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A Nuclear Localization Signal and a Membrane Association Domain Contribute to the Cellular Localization of the Tobacco Mosaic Virus 126-kDa Replicase Protein

Antonia dos Reis Figueira,* Sheetal Golem,† Sameer P. Goregaoker,† and James N. Culvert†,‡,[1]

*Department of Plant Pathology, Federal University of Lavras, C.P. 37, Lavras, Brazil, 37200; †Molecular and Cell Biology Program, University of Maryland, College Park, Maryland 20742; and ‡Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, Maryland 20742

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A transient expression system using onion epidermal cells was used to investigate domains of the *Tobacco mosaic virus* (TMV) 126-kDa replicase protein involved in cellular localization. Initially, a nuclear localization signal (NLS), identified within the amino-terminus of the 126-kDa protein, was investigated for its functionality using fusion constructs containing the green fluorescent protein (GFP). Fusion of the amino-terminal 70 amino acids of the 126-kDa protein, containing the NLS, to a β-glucuronidase-GFP open reading frame (ORF), directed the accumulation of fluorescence to the nucleus. In contrast, similar constructs lacking the NLS or containing a mutated NLS sequence failed to accumulate within the nucleus. Additional investigations using GFP fusion constructs containing the first 178 or 388 amino acids of the 126-kDa protein also displayed nuclear localization. However, fusion constructs encoding the first 781 amino acids or the entire 126-kDa ORF did not accumulate within the nucleus but instead associated with the endoplasmic reticulum (ER), forming spot-like inclusions. Thus, a dominant ER association domain exists between amino acids 388 and 781 of the 126-kDa protein. Interestingly, a full-length 126-kDa GFP fusion construct encoding a nonfunctional NLS mutation also localized to the ER but did not form inclusions. Furthermore, a TMV mutant containing the same nonfunctional NLS mutation failed to replicate in protoplasts. Together these findings suggest that both the NLS and the ER retention domain contribute to the functional localization of the 126-kDa protein. © 2002 Elsevier Science (USA)

INTRODUCTION

Positive-strand RNA viruses require the assembly of cytoplasmic membrane-bound replicase complexes to facilitate their replication (Strauss and Strauss, 1994; Buck, 1996; Agol *et al.,* 1999). The assembly of these complexes likely involves specific interactions between virus and host components and often results in the proliferation and rearrangement of cellular membranes within the infected cell. The importance of membrane rearrangements in virus replication is not yet clear; however, it has been suggested that this process may increase the total surface area available for replication and/or provide a means of increasing local concentrations of replicase complexes and their associated components (Schaad *et al.,* 1997). Membrane rearrangements may also reflect a strategy for avoiding host defense responses through compartmentalization. Determining the mechanisms responsible for controlling the association of virus replicase complexes with cellular membranes will be an important step in understanding the infection process.

Tobacco mosaic virus (TMV) is the type member of the genus *Tobamovirus* and a member of the "alphavirus supergroup." The TMV genome encodes at least four proteins (Goelet *et al.,* 1982) of which the 5′ proximal open reading frame (ORF) encodes a 126-kDa protein terminated by an amber stop codon that when suppressed yields a read-through 183-kDa protein (Pelham, 1978). Both the 126- and the 183-kDa proteins represent TMV replicase components, containing methyl-transferase (MT) and helicase-like (HEL) domains as well as a polymerase domain on the read-through portion of the 183-kDa ORF (Koonin, 1991; Koonin and Dolja, 1993; Kadare and Haenni, 1997). Two additional TMV proteins, a 30-kDa protein involved in cell-to-cell movement and the 17.5-kDa coat protein, are encoded by 3′ coterminal subgenomic mRNAs (Hunter *et al.,* 1976; Meshi *et al.,* 1987; Deom *et al.,* 1987).

For TMV, membrane rearrangements consisting of amorphous proliferations of the endoplasmic reticulum (ER), previously termed either "X-bodies" or "viroplasms," have been observed in infected plant cells (Goldstein, 1924; Matthews, 1981; Shalla, 1964; Kohlemainen *et al.,* 1965, Milne, 1966, Esau and Cronshaw, 1967; Beachy and Zaitlin, 1975; Heinlein *et al.,* 1998). The remodeled membranes found in TMV-infected cells contain both virus RNA and protein, including the 126/183-kDa replicase proteins and the 30-kDa movement protein (Hills *et al.,*

[1] To whom correspondence and reprint requests should be addressed at Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, College Park, MD 20742. Fax: (301) 314-9075. E-mail: jc216@umail.umd.edu.

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            29                 47
TMV     RRLYDTAVEEFNARDRRPK
TVCV    RRVYDNAVEELNARSRRPK
CGMMV   KRVYDEAVRSLDHQDRRPK
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FIG. 1. Sequence comparison of the predicted NLS motifs from the tobamoviruses TMV, *Turnip vein clearing virus* (TVCV), and *Cucumber green mottle mosaic virus* (CGMMV).

