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Complementation of tobacco etch potyvirus mutants by active RNA polymerase expressed in transgenic cells

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ABSTRACT **A genetic complementation system was developed in which tobacco etch virus (TEV) polymerase (NIb)-expressing transgenic plants or protoplasts were inoculated with NIb-defective TEV mutants. A β-glucuronidase (GUS) reporter gene integrated into the genomes of parental and four mutant viruses was used to assay RNA amplification. Two mutants (termed VNN and EDE) contained substitutions affecting the conserved "GDD" polymerase motif or a nuclear localization signal sequence, respectively; one (aD/b) contained a mutation debilitating the NIb N-terminal cleavage site, whereas the other (Δb) lacked the entire NIb sequence. Each mutant was unable to amplify in nontransformed tobacco protoplasts. In contrast, the VNN, EDE, and Δb mutants were complemented to various degrees in NIb-expressing cells, whereas the aD/b mutant was not complemented. The VNN mutant was complemented most efficiently, reaching an average of 11–12% the level of parental TEV-GUS, although in some experiments the level was near 100%. This mutant also replicated in, and spread through, whole transgenic plants to the same level as parental virus. The EDE mutant was complemented relatively poorly, reaching 1% or less of the level of parental TEV-GUS. Despite the close proximity of the EDE substitution to the N-terminal cleavage site, proteolytic processing of NIb was unaffected in an *in vitro* assay. The Δb mutant was complemented to an intermediate degree in protoplasts, reaching 3.5% the level of parental virus, and replicated and moved systemically in transgenic plants. These data indicate that free NIb supplied entirely in trans can provide all NIb functions essential for RNA amplification. The relative inefficient complementation of the EDE mutant suggests that the resulting mutant protein was trans-inhibitory.**

Potyviruses belong to the picornavirus-like supergroup of (+)-strand RNA viruses and contain a protein (VPg)-linked genome encoding a single polyprotein that undergoes proteolytic processing (1). Several potyviral proteins may be involved directly in viral RNA replication. The cytoplasmic inclusion protein is a RNA helicase (2), whereas the 6-kDa protein is an essential membrane-associated factor (3). The NIa protein carries out VPg and proteinase functions (4, 5), whereas the NIb protein is the putative RNA polymerase (6). These four proteins are clustered in the order cytoplasmic inclusion protein/6/NIa/NIb within the polyprotein and are processed by the NIa proteinase. Both NIa and NIb of several potyviruses, including tobacco etch virus (TEV), accumulate predominantly in the nucleus (7), although potyvirus replication likely occurs in a membrane-associated complex in the cytoplasm (8). How these replication-associated proteins interact in the cytoplasm remains to be determined.

For two other picornavirus-like supergroup members, picornaviruses and comoviruses, functional polyproteins consisting of NIa/NIb homologues have been characterized. Initiation of poliovirus (+)-RNA synthesis may involve binding of the 3CD (proteinase/polymerase) polyprotein to sequences near the genome 5′ end (9). The 3CD polyprotein also is required for capsid polyprotein processing (10, 11). A proteinase-polymerase precursor was identified as the major viral protein in active replication complexes isolated from cowpea mosaic virus-infected cells (12).

To study cis/trans requirements of the NIb polymerase during TEV RNA replication, a genetic complementation system based on transgenic expression of NIb was developed. Our results show that point and deletion mutants lacking functional NIb can be complemented at the single-cell and whole-plant levels and that all essential NIb activities can be provided in trans by the mature transgenic protein.

