VIROLOGY 224, 368–379 (1996)
ARTICLE NO. 0544

NIa and NIb of Peanut Stripe Potyvirus Are Present in the Nucleus of Infected Cells, but Do Not Form Inclusions

M. R. HAJIMORAD,*,[1] X. S. DING,* S. FLASINSKI,* S. MAHAJAN,† E. GRAFF,† R. HALDEMAN-CAHILL,† J. C. CARRINGTON,† and B. G. CASSIDY*,[2]

*Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, Oklahoma 73402; and †Department of Biology, Texas A&M University, College Station, Texas 77843

Received June 12, 1996; accepted August 5, 1996

We investigated, by immunological and gene-fusion methods, whether the failure of peanut stripe potyvirus (PStV)-encoded nuclear inclusion proteins a (NIa) and b (NIb) to form nuclear inclusions is due to the lack of their *in vivo* accumulation or the inability of one or both proteins to be transported into the nucleus. NIa domains (NIa-VPg and NIa-proteinase), full-length NIb, and full-length cylindrical inclusion (CI) protein of PStV were cloned, expressed in *Escherichia coli,* and used for antisera production. Immunoblot analysis of accumulation of NIa, NIb, and CI in time course experiments revealed that they accumulated to similar levels in PStV-infected *Nicotiana benthamiana.* In immunocytochemical studies with electron microscopy, antiserum against NIa-VPg, NIa-Pro, and NIb specifically labeled NIa and NIb proteins throughout the nuclei of PStV-infected cells, in the absence of nuclear inclusions. Translational fusions were made between NIa and NIb to either the green fluorescence protein or the β-glucuronidase in vectors for transient gene expression or stable expression in transgenic plants, respectively. Fusion proteins containing NIa accumulated in the nucleus, whereas fusion proteins containing NIb accumulated in a punctate pattern in the cytoplasm. These data indicate that at least NIa possesses a nuclear localization signal.

INTRODUCTION

The *Potyviridae* represent the largest and most economically important family of plant viruses (Ward and Shukla, 1991). Virion particles are composed of a single-stranded positive-sense RNA of nearly 10 kb encapsidated by approximately 2000 copies of capsid protein monomers in a helical fashion (Lindbo and Dougherty, 1994). The potyviral genome is covalently bound at the 5′ end to a virally encoded protein designated VPg (Hari, 1981; Siaw *et al.,* 1985; Riechmann *et al.,* 1989; Murphy *et al.,* 1990) and has a poly(A) sequence at the 3′ end (Hari *et al.,* 1979). The genome contains a single open reading frame that is translated into a large polyprotein and processed co- and posttranslationally into eight or more proteins by three virally encoded proteinases (Dougherty and Semler, 1993).

Some of the potyviral proteins can aggregate within different subcellular compartments, forming stable structures termed inclusion bodies (Edwardson *et al.,* 1993). However, the function(s) of these inclusions, as well as the mechanism(s) by which they are formed, has not been elucidated. Cylindrical (also known as pinwheel or cytoplasmic) inclusions (Edwardson, 1966) are formed by aggregation of the 70-kDa cylindrical inclusion (CI) protein in the cytoplasm of infected cells (Edwardson *et al.,* 1993). This protein has ATPase and RNA helicase activities (Lain *et al.,* 1990, 1991). All potyviruses induce the formation of characteristic cylindrical inclusions in the cytoplasm of infected cells (Edwardson, 1974), but only some induce nuclear inclusions. Nuclear inclusions are composed of two virally encoded nonstructural proteins termed nuclear inclusion protein a (NIa) and nuclear inclusion protein b (NIb) (Knuhtsen *et al.,* 1974; Dougherty and Hiebert, 1980; Baunoch *et al.,* 1988). The NIa protein contains N-terminal (NIa-VPg) and C-terminal (NIa-Pro) domains that function during genome replication and polyprotein processing (Carrington and Dougherty, 1987; Shahabuddin *et al.,* 1988; Klein *et al.,* 1994). The NIb protein contains the conserved motifs that are characteristic of RNA-dependent RNA polymerase of positive-strand RNA viruses (Koonin, 1991; Allison *et al.,* 1986; Domier *et al.,* 1987). Although some potyviruses are capable of inducing nuclear inclusions (Lesemann, 1988), several including tobacco vein mottling virus (TVMV) and peanut stripe virus (PStV) do not (Edwardson and Christie, 1991; Cassidy *et al.,* unpublished), despite the similarity in their genome organization and sequences to potyviruses that form nuclear inclusions (Robaglia *et al.,* 1989; Allison *et al.,* 1986; Domier *et al.,* 1986; Gunasinghe *et al.,* 1994; Maiss *et al.,* 1989; Puurand *et al.,* 1994; Vance *et al.,* 1992).

Studies on immunolocalization and physical and biochemical properties including the identification of nu-

[1] Current address: Department of Plant Pathology, University of Kentucky, Lexington, KY 40546.

[2] To whom reprint requests should be addressed. Fax: (405) 221-7380; E-mail: bgcassidy@noble.org.

0042-6822/96 $18.00

clear localization signals within NIa and NIb of potyviruses have been concentrated on those from tobacco etch virus (TEV) (Baunoch *et al.,* 1988, 1991; Knuhtsen *et al.,* 1974; Dougherty and Hiebert, 1980; Restrepo *et al.,* 1990; Carrington *et al.,* 1991; Restrepo-Hartwig and Carrington, 1992; Li and Carrington, 1993). However, studies on the proteins from potyviruses lacking the ability to form nuclear inclusions have not been reported. We hypothesized that the failure of PStV to form nuclear inclusions was due to either the lack of accumulation of NIa and NIb in infected tissues or the inability of one or both of the proteins to be transported to the nucleus. In this paper, we demonstrate that both NIa and NIb of PStV accumulate in infected tissues and are present in the nuclei of the infected cells. We also show that NIa, but not NIb, can direct the transport of reporter proteins to the nucleus.

MATERIALS AND METHODS

PCR amplification and cloning into pET-15b

To clone the coding regions of CI, NIa, NIa-VPg, NIa-Pro, and NIb, forward and reverse oligonucleotide primers flanking each gene were designed (Fig. 1) according to the published sequence of the blotch isolate of PStV (Gunasinghe *et al.,* 1994). Forward primers (except for primer E) contained an ATG initiation codon within a *Nco*I restriction site, and reverse primers (except for primer D) contained stop codons. All primers contained restriction sites for subcloning except for primers D and E (Fig. 1B). The primers were synthesized using an Applied Biosystems Synthesizer 392. PStV-IT (Flasinski *et al.,* 1996) which contains an infectious full-length cDNA copy of the genomic RNA of a blotch isolate of PStV (Demski *et al.,* 1984) was used as a template for PCR amplification using *Taq* or *Pfu* under standard conditions (Innis *et al.,* 1990). The CI and NIa PCR fragments were cloned into the *Sma*I site of pUC19. The CI fragment was then released by *Nco*I and *Bam*HI restriction enzyme digestions and subsequently cloned into the pET-15b expression vector (Novagen, Madison, WI) digested with *Nco*I and *Bam*HI. The NIa-VPg PCR fragment was digested with *Nco*I and cloned into pET-15b digested with *Xho*I (filled in with Klenow fragment DNA polymerase) and *Nco*I restriction enzymes. The NIa-Pro PCR fragment was digested with *Bam*HI and cloned into *Nco*I (filled in with Klenow fragment DNA polymerase) and *Bam*HI sites of pET-15b. The NIb PCR fragment was cloned into an *Eco*RV-digested, T-tailed pBluescript (SK$^-$) (Stratagene, La Jolla, CA). A fragment was then released after restriction digestion of the plasmid with *Nco*I (partially digested) and *Bam*HI. The fragment was subcloned into pET-15b digested with *Nco*I and *Bam*HI. *Escherichia coli* strains DH5α or JM101 (Gibco BRL, Gainthersburg, MD) were used for cloning. The presence of CI, NIa, NIa-VPg, NIa-Pro, and NIb genes in *E. coli* were confirmed by restriction enzyme digestion and sequencing.

Protein overexpression and purification

Proteins were expressed from pET-15b plasmids in *E. coli* strains BL21(DE3) or HMS174(DE3) (Novagen). Following isopropyl β-thiogalactopyranoside induction, bacterial inclusion proteins were purified (Harlow and Lane, 1988). The final pellet was dissolved in 100 m*M* NaCl, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 8.0. The proteins were diluted in an equal volume of 2$\times$ SDS sample buffer (Laemmli, 1970) and electrophoresed in 12% SDS polyacrylamide gel (SDS–PAGE) using discontinuous buffer system (Laemmli, 1970). The separated proteins were detected either by staining with Coomassie brilliant blue or with a cold solution of 0.2 *M* KCl containing 1 m*M* DTT (Harlow and Lane, 1988). The protein was electroeluted from KCl-stained polyacrylamide gels with the aid of a microelectroeluter (Amicon, Beverly, MA) in the presence of SDS–PAGE running buffer (Laemmli, 1970). The protein solutions were concentrated by a microconcentrator (Amicon) and the proteins were washed six times in phosphate-buffered saline (173 m*M* NaCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, 2.7 m*M* KCl, pH 7.4). The concentrations of the proteins were determined by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad). The N-terminal sequences of the purified *E. coli*-expressed CI, NIa-VPg, NIa-Pro, and NIb were determined using an Applied Biosystems peptide sequencer (477A).