1987; Osman and Buck, 1996; Heinlein *et al.,* 1998; Reichel and Beachy, 1998; Más and Beachy, 1999). Additionally, membrane fractions from TMV-infected cells also contain active replicase complexes capable of synthesizing both plus- and minus-strand viral RNAs in a template-dependent fashion (Osman and Buck, 1996). The mechanisms responsible for the association of TMV replicase and movement proteins with the ER are not yet known. However, Yamanaka *et al.* (2000, 2002) have identified two Arabidopsis genes, *TOM1* and *TOM3*, that are required for efficient tobamovirus replication. Both these host genes encode similar membrane spanning proteins that interact with the helicase-like domain of the TMV 126/183-kDa protein. Thus, *TOM1* and *TOM3* may act as tethers for the membrane attachment of TMV replicase proteins.

Homology searches of the 126-kDa protein failed to identify any potential ER retention motifs or obvious membrane spanning domains (Nakai and Horton, 1999; Gomord *et al.,* 1999). However, the TMV 126/183-kDa replicase proteins encode a predicted bipartite nuclear localization signal (NLS) at amino acids 29 to 47 (Robbins *et al.,* 1991). Interestingly, this NLS is conserved within the genus *Tobamovirus*, suggesting a function for this motif (Fig. 1). Similar NLS motifs can also be found within the replicase proteins of other cytoplasmically replicating RNA viruses, such as the helicase protein nsP2 of the alphavirus *Semliki forest virus* (SFV) as well as the polymerase proteins of the flavivirus *Dengue virus* and the potyvirus *Tobacco etch virus* (Rikkonen *et al.,* 1994; Restrepo *et al.,* 1990; Forwood *et al.,* 1999). Thus, the presence of NLS motifs within RNA viral replicase proteins appears to be a repeated phenomena whose function is at present unclear.

Within this study, gene fusion methods were used to investigate the cellular localization of the TMV 126-kDa replicase protein within an onion epidermal host cell system. Confocal microscopy demonstrated that the putative 126-kDa NLS can function to target large proteins, >100 kDa, to the nucleus. However, a second domain within the full-length 126-kDa protein was found to be dominant over the NLS, leading to the targeting of protein to the ER, primarily in the form of spot-like inclusions. The appearance of 126-kDa inclusions was dependent upon the presence of the NLS, indicating a role for this motif in cellular localization. The functional significance of the observed nuclear and ER localization activities within the TMV replicase protein is discussed.

RESULTS

Replication of TMV within onion epidermal cells

The ability of onion epidermal cells to support TMV replication was examined by mechanically inoculating sections of sliced onion bulbs with purified TMV30BGFP virions (Shivprasad *et al.,* 1999). This virus construct expresses the green fluorescent protein (GFP) of *Aequorea victoria* from a subgenomic mRNA derived from an engineered subgenomic promoter. Observations of cell layers inoculated with TMV30BGFP revealed the appearance of GFP fluorescence in single cells at 2 to 3 days post-inoculation (Fig. 2). No fluorescence was observed in mock-inoculated cell layers. Observations at 5 days post-inoculation revealed that the TMV30BGFP fluorescence did not move beyond the initially infected cell. Thus, onion epidermal cells support TMV infection at the single-cell level but do not permit virus movement.

The 126-kDa NLS functions in nuclear localization

A transient expression system was used to determine whether the identified bipartite NLS located within the TMV 126-kDa protein functions in the nuclear localization of proteins in onion epidermal cells (Finer *et al.,* 1992; Scott *et al.,* 1999). This system allows the expression of large GFP fusion constructs, >40 kDa in size, negating problems associated with the passive nuclear localization of smaller GFP fusion proteins (Grebenok *et al.,* 1997). A control construct, containing the β-glucuronidase (GUS) ORF fused to the N-terminus of the GFP ORF (GUS-GFP, MW = 95.2 kDa), displayed fluorescence throughout the cytoplasm but not within the nucleus (Fig. 3). However, a similar construct containing the first 70

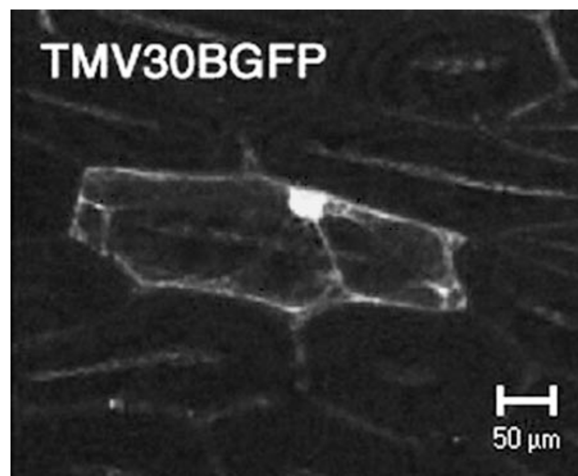

FIG. 2. GFP fluorescence demonstrating the replication of TMV30BGFP in an inoculated onion epidermal cell. Photograph taken 2 days postinoculation.