MATERIALS AND METHODS

Construction of Plasmids. Plasmids containing a full-length cDNA of the TEV genome, including the β-glucuronidase (GUS) sequence between the protein 1 and helper component-proteinase regions, were named with the prefix pTEV7DAN-GUS. Virus derived from *in vitro*-synthesized transcripts of parental pTEV7DAN-GUS (13) was designated TEV-GUS. pTEV7DAN-GUS/VNN contains a mutation substituting a sequence coding for Val-Asn-Asn in place of Gly-347–Asp-348–Asp-349 in NIb [numbering according to Allison *et al.* (6); Fig. 1]. pTEV7DAN-GUS/EDE contains a substitution of a Glu-Asp-Glu coding sequence for Lys-3–Arg-4–Lys-5 in NIb. pTEV7DAN-GUS/aD/b contains a mutation substituting an aspartate codon for the Gln-430 codon of NIa at the P1 position of the NIa/NIb cleavage site. pTEV7DAN-GUS/Δb lacks the entire NIb sequence. Genomes and viruses derived from these plasmids were designated as the VNN, EDE, aD/b, and Δb mutants. Mutations were generated by oligonucleotide-directed mutagenesis (14) and were confirmed by nucleotide sequence analysis.

The binary plasmid pGA-NIb, containing the 35S promoter and terminator of cauliflower mosaic virus, the 5′ nontranslated region of TEV, and the NIb coding region, was constructed as described (15). pGA-NIb/VNN is similar to pGA-NIb, except for the VNN substitution described above.

Plant Transformation and Immunoblot Analysis. *Nicotiana tabacum* cv. 'Xanthi nc' and 'Havana$_{425}$' plants were transformed as detailed (15). Immunoblot analysis using anti-NIb serum was done as described (15).

***In Vitro* Transcription and Protoplast Inoculation.** Full-length TEV transcripts were produced by using SP6 polymerase (Ambion) and were concentrated by precipitation in 2M LiCl (13). Leaf protoplasts ($3–8 \times 10^5$) were inoculated with transcripts (≈5–10 μg) and cultured as described (16), and GUS activity was analyzed as in previous studies (17).

Bioassay of VNN and Δb Mutants. Lysates from $\approx 2.5 \times 10^5$ VNN mutant- or TEV-GUS-infected protoplasts, or Δb mutant transcripts (20–40 μg), were applied to carborundum-

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Abbreviations: TEV, tobacco etch virus; GUS, β-glucuronidase; d.p.i., days postinoculation.

dusted leaves of NIb$^+$ transgenic plants. Systemically infected leaves were harvested at 8 days postinoculation (d.p.i.), ground in 5 vol of 10 mM Tris·HCl/1 mM EDTA, pH 7.6, and applied to another set of NIb$^+$ plants. GUS activity in inoculated leaves was analyzed histochemically by using 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc) and in systemically infected leaves by using the fluorometric substrate 4-methylumbelliferyl glucuronide. Reverse transcriptase-PCR was conducted as described (18).

Polyprotein Processing *in Vitro*. To analyze NIa/NIb polyprotein processing, transcripts from pTL7SN-a/b and pTL7SN-a/b-EDE, coding for a polyprotein composed of NIa and 63 residues of NIb, were translated in a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-labeled methionine (DuPont/NEN). After 10 min at 30°C, nonlabeled methionine (20 mM) was added, and products were withdrawn at various times.

RESULTS

NIb-Defective Mutants in Nontransformed Tobacco Protoplasts. Four TEV-GUS mutants containing sequence alterations affecting NIb or its N-terminal cleavage site were analyzed (Fig. 1). The VNN mutant contained a substitution affecting the conserved NIb "GDD" motif and has been reported (17) to be replication-defective. The EDE mutant possessed a mutation-affecting NIb Lys-3–Arg-4–Lys-5, a sequence identified as part of a nuclear localization signal (15) (unpublished data). The aD/b mutant contained a substitution affecting the P1 position of the NIa/NIb cleavage site, rendering the site proteolytically inactive (19). The Δb mutant lacked the NIb sequence entirely and encoded a polyprotein with a functional NIa/capsid protein cleavage site.

Protoplasts from *N. tabacum* 'Xanthi nc' plants were inoculated with transcripts representing TEV-GUS and the four mutants. Genome replication was measured with the GUS reporter, which has proven useful for quantitative analysis of TEV mutants (3, 17, 20). GUS activity increased steadily between 24 and 72 hr postinoculation in cells inoculated with TEV-GUS transcripts (Fig. 2). No GUS activity was stimulated by any of the mutants, suggesting that the modified genomes were amplification-defective.