Production of polyclonal antibodies

Antisera against the proteins were produced in rabbits by contract with HRP, Inc. (Denver, PA). Each rabbit was initially injected intranodally with the purified protein and thereafter five more times subcutaneously at 21, 42, 62, 82, and 104 days postimmunization. For each injection, 250 μg protein was emulsified with an equal volume of Freund's complete adjuvant for the intranodal injections and Freund's incomplete adjuvant for the subcutaneous injections. The rabbits were bled prior to immunization and also at 31, 52, 72, 93, and 114 days postimmunization. Antisera titrations were determined by immunoblotting against gel-purified *E. coli*-expressed homologous antigen or a preparation of partially purified proteins from PStV-infected or healthy *Nicotiana benthamiana*.

Protein isolation from plants and immunoblot analysis

N. benthamiana were inoculated as described (Cassidy and Nelson, 1995) using *in vitro*-synthesized PStV RNA from the full-length cDNA copy of PStV-IT or progeny virus from infectious transcripts (Flasinski *et al.,* 1996). Proteins from PStV-infected and healthy plants were partially purified by differential centrifugation as described by Hiebert *et al.* (1984). The final pellets were dissolved in 200 μl of 20 m*M* Tris–HCl, pH 8.2, and mixed with an equal volume of 2$\times$ SDS sample buffer and analyzed by 12% SDS–PAGE. Separated proteins were detected by staining with Coomassie brilliant blue or were transferred

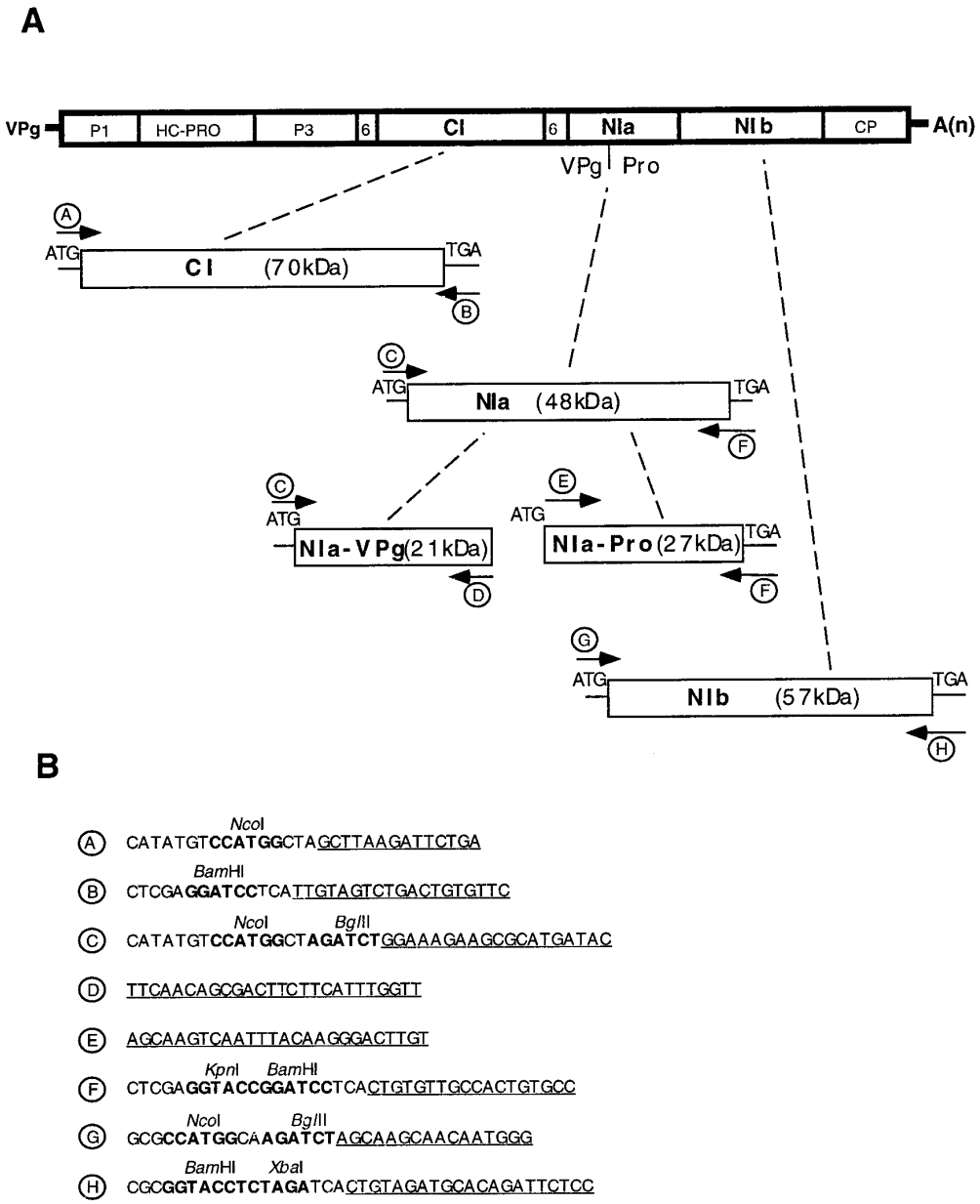


FIG. 1. (A) The proposed genomic map of PStV (Gunasinghe *et al.,* 1994) and the schematic representation of PCR-amplified coding sequences of CI, NIa, NIa-VPg, NIa-Pro, and NIb. Forward and reverse oligonucleotide primers shown as arrows. (B) Sequences of forward and reverse oligonucleotide primers used to generate each fragment. Encircled letters designate each oligonucleotide primer, nucleotides identical or complementary to PStV sequences are underlined, and recognition sequences for restriction enzymes used for cloning are shown in bold.

to nitrocellulose membranes for immunoblot analysis (Sambrook *et al.,* 1989). Bound antibodies were detected with affinity-purified goat anti-rabbit IgG(FC) conjugated with alkaline phosphatase (Promega, Madison, WI) diluted 1:7000 followed by incubation with nitroblue tetrazolium/5-bromo-4-choloro-3-indolyl phosphate. To determine the time of appearance and accumulation of PStV-encoded proteins in infected tissues, leaves were harvested from 0 to 15 days postinoculation (dpi), pulverized in liquid nitrogen, and extracted in three volumes (w/v) of a buffer containing 2% (w/v) SDS, 2% (w/v) sodium deoxycholate, 2% (v/v) Triton X-100, 20 m*M* methionine (Graybosch *et al.,* 1989) or 0.625 *M* Tris–HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol (Dolja *et al.,* 1994). The homogenates were boiled for 10 min and centrifuged at 16,000 *g* for 5 min. The supernatants were mixed with equal volumes of 2× SDS sample buffer, boiled for another 5 min, centrifuged again, and finally analyzed by 12% SDS–PAGE followed by immunoblot analysis.

Imunolocalization by electron microscopy

Tissues (6 × 3 mm) were randomly sampled from healthy or PStV-infected *N. benthamiana* leaves at 10, 15, or 35 dpi. The tissues were fixed for 3 hr in either a

mixture of 3% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.4, or the above solution without glutaraldehyde. Tissues were then washed, dehydrated, and embedded in LR White resin as described (Ding *et al.,* 1996). Thin sections were mounted on slot nickel grids and probed with each antiserum diluted in a solution containing 2% (w/v) bovine serum albumin (BSA) in 0.01 *M* phosphate buffer, pH 7.4 (BSA solution). The grids were washed in 20 m*M* Tris–HCl, 0.5 *M* NaCl, pH 7.5, containing 0.05% (v/v) Tween 20 and further incubated for 1 hr in goat anti-rabbit IgG gold (20 nm) conjugate (Bio Cell Research Laboratories, Cardiff, UK) diluted 1:200 in BSA solution. Control samples were prepared by incubating sections from infected tissues in the presence of preimmune serum or sections from healthy tissues in the presence of antiserum. Samples were examined using a Zeiss 10 A electron microscope operating at 80 kV. As the total number of gold particles in each nuclei was generally low, 30 similarly sized nuclei in each section from infected tissues and 10 in each section from healthy tissues were randomly chosen and the number of gold particles within each nuclei counted. For ultrastructural studies, PStV- or TEV-infected tissues (7 and 35 dpi, respectively) were fixed for 2 hr in 2% (v/v) glutaraldehyde in 0.1 *M* cacodylate buffer at room temperature followed by 2 hr postfixation in 1% (v/v) osmium tetroxide solution. The sections were stained for 15 min in 4% (v/v) uranyl acetate followed by 1 min in Reynold's lead citrate at room temperature and observed under the electron microscope.