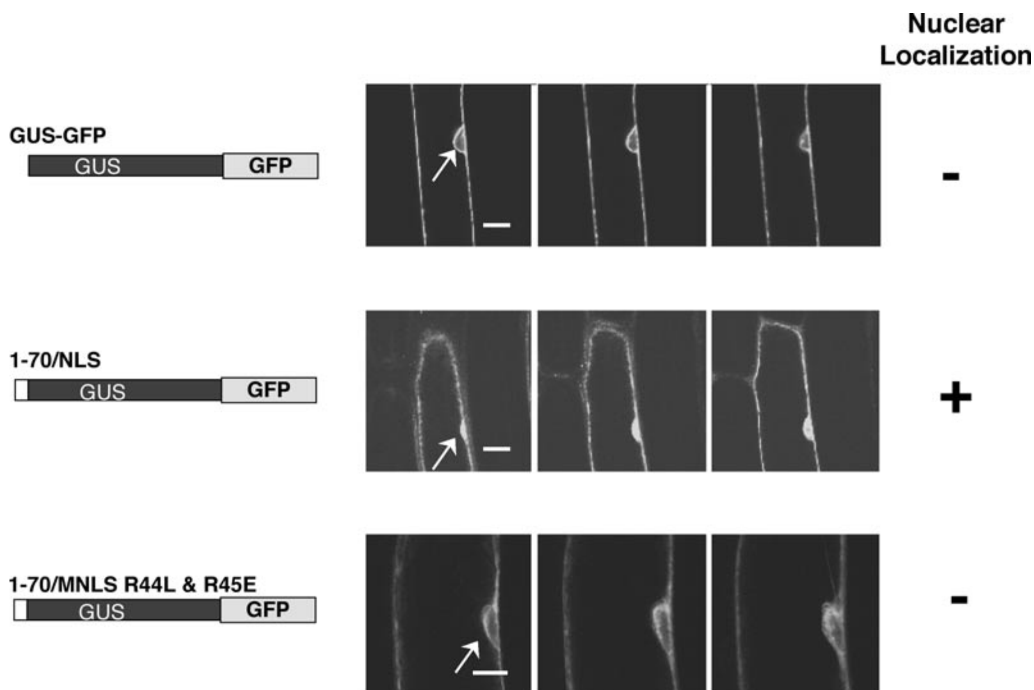

FIG. 3. Fluorescence showing the ability of different GUS–GFP fusion constructs to localize to the nucleus. Arrows indicate the location of nuclei. Constructs contain the GUS–GFP open reading frames alone or fused to either a wild-type (1–70/NLS) or a mutated (1–70/NLS–R44L and R45E) TMV 126-kDa nuclear localization signal. Pictures were captured at different focal planes within the cell. Bars represent 20 μm.

amino acids of the 126-kDa protein, including the NLS, fused to the N-terminus of the GUS-GFP construct (126^{1-70}-GUS-GFP, MW = 104.6 kDa), permitted the localization of fluorescence within the nucleus.

The functionality of the 126-kDa NLS was also examined by the introduction of two mutations, R44L and R45E, to the NLS of the 126^{1-70}-GUS-GFP construct. Similar mutations within other bipartite NLS sequences have been shown to disrupt the ability of these signals to target proteins to the nucleus (Kinkema *et al.,* 2000). This mutant construct lacked the ability to localize fluorescence to the nucleus. Taken together, these data demonstrate that the 126-kDa NLS can function in the localization of proteins to the nucleus.

An additional 126-kDa domain(s) functions to prevent nuclear localization

To further examine the cellular localization of the TMV 126-kDa protein, a set of C-terminal deletion constructs were created and fused to the N-terminus of the GFP ORF. The NLS is maintained within each of the tested fusion constructs. Two GFP fusion constructs (126^{1-178}-GFP, MW = 47.2 kDa and 126^{1-388}-GFP, MW = 71.9 kDa) containing the first 178 and 388 N-terminal amino acids of the 126-kDa protein, respectively, were found to primarily localize to the nucleus and to a lesser extent within the cytoplasm (Fig. 4). In contrast, fusion constructs carrying the first 781 amino acids or the full-length 126-kDa ORF (126^{1-781}-GFP, MW = 115.7 kDa and FL126-GFP, MW = 152.9-kDa) did not localize to the nucleus but instead accumulated within the cytoplasm as both strands and discrete spot-like inclusions (Figs. 4 and 5). Taken together, these findings indicate that the region of the 126-kDa protein between amino acid 388 and 781 functions in a dominant fashion over the N-terminal NLS to prevent the nuclear localization of the 126-kDa protein.