Amplification of the VNN Mutant in NIb-Expressing Transgenic Tobacco Protoplasts. Transgenic plants designated NIb$^+$ encoded NIb that was identical to the authentic protein, except for the addition of methionine in the first position. Transgenic

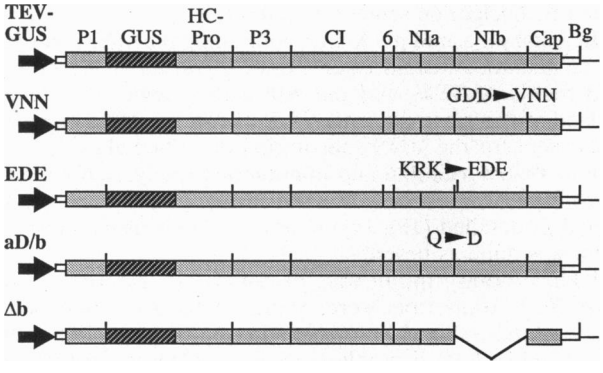

FIG. 1. Diagrammatic representation of essential portions of pTEV7DAN-GUS and its derivatives. The TEV (shaded) and GUS (hatched) coding sequence are indicated. Vertical lines represent sequences coding for proteolytic cleavage sites. Bg, *Bgl* II restriction site; P1, protein 1; HC-Pro, helper component-proteinase; P3, protein 3; CI, cylindrical inclusion protein; 6, -6-kDa protein; NIa, nuclear inclusion "a" protein; NIb, nuclear inclusion "b" protein; CP, capsid protein.

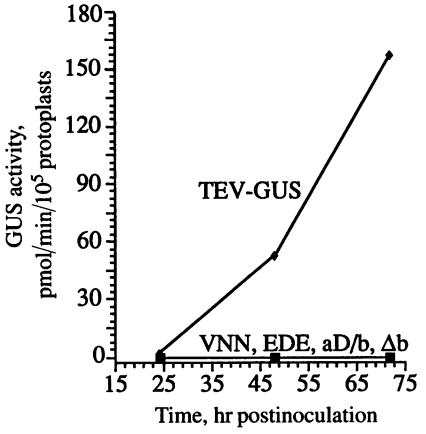

FIG. 2. Infection of nontransformed *N. tabacum* 'Xanthi nc' protoplasts with TEV-GUS and mutant transcripts. GUS activity (pmol of substrate cleaved per min/μg of protein) was measured at 24, 48, and 72 hr postinoculation. Mean values of activity from two contemporaneous infections from a representative experiment are plotted.

plants designated NIb/VNN produced a protein containing the VNN substitution described above. 'Havana$_{425}$' and 'Xanthi nc' lines expressing comparable levels of NIb (Fig. 3) and NIb/VNN (data not shown) were used.

Only parental TEV-GUS was amplified in protoplasts from nontransformed or vector-transformed plants (Fig. 4 *Left*). In striking contrast, both parental and VNN mutant genomes were amplified in NIb$^+$ cells (Fig. 4 *Right*). In some experiments (Fig. 4, experiment 1), the level of amplification of the mutant was comparable to that of TEV-GUS. Transgenic NIb$^+$ lines from both 'Xanthi nc' (experiment 1 and 2) and 'Havana$_{425}$' (experiment 3) cultivars promoted VNN genome amplification. No transgenic NIb/VNN lines supported replication of the VNN mutant (data not shown). It should be noted that the mean level of GUS activity induced by parental TEV-GUS varied considerably between different lines and protoplast preparations. Therefore, comparisons between parental and mutant infections were made only within the same experiment using the same protoplast preparation.

Infection of NIb$^+$ Transgenic Plants by the VNN Mutant. Amplification of the VNN mutant in NIb$^+$ protoplasts may have been due to complementation in trans by the transgenic protein or to restoration of the wild-type sequence by recombination with the transgene mRNA or by reversion. To distinguish between these possibilities, progeny TEV-GUS and

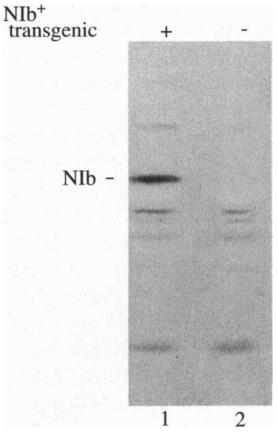

FIG. 3. Immunoblot analysis of NIb$^+$ transgenic plants. Extracts from NIb$^+$ transgenic (lane 1) or nontransgenic (lane 2) *N. tabacum* 'Xanthi nc' plants were subjected to immunoblot analysis with anti-NIb serum.