Expression of β-glucuronidase (GUS) and green fluorescence protein (GFP) fusion proteins

The PStV NIa and NIb nucleotide sequences were subcloned in frame to the 3′ end of the GUS sequence in the plant expression vector pRTL2-GUS (Restrepo *et al.,* 1990), generating pRTL2-GUS/NIa.P and pRTL2-GUS/NIb.P. The NIa sequence was transferred using *Nco*I and *Kpn*I sites at the 5′ and 3′ ends of the coding region, respectively, and the NIb sequence was transferred using *Bgl*II and *Xba*I sites (Fig. 1B). The junction between GUS and NIa and NIb in each plasmid was sequenced to ensure that the open reading frame had been maintained. Expression cassettes containing the enhanced cauliflower mosaic virus 35S promoter and 35S terminator, and the GUS/NIa or GUS/NIb fusion sequences were isolated by restriction enzyme digestion of pRTL2-GUS/NIa.P and pRTL2-GUS/NIb.P and inserted into the binary vector pGA482 (An, 1986). These plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 and used to produce transgenic *N. tabacum* cv. Xanthi nc plants as described (Carrington and Freed, 1990). Transgenic plants expressing GUS and a fusion of GUS with the NIa from TEV (GUS/NIa) were described previously (Restrepo *et al.,* 1990). For the purposes of this presentation, the GUS/NIa (TEV) fusion protein will be referred to as GUS/NIa.T. *In situ* localization of GUS activity in epidermal cells using the colorimetric substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide was carried out as described (Restrepo *et al.,* 1990).

The gene encoding a modified form of green fluorescent protein (mGFP, obtained from Dr. Jim Haseloff) was amplified by PCR and inserted between the *Nco*I and *Xba*I sites of pRTL2 (Restrepo *et al.,* 1990), producing pRTL2-GFP. A *Bgl*II site was introduced immediately after the last GFP codon during PCR amplification to permit the in-frame insertion of coding sequences. The NIa and NIb coding sequences from PStV and TEV were inserted into pRTL2-GFP using *Bgl*II and *Xba*I sites, generating pRTL2-GFP/NIa.P, pRTL2-GFP/NIa.T, pRTL2-GFP/NIb.P, and pRTL2-GFP/NIb.T. The junction between GFP and NIa and NIb in each plasmid was sequenced to ensure that the open reading frame had been maintained. The pRTL2-GFP plasmids were introduced into tobacco epidermal cells in intact leaves by biolistic delivery using a helium-driven PDS-100/He system (Bio-Rad). Microcarrier gold particle preparation and DNA precipitation onto microcarriers was performed as suggested by the manufacturer. Leaves were bombarded with DNA-coated particles at 500–900 psi and incubated in water 12–20 hr under constant fluorescent light at room temperature. Leaf pieces were vacuum-infiltrated with water and placed on a microscope slide. Fluorescence was observed with an Olympus BX50 microscope with the DM 500 dichroic mirror/BP470-490 exciter filter/BA515 barrier filter set. Photomicrographs were taken with Kodak Ektachrome 100 film.

RESULTS

Cloning and expression of CI, NIa-VPg, NIa-Pro, and NIb in *E. coli*

Coding sequences for NIa, NIb, and CI were cloned and expressed in *E. coli* to obtain immunogens for antibody production. Our attempts to express the full-length NIa in *E. coli* were unsuccessful. Hence, we cloned and expressed the VPg and proteinase domains of NIa individually. Complementary DNAs corresponding to nucleotides 4031–5932 (CI), 6091–6658 (NIa-VPg), 6659–7390 (NIa-Pro), and 7391–8938 (NIb) of the PStV genome (Fig. 1) were selected based upon the location of the conserved TEV NIa proteinase recognition sites (Carrington and Dougherty, 1987). The PCR-amplified cDNAs were cloned into the pET-15b expression vector and the recombinant plasmids pET-VPg, pET-Pro, pET-NIb, and pET-CI were obtained. Sequencing of the plasmids showed that the reading frame encoding each protein was correct (data not shown). pET-VPg and pET-NIb were transferred into HMS174 and pET-Pro and pET-CI into BL21 strains. IPTG induction of cells containing pET-Pro, pET-NIb, and pET-CI plasmids resulted in the expression of proteins with expected molecular weights as judged

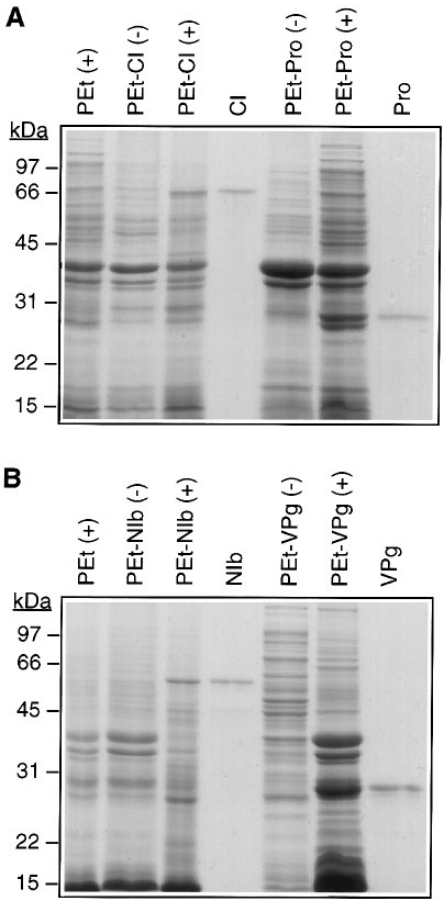


FIG. 2. SDS–PAGE analysis of CI and NIa-Pro (A) and NIb and NIa-VPg (B) expressed in *E. coli.* Insoluble inclusion proteins were prepared from cells containing pET-15b plasmid with or without a cDNA insert and prior to (−) or 3–4 hr after (+) induction with IPTG. Gel-purified *E. coli* expressed viral proteins (3 μg each) were also coelectrophoresed with the inclusion proteins and the separated proteins were detected by Coomassie brilliant blue staining. The sizes of protein standards (Bio-Rad) are given at the left.

by SDS–PAGE (Fig. 2) and in agreement with their predicted sizes (Gunasinghe *et al.,* 1994). However, cells containing pET-VPg plasmid expressed a protein with an apparent molecular weight of between 29 and 32 kDa (Fig. 2B), much higher than the predicted 21 kDa (Gunasinghe *et al.,* 1994). The reverse primer used to amplify NIa-VPg lacked a stop codon (Fig. 1B), hence some additional size could be expected. Through the use of an oligonucleotide primer identical to nucleotides 6615–6633 of PStV (Flasinski *et al.,* 1996), the pET-VPg plasmid was sequenced and the first stop codon was identified 69 nucleotides downstream from the VPg 3′ end within the pET vector. This fragment would encode an additional 23 amino acids adding approximately 2.7 kDa to the predicted molecular weight of expressed NIa-VPg. The higher molecular weight of the expressed VPg judged by SDS–PAGE could be due to its anomalous mobility. Analysis of the N-terminal amino acid sequences of *E. coli*-expressed CI, NIa-VPg, NIa-Pro, and NIb further verified their identities. The first amino acid residue of CI and the first to third residue of NIa-VPg and NIb (Gunasinghe *et al.,* 1994) were derived from their respective forward primer sequences (Fig. 1B). The N-terminal amino acid residues of *E. coli*-expressed NIa-Pro were identical to those of the expected engineered protein (Gunasinghe *et al.,* 1994).

Characterization of polyclonal antibodies

Each rabbit received a total of 1.25 mg gel-purified protein in five injections over a period of 115 days. Preimmune sera did not react with partially purified PStV-encoded proteins from infected plants or overexpressed proteins from *E. coli* (data not shown). The reciprocal titers of all the antisera exceeded 2×10^4 in the second bleeding and 10^5 in the last bleeding when tested against gel-purified homologous antigens or a preparation of partially purified PStV-encoded proteins from infected plants (data not shown). In all the immunological tests conducted in this study, antisera collected in the last bleedings and without any further treatment were used.