Mutation of the NLS alters the localization pattern of the 126-kDa protein

To further investigate the importance of the NLS motif on the overall localization of the 126-kDa protein, mutations R44L and R45E, which render the NLS nonfunctional (Fig. 3), were moved into the FL126-GFP fusion protein to create $FL126^{MNLS}$-GFP. Transient expression of $FL126^{MNLS}$-GFP resulted in a significantly different localization pattern when compared to the unmutated 126-kDa fusion construct (Fig. 5). Most notable was the inability of $FL126^{MNLS}$-GFP to form spot-like inclusion bodies. Primarily the $FL126^{MNLS}$-GFP was only observed associated as strands within the cytoplasm.

TMV replicase proteins have been shown to associate with the ER in infected cells. To determine whether FL126-GFP or $FL126^{MNLS}$-GFP associated with the ER, transformed cells were stained with rhodamine B hexyl ester, an ER/mitochondria stain that fluoresces orange-red when excited, providing a means of multicolor labeling within GFP-expressing cells (Rashid and Horobin,

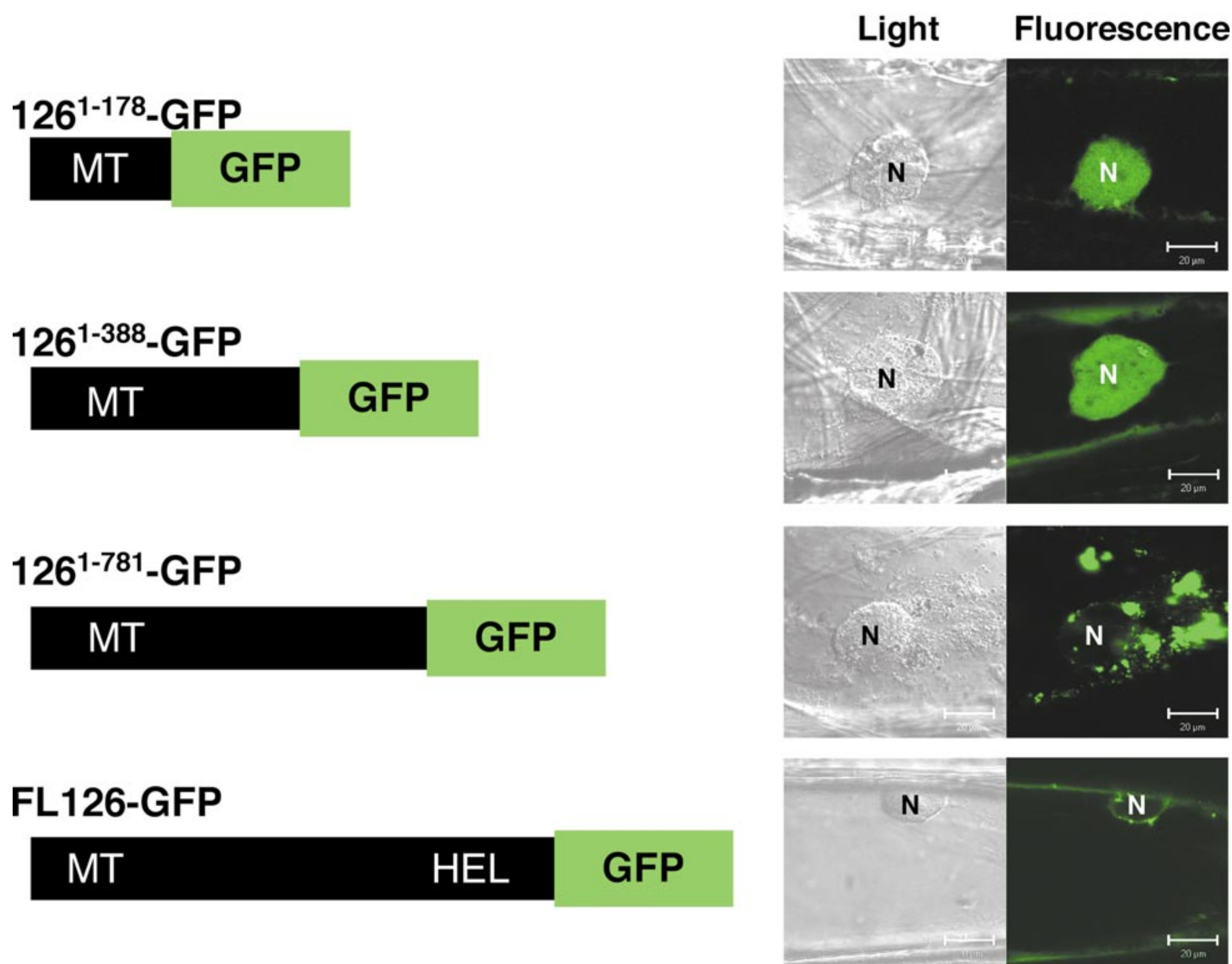

FIG. 4. Nuclear localization of 126-kDa C-terminal deletion constructs in onion epidermal cells. N marks the same nucleus in both light and fluorescent microscopy.