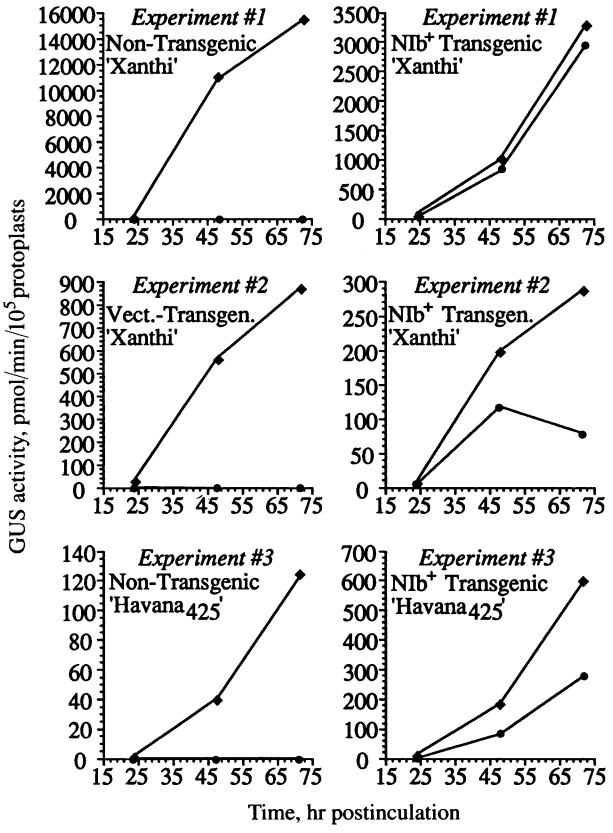

FIG. 4. Complementation of the VNN mutant in NIb$^+$ transgenic protoplasts. Data from three independent experiments are shown. Nontransgenic or vector-transgenic (*Left*) and NIb$^+$ transgenic (*Right*) protoplasts were inoculated with TEV-GUS (◆) and VNN mutant (●) transcripts. GUS activity was measured at 24, 48, and 72 hr postinoculation. Each data point represents the mean from two contemporaneous infections.

VNN mutant viruses derived from infected NIb$^+$ protoplasts were used to inoculate a test series of NIb$^+$, NIb/VNN, and vector-transformed plants. If amplification resulted from complementation, the VNN mutant progeny should infect only the NIb$^+$ transgenic plants. If amplification resulted from wild-type sequence restoration, the progeny should infect each type of plant.

Both the parental and VNN mutant viruses from inoculated NIb$^+$ protoplasts induced systemic infection in NIb$^+$ transgenic plants. Extracts from these plants were used to inoculate the test series. Inoculated leaves were infiltrated with a GUS histochemical substrate at 3 d.p.i. TEV-GUS infection foci were visible in leaves of vector-transformed (Fig. 5*A*), NIb$^+$ (Fig. 5*C*), and NIb/VNN plants (Table 1). Foci of VNN mutant infection were visible in leaves of NIb$^+$ transgenic plants (Fig. 5*D*), but not in leaves of vector-transformed (Fig. 5*B*) or NIb/VNN plants (Table 1). NIb$^+$ transgenic plants infected by either parental TEV-GUS or the VNN mutant exhibited typical vein-clearing symptoms by 6 d.p.i. The mean levels of GUS activity in leaves systemically infected by TEV-GUS or the VNN mutant were comparable (Table 1). Activity levels induced by parental and VNN mutant viruses varied over a 6-fold range between individual plants within a set, and this was reflected in the relatively high SD values. Nucleotide sequence analysis of reverse transcription-PCR products generated from extracts of upper noninoculated leaves of VNN mutant-infected NIb$^+$ transgenic plants revealed that the VNN mutation was preserved in the progeny virus. These data demonstrate that the NIb-defective VNN mutant was rescued by trans-complementation in single cells and whole plants.