Each antiserum specifically detected its corresponding protein in partially purified protein preparation from PStV-infected plant tissues (Figs. 3A–3D, lanes I). None of the antisera reacted with proteins in partially purified proteins from healthy tissues (Figs. 3A–3D, lanes H). Antisera raised against both NIa-Pro and NIa-VPg detected a protein having a molecular weight equal to that of an uncleaved NIa protein (~48 kDa; Figs. 3B and 3C, lanes I). Additional protein species, antigenically related to NIa and migrating faster than 48 kDa, were detected by antisera to both NIa-Pro

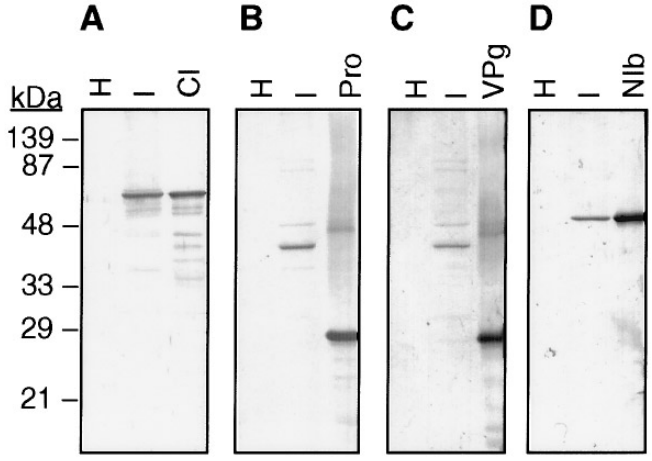


FIG. 3. Immunodetection of CI, NIa, and NIb in extracts from PStV-inoculated and healthy *N. benthamiana.* Partially purified plant proteins equivalent to 72 mg fresh tissue from healthy or PStV-infected plants, respectively, were loaded in lanes H and I. The samples were coelectrophoresed in 12% polyacrylamide gel with 3 μg of *E. coli*-expressed proteins CI [A, lane CI (70 kDa)], NIa-Pro [B, lane Pro (27 kDa)], NIa-VPg [C, lane VPg (28 kDa [predicted size 21 kDa])], and NIb [D, lane NIb (57 kDa)]. Following transfer of the separated proteins to nitrocellulose, blots were probed with antiserum to CI (A), NIa-Pro (B), NIa-VPg (C), and NIb (D). All the antisera were used at reciprocal dilution of 2×10^4. The sizes of prestained protein standards (Bio-Rad) are given at the left.

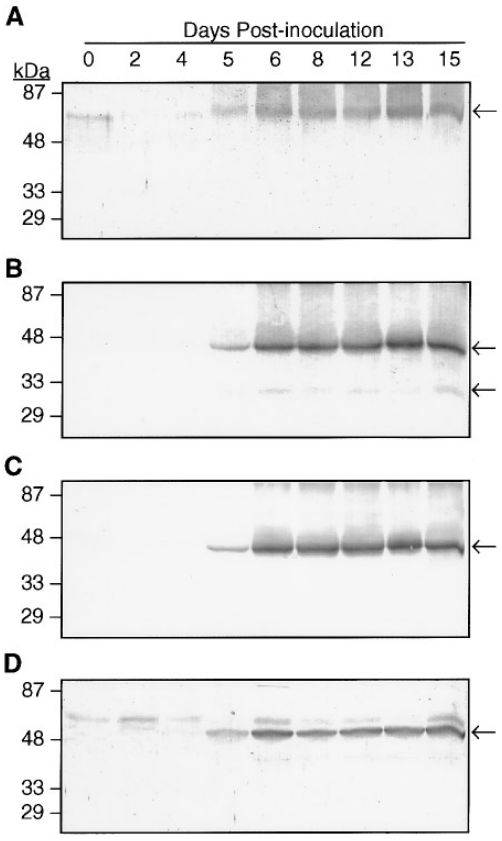


FIG. 4. Accumulation of CI, NIa, and NIb in PStV-infected *N. benthamiana* tissue. Leaf tissue was harvested 0, 2, 4, 5, 6, 8, 12, 13, and 15 dpi and soluble proteins were extracted from 0.3 g of fresh weight tissue as described in the text. Total plant protein equivalent to 2.5 mg of fresh tissue was loaded per lane and subjected to SDS–PAGE. Following transfer of the proteins to nitrocellulose, the blots were probed with anti-CI (A), anti-NIa-VPg (B), anti-NIa-Pro (C), and anti-NIb (D). Anti-CI and Anti-NIb were used at reciprocal dilutions of 2×10^4 and anti-NIa-VPg and anti-NIa-Pro at 1×10^4 and 5×10^3, respectively. Arrows indicate the position of each of the PStV proteins. The bottom arrow in B indicates the position of the anomalously migrating ~30-kDa VPg. The sizes of protein standards (Bio-Rad) are given at the left.

and NIa-VPg (Figs. 3B and 3C, lanes I). Antiserum against NIa-VPg detected multiple bands with molecular weights of ~28 kDa in partially purified protein preparations (Fig. 3C, lane I) and ~32 kDa in total protein preparations from virus infected tissue (Fig. 4B).

Antisera raised against NIb and CI detected proteins in partially purified PStV-encoded proteins from plants which comigrated with *E. coli*-expressed NIb and CI, respectively (Figs. 3A and 3D, lanes NIb and CI). Anti-CI and anti-NIb sera reacted poorly with plant antigens in total soluble protein preparations from uninoculated tissues (Figs. 4A and 4D, lanes 0).

Time course of accumulation of CI, NIa, and NIb in PStV-infected tissues

Immunoblot analysis of total soluble proteins from tissues inoculated 0–15 dpi showed that CI, NIa, and NIb appeared at 5 dpi in systemically infected leaves and continued to accumulate through the sampling period (Figs. 4A–4D). The concentration of all the proteins increased until 6 dpi but remained almost at the same level thereafter up to 15 dpi tested (Figs. 4A–4D). Antiserum to NIa-VPg detected a protein corresponding to the full-length NIa and a smaller protein possibly corresponding to VPg (Fig. 4B). The smaller protein appeared as early as 6 dpi and was detected thereafter up to 15 dpi tested. Antiserum to NIa-Pro only detected the full-length NIa (Fig. 4C).

Nuclear inclusion body detection and immunolocalization of NIa and NIb

Nuclear inclusion bodies were not observed in thin sections of PStV-infected tissues under the electron microscope up to 35 dpi (Fig. 5A). However, nuclear inclu-

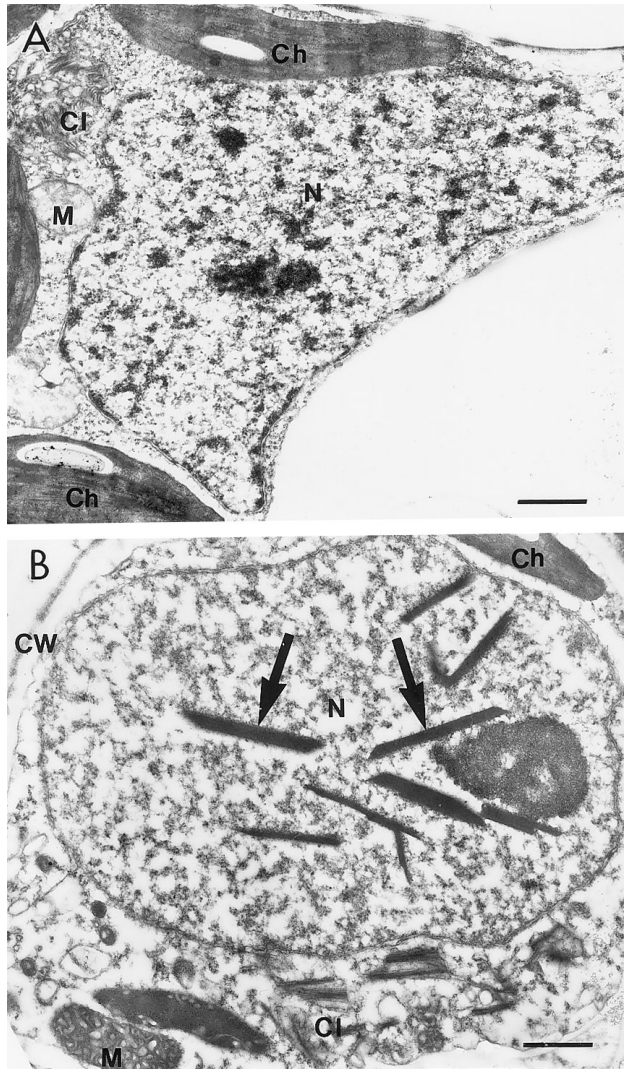


FIG. 5. Electron micrographs of LR White resin-embedded sections from virus-infected *N. benthamiana* leaf tissues harvested, respectively, at 7 and 35 dpi. (A) PStV-infected tissue 35 dpi. (B) TEV-infected tissue 7 dpi. The tissues were fixed in 2% glutaraldehyde and then in 1% osmium tetroxide solution followed by staining with uranyl acetate and Reynold's lead citrate. Arrows indicate the locations of TEV nuclear inclusions. Ch, chloroplast; CI, cytoplasmic inclusions; CW, cell wall; M, mitochondria, N, nucleus. Bars represent (A) 0.4 μm or (B) 1.4 μm.