1990; Terasaki and Reese, 1992). Results demonstrated that the fluorescence derived from FL126^{MNLS}-GFP consistently overlapped with the rhodamine-derived fluorescence, revealing a network of interconnected tubular strands and sheets consistent with previous descriptions of onion epidermal cell ER (Knebel *et al.,* 1990). Similarly, the majority of the fluorescence derived from FL126-GFP also overlapped with the rhodamine fluorescence. However, some of the brightest spot-like inclusions produced by FL126-GFP did not completely correspond with rhodamine-stained ER. We suspect that fluorescence from highly localized concentrations of FL126-GFP present within some inclusions overshadowed the weaker fluorescing rhodamine-stained membranes. This possibility is supported by the fact that weaker fluorescing FL126-GFP inclusions do overlap with the rhodamine fluorescence.

Mutation of the NLS within TMV inhibits replication

The importance of the NLS motif in virus replication was examined by engineering mutations R44L and R45E into the 126-kDa ORF of the full-length TMV cDNA constructs pSNC004 and pTMV30BGFP (Dawson *et al.,* 1986; Turpen *et al.,* 1995; Shivprasad *et al.,* 1999). As shown above, these mutations disrupt the ability of the NLS to function in nuclear localization as well as affect the cellular localization pattern of the 126-kDa protein in onion epidermal cells. Transcripts derived from the NLS mutant TMV cDNA constructs repeatedly failed to infect either a systemic host, *Nicotiana benthamiana,* or a local lesion host, *Nicotiana tabacum* cv Xanthi nc, as determined by the lack of symptoms or the expression of GFP. Additionally, no detectable levels of virus replication were observed in *N. benthamiana* protoplasts electroporated with NLS mutant transcripts (Fig. 6). Thus, mutation of the 126-kDa NLS motif appears to fatally disrupt TMV replication.

DISCUSSION

In this study, domains of the TMV 126-kDa protein fused to GFP were investigated for their role in cellular localization in onion epidermal cells. GFP-fusion proteins