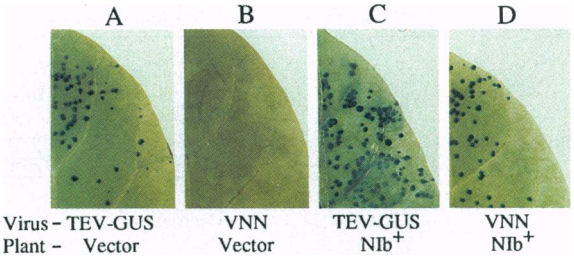

FIG. 5. Infection of vector-transformed and NIb$^+$ transgenic plants with the VNN mutant. Inocula were derived from TEV-GUS (*A* and *C*) or VNN mutant (*B* and *D*) that had been propagated in NIb$^+$ protoplasts and passaged once in NIb$^+$ transgenic plants. Infection foci were visualized 3 d.p.i. using a GUS histochemical substrate.

Complementation of Other NIb-Defective Mutants in Protoplasts and Plants. Protoplasts from NIb$^+$ and vector-transformed plants were inoculated with transcripts of the NIa/NIb cleavage site-defective aD/b mutant and the nuclear localization signal-defective EDE mutant genomes. GUS activity was not detected in either cell type inoculated with the aD/b mutant transcripts (Table 2), suggesting that NIb in trans was incapable of complementing this mutant. In contrast, GUS activity was detected consistently in NIb$^+$ transgenic, but not vector-transformed, cells inoculated with the EDE mutant transcripts. However, the mean percentage of EDE mutant activity relative to TEV-GUS in two sets of experiments was 1 and 0.7% (Table 2), which was only 8.4 and 5.7%, respectively, the level of VNN mutant activity using the same protoplast preparations. To determine whether the low complementation levels of the aD/b and EDE mutants were due to other changes unknowingly introduced into the genome, the mutations were changed back to their respective wild-type sequences by site-directed mutagenesis. In both cases, infectivity was restored in nontransgenic protoplasts, arguing against the presence of other debilitating mutations in either of the mutants.

The low complementation level of the EDE mutant could have been due to defective NIa-mediated processing at the nearby NIa/NIb cleavage site. To determine the effect of the EDE mutation on proteolysis, transcripts coding for a polyprotein consisting of NIa and part of NIb (wild-type or EDE mutant) were translated *in vitro*, and a pulse-chase analysis was conducted. Proteolysis resulting in free NIa was nearly complete after the 10-min pulse period for both wild-type and mutant polyproteins (Fig. 6). The wild-type and mutant precursors remaining after the 10-min pulse were processed similarly over the 65-min chase.

To determine whether a cis-required RNA or protein function was affected by the EDE mutation, amplification of the Δb mutant was tested in NIb$^+$ and vector-transformed cells. If the EDE substitution affected a cis-required activity of NIb, the Δb mutant lacking NIb entirely should have been complemented no better than, or worse than, the EDE mutant. The Δb mutant was complemented in NIb$^+$ protoplasts at 3.5% the level measured with parental TEV-GUS (Table 2). This was 30% the level detected with the VNN mutant using the same protoplast preparations, but 5-fold higher than the level measured with the EDE mutant. The Δb mutant was also capable of infecting NIb$^+$ plants systemically. At 8 d.p.i., GUS activity in leaves systemically infected by the Δb mutant was comparable to the activity level of parental TEV-GUS, although variation was high between plants (Table 1). These data indicate that all essential NIb functions can be provided by mature NIb expressed outside the context of the TEV polyprotein.