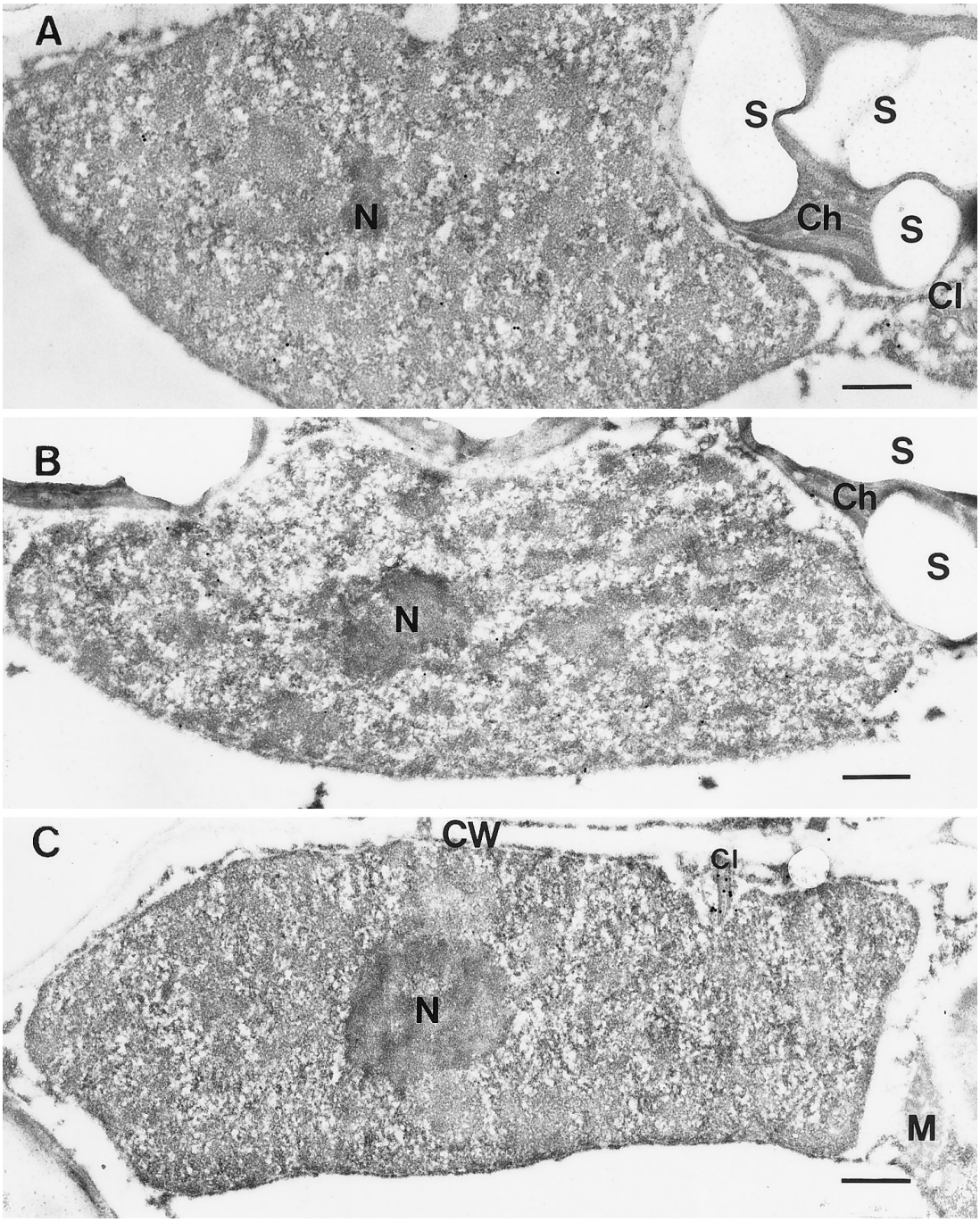


FIG. 6. Immunolocalization of NIa, NIb, and CI in sections from PStV-infected *N. benthamiana* leaf tissues. Sections were probed with antiserum to NIa-Pro (A), NIb (B), and CI (C). Ch, chloroplast; CI, cytoplasmic inclusions; CW, cell wall; M, mitochondria, N, nucleus; S, starch. Bars represent (A and C) 0.5 μm or (B) 0.6 μm.

sions were observed as early as 7 dpi in TEV-infected tissues (Fig. 5B). After treatment with antiserum or preimmune serum the number of gold particles was counted on the entire surface of 30 or 10 similarly sized nuclei (average 12-μm^2 area) from infected or healthy tissues, respectively. Antisera against NIa-Pro and NIb, but not CI, specifically labeled proteins randomly throughout the nuclei of PStV-infected cells. Even in heavily labeled nuclei [NIa-Pro, 12 gold grains (Fig. 6A); NIb 24, gold grains (Fig. 6B)] no crystalline nuclear inclusion structures could be detected. The antiserum against NIa-VPg produced results similar to those of antiserum to NIa-Pro (data not shown). The average number of gold particles in nuclei probed with NIb antiserum was comparable to those probed with NIa-VPg antiserum, but was less than those probed with NIa-Pro antiserum (Table 1). The number of gold particles averaged less than 1 when healthy sections were probed with immune sera or when infected

TABLE 1

Number of Gold Particles Present in Nuclei of Healthy or PStV-Inoculated *N. benthamiana* Treated with Preimmune Sera or Antisera against NIa-VPg, NIa-Pro, NIb, and CI

	Experiment I[b]		Experiment II	
Treatment[a]	Healthy	PStV-inoculated	Healthy	PStV-inoculated
Preimmune	ND[c]	0.9 ± 0.5[d] (4)	ND	0.2 ± 0.1
Anti-NIa-VPg	0.4 ± 0.3 (2)	6 ± 0.6 (14)	0.3 ± 0.3 (1)	5 ± 1.9 (11)
Preimmune	ND	0 (0)	ND	0.3 ± 0.2 (1)
Anti-NIa-Pro	0.2 ± 0.2 (1)	12 ± 1.6 (38)	0 (0)	11 ± 1.6 (31)
Preimmune	ND	0.2 ± 0.1 (1)	ND	0.3 ± 0.2 (1)
Anti-NIb[e]	0.6 ± 0.3 (2)	4 ± 0.3 (7)	0.3 ± 0.2 (1)	3 ± 0.4 (9)
Preimmune	ND	0.4 ± 0.2 (1)	ND	0.5 ± 0.2 (2)
Anti-NIb[f]	0.2 ± 0.2 (2)	5 ± 0.5 (13)	ND	10 ± 0.8 (24)
Preimmune	ND	0.3 ± 0.5 (2)	ND	0.7 ± 0.4 (4)
Anti-CI	0.2 ± 0.1 (1)	0.1 ± 0.1 (1)	0 (0)	0.4 ± 0.1 (2)

[a] Antisera were used at the following reciprocal dilutions: anti-NIa-VPg, 10^4; anti-NIa-Pro, 10^4; anti-NIb[e], 5×10^3; anti-NIb[f], 2.5×10^3, and anti-CI, 2×10^4. Preimmune serum was used at the same dilution as its corresponding antiserum.

[b] Sections used in experiments I and II were prepared from different tissues. PStV-inoculated tissues were harvested and fixed 10 days postinoculation from systemically infected leaves.

[c] Not tested.

[d] Average number of gold particles decorating 30 equally sized nuclei from PStV-infected or 10 equally sized nuclei from healthy tissue. Numbers in parentheses indicate maximum number of gold particles observed decorating a single nucleus for that treatment.