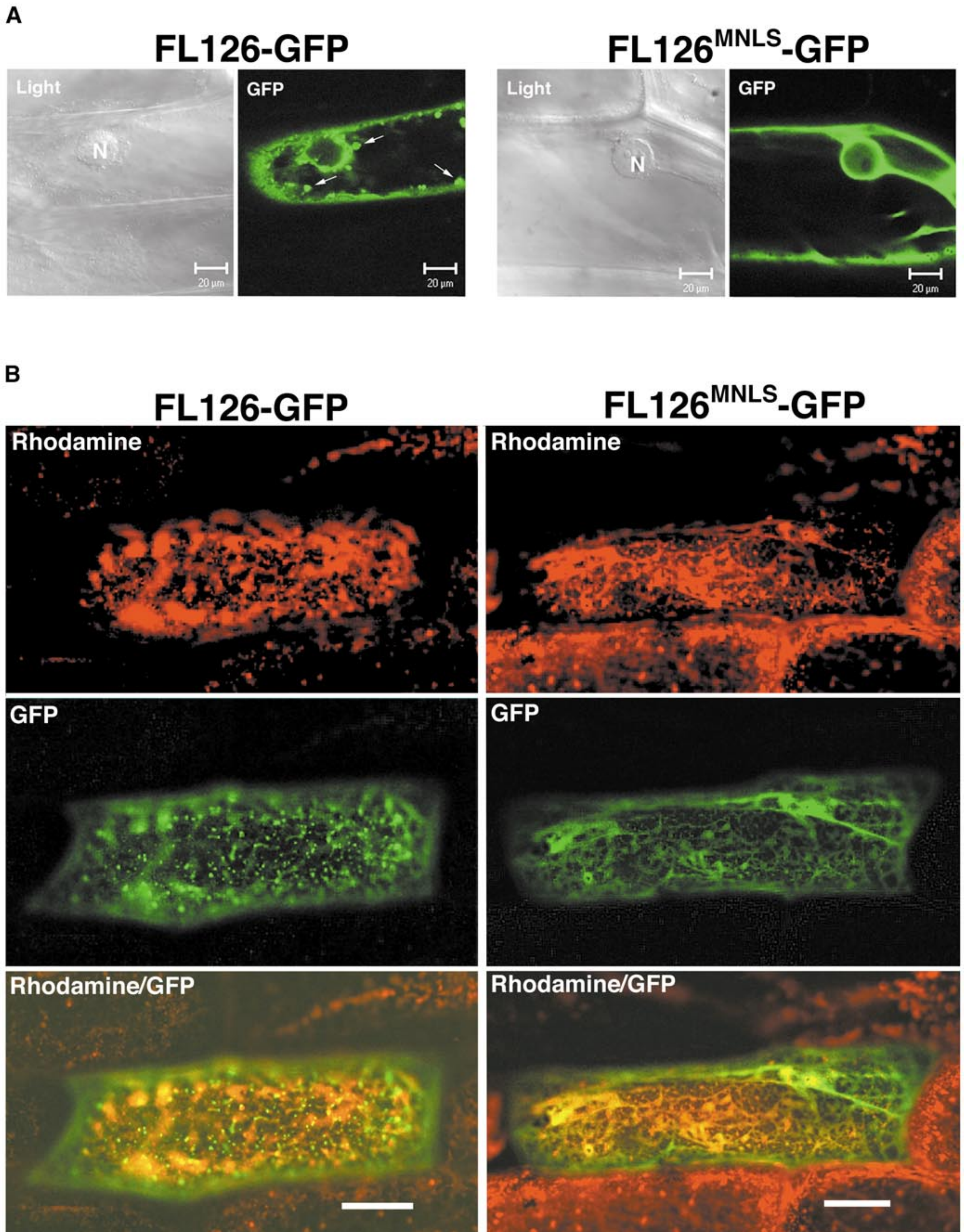

FIG. 5. Localization patterns of the wild-type (FL126-GFP) and NLS mutant ($FL126^{MNLS}$-GFP) 126-kDa proteins fused to GFP. (A) Visible and fluorescence images showing the inability of these constructs to localize to the nucleus as well as the spot-like inclusions (arrows) produced by FL126-GFP. (B) Colocalization of GFP with the rhodamine derived ER fluorescence.

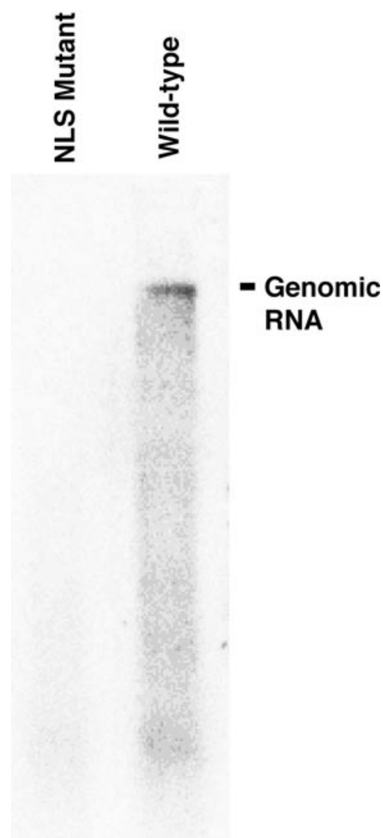

FIG. 6. Northern blot analysis for the detection of NLS mutant and wild-type TMV replication in *N. benthamiana* protoplasts.

have been used effectively to study the localization of numerous cell and viral proteins (Grebenok *et al.,* 1997). The fact that onion epidermal cells lack autofluorescing chlorophyll, have large identifiable cell structures, and are easily obtained and transformed make these cells particularly useful for studying the localization patterns of GFP-tagged proteins (Reuzeau *et al.,* 1997). Furthermore, the ability of onion epidermal cells to support TMV replication makes this system ideal for investigating the phenomena of virus-induced membrane remodeling and the assembly of replicase complexes.

Results from our experiments are consistent with previous reports that have demonstrated the predominant association of TMV replicase proteins with the ER (Hills *et al.,* 1987; Osman and Buck, 1996; Heinlein *et al.,* 1998; Reichel and Beachy, 1998). ER localization presumably represents an essential function required for the assembly of functional replicase complexes. For TMV this process is visualized as alterations in ER morphology, such as membrane aggregations and invaginations that likely lead to the formation of X-body or viroplasm-type inclusions found within TMV-infected cells (Goldstein, 1924; Matthews, 1981; Shalla, 1964; Kolemainen *et al.,* 1965, Milne, 1966, Esau and Cronshaw, 1967; Beachy and Zaitlin, 1975; Reichel and Beachy, 1998). Results from this study indicate that the 126-kDa protein associates with the ER in the absence of other viral proteins and components. Colocalization of the 126-kDa protein to the ER was conferred by a domain(s) located between residues 388 and 781. Analysis of this region of the 126-kDa protein does not reveal an identifiable ER retention signal; however, a 21 amino acid amphipathic helix, residues 708–728, is predicted. One of two membrane-binding regions of the SFV capping protein, nsP1, consists of a 19 amino acid amphipathic helix that contains a tryptophan residue that functions to anchor the protein into the lipid bilayer (Lampio *et al.,* 2000). A similar helix has also been identified within the membrane association segment of the *Brome mosaic virus* 1a replicase protein (den Boon *et al.,* 2001). The identified 126-kDa helix contains a phenylalanine at one end of the helix that could function in membrane attachment in a fashion similar to the SFV nsP1 tryptophan residue. Alternatively, Yamanaka *et al.* (2000, 2002) identified two homologous *Arabidopsis* transmembrane proteins, *TOM1* and *TOM3*, that are required for efficient tobamovirus replication. Using the yeast-based Sos-membrane recruitment system (Aronheim *et al.,* 1997), it was also demonstrated that both *TOM1* and *TOM3* interact directly with a portion of the 126-kDa replicase protein. Interestingly, the residues essential for *TOM1* interaction are contained within the nonnuclear localizing constructs, 126^{1-781}-GFP and FLTMV-GFP, but are not present within nuclear localizing constructs, 126^{1-388}-GFP or 126^{1-178}-GFP (Fig. 4). Thus, findings in this study are consistent with the membrane retention of the 126-kDa protein via either a membrane bound host protein, such as *TOM1*, and/or a membrane insertion of an amphipathic helix. Further experiments are needed to more precisely characterize the 126-kDa sequences involved in membrane association and to decipher the mechanisms through which these sequences specifically target the protein to the ER.

