terminal nuclear localization signal. The NIa proteinase is fully functional as part of the 49-kDa form and as a 27-kDa processed product (25). The smaller proteinase form might be freely diffusible in the cytoplasm and necessary to catalyze one or more of the *trans*-processing reactions. By detecting NIa-mediated proteolysis at a novel cleavage site in the GUS/HC-Pro polyprotein in vivo, we provide evidence for a cytoplasmic pool of NIa proteinase with processing capacity in excess of that needed to carry out all required cleavage reactions. In addition, processing at the introduced site had no detrimental effect on virus replication in protoplasts, further supporting the idea of a surplus cytoplasmic NIa proteinase pool.

The replication-debilitated phenotype of the processing mutant may also be explained if internal NIa cleavage activates or deactivates essential replication-associated functions that have yet to be identified. In this context, it is interesting to note that the poliovirus 3C proteinase, a homolog of the potyvirus NIa proteinase domain, performs a role in RNA replication through formation of a noncovalent complex with sequences near the 5′ end of genomic RNA (2, 3). If this function is conserved between the picornaviruses and potyviruses, separation of the proteinase domain from the VPg domain may be necessary for RNA-protein interaction. Although the Glu-189-to-Leu mutation clearly restricted internal processing, we cannot exclude the possibility that the virus-debilitating effect was due to inactivation of a full-length NIa function rather than inhibition of cleavage.

TEV-GUS system. Because of the relatively low specific infectivity of synthetic, m^7GpppG-capped transcripts compared with authentic, VPg-containing TEV RNA in protoplasts, results of analyses of virus activity by serological assays and bioassay methods are inconsistent (unpublished observations). This problem prohibits quantitative measurements of wild-type or mutant virus replication in cultured cells. In this study, we used a reporter protein (GUS) encoded by an engineered TEV genome as a probe for viral RNA amplification. The fluorometric GUS assay is extremely sensitive, rapid, and quantitative, thereby providing a means to overcome the difficulties associated with infection of protoplasts by transcripts. Because of the production of GUS in amounts equimolar to those of all other TEV proteins and the high stability of GUS (30), measurement of GUS activity in protoplasts serves as a reliable indicator of viral protein accumulation over time. Increase of GUS activity over time indirectly correlates with virus replication activity, since TEV-encoded proteins and genomic RNA accumulate in proportion to one another (18). As demonstrated here, the TEV-GUS/protoplast system should permit extensive genetic analysis of each TEV-encoded protein. Finally, the ability to insert functional cleavage sites around the GUS sequence increases the potential applications for expression of engineered viral and nonviral proteins in plants.

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