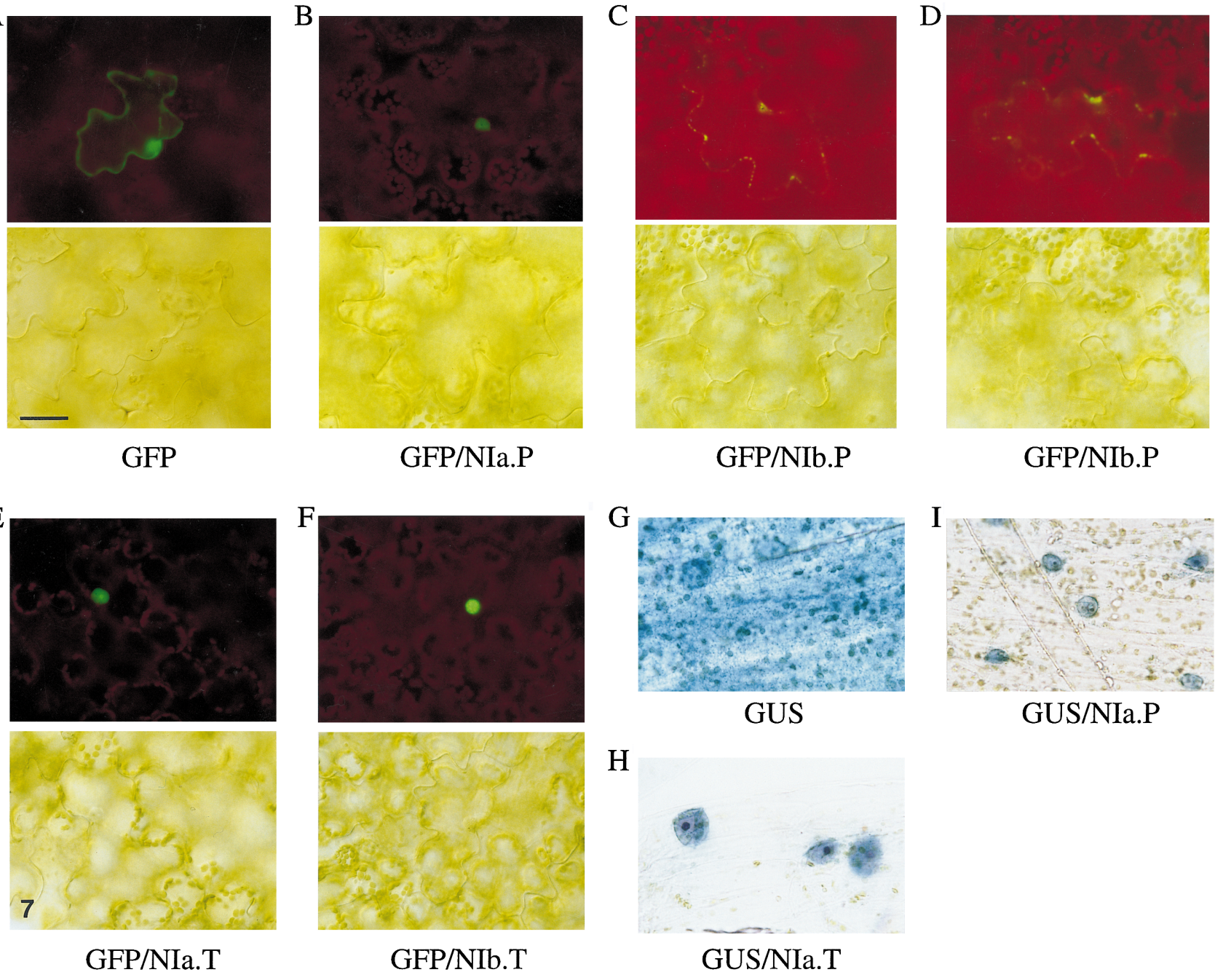
sections were probed with preimmune sera (Table 1). When the concentration of NIb antiserum increased to reciprocal dilution of 10^3, the average numbers of gold particles per nuclei from PStV-inoculated and healthy tissues increased to 8.57 ± 0.9 and 1.8 ± 0.6, respectively. PStV-infected sections probed with the preimmune serum at the same dilution had an average 1.6 ± 0.5 gold particles per nuclei.

The labeling of infected nuclei with antiserum against CI was similar to that observed for the preimmune serum (Table 1). When PStV-infected *N. benthamiana* sections were probed with antiserum to CI, cytoplasmic pinwheel inclusions were specifically labeled with gold particles in infected cells (Fig. 6C) indicating that the antigenicity of the CI protein was retained during fixation. Occasionally a few gold particles were observed in cytoplasmic areas of infected cells containing cytoplasmic inclusions and treated with antiserum to NIa-Pro (Fig. 6A), NIb, or NIa-VPg (data not shown). However, when 10 randomly selected cytoplasmic areas (~4 μm^2 each) containing no pinwheel inclusions were examined after probing with antisera against NIa-VPg, NIa-Pro, or NIb, the total number of gold particles counted did not exceed 2 (data not shown). Similarly, small concentrations of NIa and NIb of TEV have been detected in the cytoplasm after infection (Baunoch *et al.,* 1991).

Tissues from PStV-infected and healthy *N. benthamiana* (15 dpi) were also fixed using paraformaldehyde only and probed with an antiserum to NIb at the dilution of 1:5000. The average numbers of gold particles in each nucleus from infected or healthy tissues were, respectively, 5 ± 0.7 and 0.4 ± 0.2. The maximum numbers of gold particles decorating a nucleus from infected and healthy sections were, respectively, 14 and 2.

Localization of PStV NIa- and NIb-containing fusion proteins

Plant expression plasmids encoding fusion proteins containing GFP and PStV NIa (GFP/NIa.P) or NIb (GFP/NIb.P) were constructed and bombarded into epidermal cells of intact tobacco leaves. As controls, plasmids encoding nonfused GFP or GFP fusions containing TEV NIa (GFP/NIa.T) or NIb (GFP/NIb.T) were also analyzed. The GFP fluorescence was clearly visible against a background of red autofluorescence from chloroplasts after bombardment with each construct. Nonfused GFP was present throughout the cytosol, although some nuclear accumulation was always visible (Fig. 7A). Consistent with previous results (Restrepo *et al.,* 1990; Li and Carrington, 1993), both the GFP/NIa.T and the GFP/NIb.T fusion proteins were localized to the nucleus with little or no fluorescence detected in the cytoplasm (Figs. 7E and 7F). The GFP/NIa.P fusion protein was detected only in the nucleus of bombarded cells (Fig. 7B), indicating that the PStV NIa protein likely contains an independent nuclear localization signal (NLS). In contrast, GFP/NIb.P fusion protein accumulated predominantly in the cytoplasm (Figs. 7C and 7D). Unlike nonfused GFP, however, the GFP/NIb.P fusion protein accumulated in a punctate pattern throughout the cytoplasm. Expression of the GFP/NIb.P fusion protein was relatively low compared with expression of the other proteins, requiring longer photomicrographic exposures to detect GFP fluorescence and, thus, a brighter red autofluorescent background.


A
B
C
D
GFP
GFP/NIa.P
GFP/NIb.P
GFP/NIb.P
E
F
G
I
7
GUS
GUS/NIa.P
H
GFP/NIa.T
GFP/NIb.T
GUS/NIa.T

Transgenic tobacco plants expressing GUS/NIa.P fusion protein were also analyzed. We were unable to generate plants expressing GUS/NIb.P, possibly due to toxicity or instability of the fusion protein. Similarly, transgenic plants expressing GUS/NIb.T fusion protein were extremely difficult to obtain (Li and Carrington, 1993). In parallel with GUS (cytosolic)- and GUS/NIa.T (nuclear)-expressing transgenic controls, the subcellular location of GUS/NIa.P in epidermal cells was analyzed using a colorimetric GUS substrate. This fusion protein was consistently detected in the nucleus of transgenic cells (Fig. 7I). As shown previously (Restrepo *et al.,* 1990), nonfused GUS activity was detected uniformly in the cytosol while GUS/NIa.T activity was confined to the nucleus (Figs. 7G and 7H, respectively). Nontransgenic plants exhibited no GUS activity (data not shown.)

DISCUSSION

We raised antisera against *E. coli*-expressed CI, NIb, and the two domains, VPg and proteinase, of NIa of PStV to study the accumulation and *in vivo* localization of the proteins in systemically infected leaves of *N. benthamiana.* In time course experiments, the CI, NIa, and NIb proteins accumulated to similar levels in PStV-infected tissues (Figs. 4A–4D). This is similar to the proteins encoded by TEV (Restrepo *et al.,* 1990; Baunoch *et al.,* 1991), which is expected due to the polyprotein strategy of gene expression by potyviruses (Riechmann *et al.,* 1992). In immunolocalization studies, NIa and NIb of PStV (but not CI) were localized in the nuclei of the PStV-infected cells (Table 1 and Figs. 6A, 6B, and 6C). This suggests NIa and NIb of PStV both accumulate in the nucleus, a feature similar to NIa and NIb encoded by TEV (Knuhtsen *et al.,* 1974). It has been demonstrated that both NIa and NIb of TEV contain NLSs (Restrepo *et al.,* 1990; Carrington *et al.,* 1991; Restrepo-Harwig and Carrington, 1992; Li and Carrington, 1993). However, the lack of nuclear accumlation of the GFP/NIb.P may indicate that the PStV NIb does not contain a NLS itself. Alternatively, fusion of heterologous sequences to the amino terminus of the NIb may interfere with its NLS function.