DISCUSSION

Active NIb polymerase expressed in transgenic tobacco cells rescued NIb-defective TEV mutants in trans. As a mutant

Table 1. Bioassay of VNN and Δ mutants in transgenic plants

Virus	Plant	Infectivity: Inoculated leaves — Infection foci*	Infectivity: Systemic leaves — Infected/inoculated†	Infectivity: Systemic leaves — GUS activity‡
Exp. 1§				
TEV-GUS	Vector	12¶	3/5	1346 ± 339
TEV-GUS	NIb+	32 ± 9	5/5	1388 ± 256
TEV-GUS	NIb/VNN	14 ± 10	5/5	1447 ± 946
VNN mutant	Vector	0	0/5	0
VNN mutant	NIb+	13 ± 1	5/5	1604 ± 1018
VNN mutant	NIb/VNN	0	0/5	0
Exp. 2§				
TEV-GUS	Vector	119 ± 57	4/4	1374 ± 1003
TEV-GUS	NIb+	125 ± 19	4/4	5861 ± 2866
TEV-GUS	NIb/VNN	137 ± 44	4/4	3792 ± 3338
Δb mutant	Vector	0	0/4	0
Δb mutant	NIb+	65 ± 18	4/4	7006 ± 7015
Δb mutant	NIb/VNN	0	0/4	0

*Infection foci per leaf were counted at 3 d.p.i. With one exception, mean values ± SD were determined using three (Exp. 1) or four leaves (Exp. 2).
†Systemically infected plants per number of plants inoculated.
‡GUS activity (pmol of substrate cleaved per min/μg of protein) was measured in a homogenate from the third leaf above the inoculated leaf at 8 (Exp. 2) or 9 (Exp. 1) d.p.i. Mean ± SD was determined with three to five plants.
§Inoculum was from infected NIb+ plants that had been inoculated with extracts from infected NIb+ protoplasts (Exp. 1) or RNA transcripts (Exp. 2).
¶As only two plants were analyzed (10 and 14 foci per leaf), SD was not calculated.

(VNN) containing a substitution of a conserved motif and a mutant (Δb) lacking the NIb sequence entirely could both be complemented, mature NIb is able to diffuse to sites of action. This result also indicates that no NIb-containing polyproteins perform essential functions during the potyviral replicative cycle, although supplementary or regulatory roles for such polyproteins are possible. The NIb polymerase stands in contrast to the picornavirus 3D polymerase, which appears to function in a polyprotein form in capsid protein processing and in a ribonucleoprotein complex proposed to be involved in initiation of (+)-RNA synthesis (9–11).

Previous reports of complementation of poliovirus 3D polymerase mutants yielded conflicting results. In one case, a 3D polymerase-defective mutant could not be rescued by coinfection with another poliovirus mutant, prompting the conclusion that 3D polymerase cannot be supplied exclusively in trans (21). Using a different approach, Charini *et al.* (22) demonstrated that the RNA chain-elongation activity of 3D polymerase could be supplied in trans by a replication-competent helper virus. In the latter study, it should be noted that all active viral nonstructural proteins could have been provided by the helper virus because complementation was detected by measurement of mutant viral RNA amplification.

The complementation system we present allows direct analysis of cis/trans relationships of viral proteins and overcomes some of the limitations of previous approaches. The use of an enzymatic reporter, as pointed out previously using TEV and poliovirus models (9, 13), provides an extremely sensitive means to measure mutant genome amplification. Complementation in this system ensures that all nontransgene-encoded viral functions are generated by the mutant virus. This technique allows analysis of individual protein functions in a manner more straightforward than in complementation systems requiring coinfection by two viruses. Although NIb was found not to require cis-linkage with other TEV proteins, this system is suited to detect those functions that do require such a linkage by transgenic expression of polyproteins.

The NIa/NIb cleavage site-defective mutant (aD/b) was not complemented in NIb+ transgenic cells. Considering that the NIb deletion mutant was complementable, it is unlikely that the replication defect of the aD/b mutant was due solely to an

Table 2. Relative amplification of NIb-defective mutants in transgenic protoplasts

Experiment set*	Plant	Virus† TEV-GUS	VNN	EDE	aD/b	Δb
1	Vector	100	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	ND
	NIb+	100	11.7 ± 6.3	1.0 ± 0.6	0.1 ± 0.1	ND
2	Vector	100	0.1 ± 0.2	0.2 ± 0.2	ND	0.0
	NIb+	100	11.6 ± 6.0	0.7 ± 0.4	ND	3.5 ± 2.4

ND, not determined.
*Mean values ± SD from at least three independent experiments were calculated for each experiment set.
†Relative mutant amplification, based on GUS activity at 72 hr postinoculation, was calculated as follows: (mutant activity per parental TEV-GUS activity) × 100. Note that values for mutants in vector-transformed cells are likely due to GUS activity induced by input, nonamplified transcripts.