Results from transient expression studies demonstrated that the NLS present within the 126/183-kDa protein could function to nuclear localize large polypeptides, $>$100 kDa in size. Furthermore, the presence of a functional NLS was required for the formation of spot-like inclusion bodies that represented localized concentrations of the 126-kDa protein. Thus, the NLS in combination with a 126-kDa membrane association region(s) functions to localize high concentrations of replicase protein to defined membrane areas. This process may enhance the cellular environment needed for the assembly of active replication complexes. The inability of the NLS TMV mutant to replicate in plants or protoplasts suggests that the maintenance of this motif is critical for virus replication, possibly in the assembly of functional replicase complexes. However, because of the multiple overlapping functions present within the TMV 126/183-kDa proteins, we cannot rule out the possibility that the NLS mutations disrupted a second replicase function,

such as RNA capping, that was ultimately responsible for the inability of this mutant to replicate.

The possibility also exists that the NLS directs the nuclear accumulation of the 126/183-kDa protein at levels below our current detection limits. What the function of this protein would be in the nucleus can only be speculated. One possibility is that this protein acts to alter the transcription of nuclear genes during infection and thereby creates a cellular environment more favorable for virus replication. Alterations in the transcription of nuclear genes have been characterized in many virus systems. For example, within SFV the presence of a functional NLS in the viral nsP2 helicase protein has been correlated with the shutoff of host DNA synthesis (Rikkonen, 1996). Another possibility is that the 126-kDa protein functions to retard host RNAi defense mechanisms. The RNAi suppressor 2b protein of *cucumber mosaic virus* requires localization to the nucleus to function (Lucy *et al.,* 2000). Furthermore, expression of the TMV 126/183-kDa proteins has been linked to the suppression of an RNAi silenced transgene (R. S. Nelson, personal communication). However, it is not yet known if the NLS affects this suppressor function of the 126/183-kDa proteins.

Effecting host cell functions may not require the 126/183-kDa to localize to the nucleus. In the family *Flaviviridae*, *Dengue virus* polymerase protein NS5 and *Hepatitis C virus* protein NS5A contain functional NLS sequences that have been shown to interact *in vivo* with importin-β homologues (Ide *et al*., 1996; Chung *et al.,* 2000; Johansson *et al.,* 2001). Chung *et al.* (2000) suggested that the NS5A-NLS may function to cytoplasmically sequester cellular components involved in nuclear localization as a means to alter the virus's cellular environment and/or evade host defense responses. Whether the TMV 126/183-kDa NLS has similar effects on host physiology remains to be determined.

MATERIALS AND METHODS

TMV30BGFP inoculations

Purified TMV30BGFP (Shivprasad *et al.,* 1999), 50 μg/ml, was used to mechanically inoculate Carborundum-dusted inner peels of sliced onion bulbs. At varying time points the inoculated inner epidermal cell layer was removed and observed for GFP fluorescence by microscopy as described below.

GFP fusion constructs

All transient expression constructs were created within a pBR322 derivative, pCMC1100, that contains the 35S promoter from *Cauliflower mosaic virus,* a polylinker domain and the nopaline synthase polyadenylation signal (McCabe *et al.,* 1988). Polymerase chain reaction amplification was used to engineer unique restriction sites into each component of the fusion constructs. The ORF of EGFP (Clontech, Palo Alto CA) was modified to contain a 5′ *BsiW*I site and a 3′ *Pst*I site. The β-glucuronidase ORF was altered to carry 3′ *BsiW*I and 5′ *Kpn*I sites. Segments of the 126-kDa protein were modified to contain a 5′ *Nco*I site and either a 3′ *Kpn*I or a *BsiW*I site, depending upon whether the segment was to be fused directly to the GFP or GUS ORFs. Individually modified components were then ligated in succession into similarly cut pCMC1100. Junctions between ORFs were sequenced to confirm reading frame maintenance.