The highest numbers of gold particles detected within a single nucleus by antisera to NIa-VPg, NIa-Pro, and NIb were 14, 38, and 24, respectively (Table 1). However, the average number of gold particles per nuclei of PStV-inoculated tissues was found to be much lower (Table 1). This could be due to the nonuniform and asynchronous infection by PStV which is a common feature of infection by most plant viruses (Matthews, 1991). We showed that tissues fixed by two different procedures labeled similarly with antiserum to NIb. This evidence together with strong labeling of cytoplasm of infected cells with antiserum to CI (Fig. 6C) indicates that it is unlikely that fixation has altered the antigenicity of the proteins. Our attempt to increase the sensitivity of the technique by increasing the antiserum concentration was not successful because no significant increase in the average numbers of gold particles was observed with lower dilutions of NIb antiserum (Table 1). Hence, we presume that either the concentrations of NIa and NIb proteins are low inside the nuclei or the proteins are not easily accessible to antibody binding.

Despite the presence of both NIa and NIb in the nucleus of PStV-infected cells, no inclusion bodies were detected even in nuclei containing the highest number of gold particles (Figs. 6A, 6B, and 5A). It was shown that the time of infection did not have any influence on inclusion formation as they were not observed in PStV-infected tissues at 10 or 35 dpi (data not shown and Fig. 5). In this study, we did not quantify the accumulation of NIa and NIb of PStV in the nuclei of infected cells and hence it is not known whether the lack of nuclear inclusions in PStV-infected cells is due to the lower accumulation of these proteins than in TEV-infected cells. It has been pointed out, however, that the ability of viral proteins to form crystalline inclusions within the infected cell does not depend on the overall concentration reached in the cell, but it depends on the properties of viral proteins (Matthews, 1991). Hence, it is reasonable to propose that the inability of PStV-encoded NIa and NIb to form nuclear inclusions is due to their failure to aggregate inside the nucleus after transport. TVMV also fails to induce nuclear inclusions in infected cells (Lesemann, 1988) and it remains to be determined if TVMV-encoded NIa and NIb are also present in the nucleus of infected cells and lack the ability to aggregate. It has been recently demonstrated that the NIa and NIb of TVMV have the potential to interact with each other in a yeast two-hybrid system (Hong *et al.,* 1995). It was noted by the authors that the observed interactions could be related to some replication events that require NIa and NIb to assemble together at an early stage. The domains responsible for this interaction and that necessary for the formation of nuclear inclusions may be different.

The observation that GFP/NIa.P and GUS/NIa.P were transported into the nucleus in transfected cells (Fig. 7B) or transgenic plants (Fig. 7I) is in agreement with the immunolocalization data (Fig. 6A and Table 1) and indicates that the PStV NIa contains a NLS. However, GFP/

FIG. 7. Localization of GFP and GUS fusion proteins in bombarded and transgenic cells. (A–F) Fluorescence of GFP and GFP fusion proteins in microprojectile bombarded tissue. Each panel shows a GFP fluorescence (top) and bright-field (bottom) image of the same field of view. Each panel contains one epidermal cell with GFP activity. The fluorescence photomicrographs shown in C and D required relatively long exposure times, resulting in the appearance of a brighter red autofluorescent background. (G–I) Colorimetric GUS activity assays with epidermal cells from transgenic plants. The indigo color reaction indicates the subcellular location of GUS activity. Bar in A represents 50 μm.

NIb.P was not detected in the nucleus of transfected cells (compare Fig. 7A with 7C and 7D). However, the GFP/NIb.P data should be interpreted with caution. It is possible that NIb of PStV contains one or more NLSs that are inhibited by GFP. Alternatively, NIb might lack a nuclear localization signal but is chaperoned into the nucleus by NIa.

The proteolysis of NIa and consequently the generation of multiple protein fragments has been reported for NIa of TEV and turnip mosaic virus (TuMV) (Dougherty and Parks, 1991; Laliberte *et al.,* 1992; Menard *et al.,* 1995; Parks *et al.,* 1995; Kim *et al.,* 1995). The protein species antigenically related to NIa that were detected in partially purified protein preparation from PStV-infected plant tissues by both antisera to NIa-Pro and to NIa-VPg (Figs. 3B and 3C, lanes I) are cleaved forms of NIa. However, we could not detect the expected 27-kDa NIa-Pro in either total SDS soluble protein (Fig. 4C) or partially purified protein preparations (Fig. 3B, lane I) from PStV-infected plants. It has been reported that the proteinase domain of TuMV was not detected by SDS–PAGE or immunoblotting analyses after expression of full-length NIa in *E. coli,* but the VPg domain was easily detected (Laliberte *et al.,* 1992). The authors proposed that either their antisera raised against full-length NIa was unable to react with the proteinase domain or following processing its complete degradation rendered its detection impossible (Laliberte *et al.,* 1992). However, our antisera detected *E. coli*-expressed NIa-Pro (Fig. 3B, lane Pro) but not in protein preparations from PStV-infected tissues (Fig. 3B, lane I and Fig. 4C). Hence, it is more likely that NIa-Pro is undetectable due to its instability in plant extracts or *in vivo.*

The role(s) of NIa and NIb of potyviruses in the nucleus is not known. It has been speculated that their transport into the nucleus is simply a means of reducing their cytoplasmic concentrations to levels that allow completion of the virus replicative cycle; however, a role in host gene expression, ribosome assembly, or another nuclear process has also been suggested (Baunoch *et al.,* 1991; Restrepo *et al.,* 1990). Our finding that PStV-encoded NIa and NIb are also transported to the nucleus suggests that this is a general feature of potyviruses regardless of their ability to form nuclear inclusions.

ACKNOWLEDGMENTS

The authors are grateful to Drs. R. S. Nelson and K. Korth for the critical reading of the manuscript. We thank Jim Haseloff for supplying the mGFP clone. We also thank V. Graves and A. Harris for assistance with DNA sequencing and oligonucleotide synthesis, J. Clouse for peptide sequencing, C. Ly and D. Boydston for illustrations and photographs, and A. Wilkins for typing the manuscript. This research was supported in part by a grant to J.C.C. from The National Institute of Health (AI27832) and by a Samuel Roberts Noble Foundation Collaborative Plant Biology Grant.

REFERENCES

Allison, R., Johnston, R. E., and Dougherty, W. G. (1986). The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: Evidence for the synthesis of a single polyprotein. *Virology* 154, 9–20.

An, G. (1986). Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. *Plant Physiol.* 81, 86–91.

Baunoch, D. A., Das, P., and Hari, V. (1988). Intracellular localization of TEV capsid and inclusion proteins by immunogold labeling. *J. Ultrastruct. Mol. Struct. Res.* 99, 203–212.

Baunoch, D. A., Das, P., Browning, M. E., and Hari, V. (1991). A temporal study of the expression of the capsid, cytoplasmic inclusion and nuclear inclusion proteins of tobacco etch potyvirus in infected plants. *J. Gen. Virol.* 72, 487–492.

Carrington, J. C., and Dougherty, W. G. (1987). Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. *J. Virol.* 61, 2540–2548.

Carrington, J. C., and Freed, D. D. (1990). Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. *J. Virol.* 64, 1590–1597.

Carrington, J. C., Freed, D. D., and Leinicke, A. J. (1991). Bipartite signal sequence mediates nuclear translocation of the plant potyviral NIa protein. *Plant Cell* 3, 953–962.

Cassidy, B. G., and Nelson, R. S. (1995). Differences in protection phenotypes in tobacco plants expressing coat protein genes from peanut stripe potyvirus with or without an engineered ATG. *Mol. Microb. Interact.* 8, 357–365.

Demski, J. W., Reddy, D. V. R., Sowell, G. J., and Bays, D. (1984). Peanut stripe virus — A new seed-borne potyvirus from China infecting groundnut (*Arachis hypogaea*). *Ann. Appl. Biol.* 105, 495–501.

Ding, X. S., Carter, S. A., and Nelson, R. S. (1996). Enhanced cytochemical detection of viral proteins and RNAs using double-sided labeling and light microscopy. *Bio/Techniques* 20, 111–115.

Dolja, V. V., Haldeman, R., Robertson, N. L., Dougherty, W. G., and Carrington, J. C. (1994). Distinct functions of capsid protein in assembly and movement of tobacco etch potyvirus in plants. *EMBO J.* 13, 1482–1491.

Domier, L. L., Franklin, K. M., Shahabuddin, M., Hellmann, G. M., Overmeyer, J. H., Hiremath, S. T., Siaw, M. F. E., Lomonossoff, G. P., Shaw, J. G., and Rhoads, R. E. (1986). The nucleotide sequence of tobacco vein mottling virus RNA. *Nucleic Acids Res.* 14, 5417–5430.

Domier, L. L., Shaw, J. G., and Rhodes, R. E. (1987). Potyviral proteins share amino acid sequence homology with picorna-, como-, and caulimoviral proteins. *Virology* 158, 20–27.