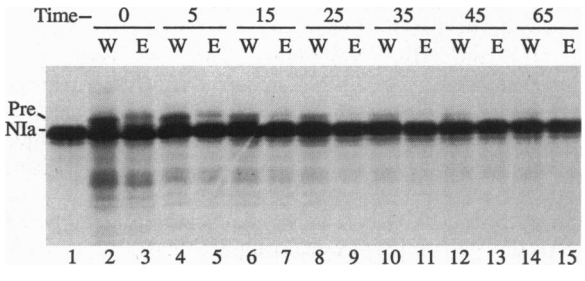

FIG. 6. Processing of the NIa/NIb cleavage site *in vitro*. Polyproteins consisting of NIa and the N-terminal 63 amino acid residues of NIb from wild-type (W) or the EDE mutant (E) were synthesized in a rabbit reticulocyte lysate for 10 min in the presence of ^{35}S-labeled methionine, after which excess nonlabeled methionine was added. Samples were withdrawn at the times (min after addition of methionine) indicated and subjected to SDS/PAGE and autoradiography. Radiolabeled NIa is shown in lane 1. Positions of the precursor (Pre) and NIa product are indicated.

inactive NIb. It is possible that NIa functions were impaired in this processing-defective mutant. The rate of NIa-mediated proteolysis might have been affected, although NIa-containing polyproteins were shown previously to retain proteolytic activity (23, 24). The VPg function of NIa may have been disrupted, even though the VPg region is located near the N terminus in a biochemically distinct domain (4). Alternatively, an undiscovered NIa function may have been inactivated.

The EDE mutant was not complemented efficiently in NIb$^+$ transgenic cells. Again, in view of the level of Δb mutant complementation, which was substantially higher than that of the EDE mutant, we argue that this was not due to a defective cis-required function. Neither is it likely that NIa/NIb proteolysis was affected significantly, even though the substitution was only three amino acid residues away from the cleavage site. NIb molecules containing the EDE substitution are unable to translocate to the nucleus in plant cells (15). Although the significance of NIb nuclear transport for the virus is not known, failure to transport could have at least two consequences. (*i*) If a NIb function is required in the nucleus, failure to translocate would essentially abolish this activity. This possibility seems unlikely given that the transgenic NIb protein, which should have been transported (16), was incapable of supporting efficient replication of the mutant. (*ii*) Excessive accumulation of NIb in the cytoplasm could be inhibitory. Irrespective of a nuclear transport defect, the EDE mutant protein may be trans-inhibitory to the complementing activity of the transgenic protein. This activity can be envisioned if NIb has distinct domains involved in, for example, protein–protein interaction and polymerase activities, as shown with brome mosaic virus (25). The EDE mutation may affect one activity but not the others, resulting in competitive interference with fully active NIb in the transgenic cells. Following this argument, NIb with the VNN substitution may be a relatively poor competitor with active transgenic NIb, allowing a higher level of complementation.

These results have implications beyond trans-activation of TEV polymerase-defective mutants. Numerous reports have suggested that expression of replicase genes from (+)-strand RNA plant viruses, including potyviruses, in plants confers high levels of resistance to virus infection (26, 27). Speculation about the mechanism of resistance has centered primarily around interfering effects of the transgenic proteins (28). On the basis of the abilities of several NIb-expressing lines to complement NIb-defective mutants, resistance observed by others may not be a simple consequence of protein-mediated interference. Finally, the ability to rescue replication-defective mutants specifically in transgenic plants may have utility for large-scale expression of commercially important products in plants using replicating viral vectors. Use of helper plant-dependent viruses in conjunction with complementing transgenic hosts would ensure that recombinant virus vectors remain contained in field situations.

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