Transient expression assays

Monolayers of epidermal cells were removed from the inner peel of white onions bulbs (*Allium cepa*) and placed on modified MS medium (1× MS salts, 3% sucrose, and 2% agar, pH 5.8). Particle bombardment was done using a particle inflow gun based on the designs of Takeuchi *et al.* (1992) and Finer *et al.* (1992). Approximately 2 μg of plasmid DNA was ethanol precipitated onto 0.5 mg of tungsten particles (1.3 μm in diameter, Bio-Rad, Hercules, CA). DNA-coated particles were resuspended in 95% ethanol by sonication in a Branson 2200 ultrasonic cleanser (Branson Equipment, Shelton, CT). DNA-coated particle suspensions were deposited onto plastic filter holder screens (Gelman Sciences, Ann Arbor, MI) and allowed to dry. Coated screens were mounted in the particle inflow gun and a 50-ms pulse of helium (50 psi) used to propel DNA-coated tungsten particles into the onion monolayers mounted 2.5 in. below the screens. Bombarded monolayers were then incubated for 12 to 16 h at room temperature.

Confocal microscopy

Bombarded onion epidermal skins were mounted in water on glass slides with coverslips and viewed by confocal fluorescence microscopy using a Zeiss LSM510 laser scanning confocal microscope system with 10× NA 0.8 dry and 63× NA 1.2 water-immersion lenses (Carl Zeiss Inc., Thonwood, NY). Excitation sources were 488 nm for GFP and 543 nm for rhodamine B hexyl ester. For ER staining, epidermal skins were floated in a water solution, 1 μg/ml, of rhodamine B hexyl ester for 10 min and then rinsed thoroughly with water. Images were modified in Zeiss LSM Imager Examiner, Version 2.80, and processed for printing in Adobe Photoshop (Grand Prairie, TX).

Construction and analysis of the TMV NLS mutant

Full-length infectious cDNA clones of the U1 strain of TMV, pSNC004, and pTMV30BGFP, joined to the T7 promoter were used as the parental constructs for the creation of TMV NLS mutants (Dawson *et al.,* 1986; Turpen *et al.,* 1995; Shivprasad *et al.,* 1999). TMV RNA numbering is from Goelet *et al.* (1982). The R44L–R45E mutations

within the 126-kDa ORF were introduced using a PCR-based mutagenesis procedure (Higuchi *et al.,* 1988). DNA sequencing was carried out to confirm the introduction of the NLS mutation into the full-length TMV cDNA constructs. Infectious RNA transcripts generated from mutant and wild-type constructs were mechanically inoculated onto both a TMV systemic host, *N. benthamiana*, and a local lesion host, *N. tabacum* cv Xanthi nc. Inoculated hosts were maintained at 25°C under a 12-h photoperiod. Plants were observed visually for the appearance of TMV-induced symptoms, and in the case of TMV30BGFP, by fluorescent microscopy, for a period of 3 weeks post-inoculation.

Preparation and inoculation of protoplasts

Protoplasts were prepared from callus tissue grown from surface sterilized seeds of *N. benthamiana* plated onto MS media (3% sucrose, 1× Murashige-Skoog salts, 1% agar, 3 mM MES pH 5.8, 1× Gamborg's vitamin solution) supplemented with 0.5 μg/mL Kinetin and 0.5 μg/mL 2,4-D (Simon *et al.,* 1992; Dardick *et al.,* 2000). Mannitol (0.6 M) was added to callus cultures and incubated for 20 min at 25°C with shaking. Callus cells were pelleted at 70 g and 50 mL of protoplast isolation medium (1× Murashige-Skoog salts, 0.0.1 M sucrose, 3 mM MES pH 5.8, 0.5 M mannitol, 1× Gamborg's vitamins, 0.4 μg/mL kinetin, 0.4 μg/mL 2,4-D) containing 10 mg/mL Cellulysin and 2 mg/mL Pectolyase (Calbiochem, San Diego, CA) added. Cells were then incubated for 3 h at 25°C with shaking. Protoplasts were passed through a 53-μm nylon mesh (Small Parts Inc., Miami Lakes, FL) and washed by centrifugation with cold 0.6 M mannitol. Protoplast concentration and viability were determined visually on a hemacytometer under a microscope.

Approximately 1.0×10^7 protoplasts were resuspended in 600 μL of electroporation buffer (5 mM MES, 70 mM KCl, 0.5 M mannitol, pH 5.8). Wild-type or NLS mutant RNA (5μg), generated from *in vitro* transcription, was added to the protoplast suspension. Protoplasts were then electroporated once (50 μF, 300 V) as described by Ishikawa *et al.* (1993). Protoplasts were immediately diluted with 5 mL of culture medium (1× Murashige-Skoog salts, 0.1 M sucrose, 3 mM MES, 0.4 M mannitol, 1× Gamborg's vitamin solution, 0.4 μg/mL kinetin, 0.4 μg/mL 2,4-D, pH 5.8) and incubated in the dark at 25°C. Total RNA was isolated at 20 h postelectroporation and subjected to Northern blot analysis using radiolabeled probes specific for sequences within the TMV 126-kDa ORF (Dardick *et al.,* 2000).

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