Dougherty, W. G., and Hiebert, E. (1980). Translation of potyviral RNA in a rabbit reticulocyte lysate: Identification of nuclear inclusion proteins as products of the *in vitro* translation of tobacco etch virus RNA and cylindrical protein as a product of the potyvirus genome. *Virology* 104, 174–182.

Dougherty, W. G., and Parks, T. D. (1991). Post-translational processing of the tobacco etch virus 49-kDa small nuclear inclusion polyprotein: Identification of an internal cleavage site and delimitation of VPg and proteinase domains. *Virology* 183, 449–456.

Dougherty, W. G., and Semler, B. L. (1993). Expression of virus-encoded proteinases: Functional and structural similarities with cellular enzymes. *Microbiol. Rev.* 57, 781–822.

Edwardson, J. R. (1966). Cylindrical inclusions in the cytoplasm of leaf cells infected with tobacco etch virus. *Science* 153, 883–884.

Edwardson, J. R. (1974). Some properties of the potato virus Y group. Fla. Agric. Exp. Stn. Monogr. Ser. 4.

Edwardson, J. R., and Christie, R. G. (1991). "The Potyvirus Group," Monogr. No. 16, Vol. IV. Agricultural Experiment Station, Institute of Food and Agricultural Science, University of Florida, Gainesville.

Edwardson, J. R., Christie, R. G., Purcifull, D. E., and Petersen, M. A. (1993). Inclusions in diagnosing plant virus diseases. *In* "Diagnosis of Plant Virus Diseases" (R. E. F. Matthews, Ed.), pp. 101–128. CRC Press, Boca Raton, FL.

Flasinski, S., Gunasinghe, U. B., Gonzales, R. A., and Cassidy, B. G. (1996). cDNA sequence and infectious transcripts of peanut stripe virus. *Gene* 17, 299–300.

Graybosch, R., Hellmann, G. M., Shaw, J. G., Rhoads, R. E., and Hunt, A. G. (1989). Expression of a potyvirus non-structural protein in transgenic tobacco. *Biochem. Biophys. Res. Commun.* 160, 425–432.

Gunasinghe, U. B., Flasinski, S., Nelson, R. S., and Cassidy, B. G. (1994). Nucleotide sequence and genome organization of peanut stripe potyvirus. *J. Gen. Virol.* 75, 2519–2526.

Harlow, E., and Lane, D. (1988). "Antibodies: A Laboratory Manual," pp. 53–91. Cold Spring Harbor Laboratory Press, New York.

Hari, V. (1981). The RNA of tobacco etch virus: Further characterization and detection of protein linked to RNA. *Virology* 112, 391–399.

Hari, V., Siegel, A., Rozek, C., and Timberlake, W. E. (1979). The RNA of tobacco etch virus contains poly(A). *Virology* 92, 568–571.

Hiebert, E., Purcifull, D. E., and Christie, R. G. (1984). Purification and immunological analyses of plant viral inclusion bodies. *Methods Virol.* 8, 225–280.

Hong, Y., Levay, K., Murphy, J. F., Klein, P. G., Shaw, J. G., and Hunt, A. G. (1995). A potyvirus polymerase interacts with the viral coat protein and VPg in yeast cells. *Virology* 214, 159–166.

Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (1990). "PCR Protocols: A Guide to Methods and Applications." Academic Press, San Diego.

Kim, D.-H., Park, Y. S., Kim, S. S., Lew, J., Nam, H. G., and Cho, K. Y. (1995). Expression, purification, and identification of a novel self-cleavage site of the NIa C-terminal 27-kDa protease of turnip mosaic potyvirus C5. *Virology* 213, 517–525.

Klein, P. G., Klein, R. R., Rodríguez-Cerezo, E., Hunt, A. G., and Shaw, J. G. (1994). Mutational analysis of the tobacco vein mottling virus genome. *Virology* 204, 759–769.

Knuhtsen, H., Hiebert, E., and Purcifull, D. E. (1974). Partial purification and some properties of tobacco etch virus-induced intranuclear inclusions. *Virology* 61, 200–209.

Koonin, E. V. (1991). The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. *J. Gen. Virol.* 72, 2197–2206.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680–685.

Lain, S., Riechmann, J. L., and Garcia, J. A. (1990). RNA helicase: A novel activity associated with a protein encoded by a positive strand RNA virus. *Nucleic Acids Res.* 18, 7003–7006.

Lain, S., Martin, M. T., Riechmann, J. L., and Garcia, J. A. (1991). Novel catalytic activity associated with positive-strand RNA virus infection: Nucleic acid-stimulated ATPase activity of the plum pox potyvirus helicase-like protein. *J. Virol.* 65, 1–6.

Laliberte, J.-F., Nicolas, O., Chatel, H., Lazure, C., and Morosoli, R. (1992). Release of a 22-kDa protein derived from the amino-terminal domain of the 49-kDa NIa of turnip mosaic potyvirus in *Escherichia coli. Virology* 190, 510–514.

Lesemann, D.-E. (1988). Cytopathology. *In* "The Plant Viruses — The Filamentous Plant Viruses" (R. G. Milne, Ed.), Vol. 4, pp. 179–235. Plenum, New York.

Li, X. H., and Carrington, J. C. (1993). Nuclear transport of tobacco etch potyviral RNA-dependent RNA polymerase is highly sensitive to sequence alterations. *Virology* 193, 951–958.

Lindbo, J. A., and Dougherty, W. G. (1994). Potyviruses. *In* "Encyclopedia of Virology" (R. G. Webster and A. Granoff, Eds.), Vol. 3, pp. 1148–1153. Academic Press, San Diego.

Maiss, E., Timpe, U., Brissk, A., Jelkmann, W., Casper, R., Himmler, G., Mattanovich, D., and Katinger, H. W. D. (1989). The complete nucleotide sequence of plum pox virus RNA. *J. Gen. Virol.* 70, 513–524.

Matthews, R. E. F. (1991). "Plant Virology," 3rd ed. Academic Press, San Diego.

Menard, R., Chatel, H., Dupras, R., Plouffe, C., and Laliberte, J.-F. (1995). Purification of turnip mosaic potyvirus viral protein genome-linked proteinase expressed in *Escherichia coli* and development of a quantitative assay for proteolytic activity. *Eur. J. Biochem.* 229, 107–112.

Murphy, J. F., Rhoads, R. E., Hunt, A. G., and Shaw, J. G. (1990). The VPg of tobacco etch virus is the 49-kDa proteinase or the N-terminal 24-kDa part of the proteinase. *Virology* 178, 285–288.

Parks, T. D., Howard, E. D., Wolpert, T. J., Arp, D. J., and Dougherty, W. G. (1995). Expression and purification of a recombinant tobacco etch virus NIa proteinase: Biochemical analyses of the full-length and a naturally occurring truncated proteinase form. *Virology* 210, 194–201.

Puurand, U., Makinen, K., Paulin, L., and Saarma, M. (1994). The nucleotide sequence of potato virus A genomic RNA and its sequence similarities with other potyviruses. *J. Gen. Virol.* 75, 457–461.

Restrepo, M. A., Freed, D. D., and Carrington, J. C. (1990). Nuclear transport of plant potyviral proteins. *Plant Cell* 2, 987–998.

Restrepo-Hartwig, M. A., and Carrington, J. C. (1992). Regulation of nuclear transport of a plant potyvirus protein by autoproteolysis. *J. Virol.* 66, 5662–5666.

Riechmann, J. L., Lain, S., and Garcia, J. A. (1989). The genome-linked protein and 5′ end RNA sequence of plum pox potyvirus. *J. Gen. Virol.* 70, 2785–2789.

Riechmann, J. L., Lain, S., and Garcia, J. A. (1992). Highlights and prospects of potyvirus molecular biology. *J. Gen. Virol.* 73, 1–16.

Robaglia, C., Durand-Tardif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S., and Casse-Delbart, F. (1989). Nucleotide sequence of potato virus Y (N strain) genomic RNA. *J. Gen. Virol.* 70, 935–947.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shahabuddin, M., Shaw, J. G., and Rhoads, R. E. (1988). Mapping of the tobacco vein mottling virus VPg cistron. *Virology* 163, 635–637.

Siaw, M. F. E., Shahabuddin, M., Ballard, S., Shaw, J. G., and Rhoads, R. E. (1985). Identification of a protein covalently linked to the 5′ terminus of tobacco vein mottling virus RNA. *Virology* 142, 134–143.

Vance, V. B., Moore, D., Turpen, T. H., Bracker, A., and Hollowell, V. C. (1992). The complete nucleotide sequence of pepper mottle virus genomic RNA: Comparison of the encoded polyproteins with those of other sequenced potyviruses. *Virology* 191, 19–30.

Ward, C. W., and Shukla, D. D. (1991). Taxonomy of potyviruses: Current problems and some solutions. *Intervirology* 32, 269–296.