Dynamic localization of two tobamovirus ORF6 proteins involves distinct organellar compartments

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ORF6 is a small gene that overlaps the movement and coat protein genes of subgroup 1a tobamoviruses. The ORF6 protein of tomato mosaic virus (ToMV) strain L (L-ORF6), interacts in vitro with eukaryotic elongation factor 1α, and mutation of the ORF6 gene of tobacco mosaic virus (TMV) strain U1 (U1-ORF6) reduces the pathogenicity in vivo of TMV, whereas expression of this gene from two other viruses, tobacco rattle virus (TRV) and potato virus X (PVX), increases their pathogenicity. In this work, the in vivo properties of the L-ORF6 and U1-ORF6 proteins were compared to identify sequences that direct the proteins to different subcellular locations and also influence virus pathogenicity. Site-specific mutations in the ORF6 protein were made, hybrid ORF6 proteins were created in which the N-terminal and C-terminal parts were derived from the two proteins, and different subregions of the protein were examined, using expression either from a recombinant TRV vector or as a yellow fluorescent protein fusion from a binary plasmid in Agrobacterium tumefaciens. L-ORF6 caused mild necrotic symptoms in Nicotiana benthamiana when expressed from TRV, whereas U1-ORF6 caused severe symptoms including death of the plant apex. The difference in symptoms was associated with the C-terminal region of L-ORF6, which directed the protein to the endoplasmic reticulum (ER), whereas U1-ORF6 was directed initially to the nucleolus and later to the mitochondria. Positively charged residues at the N terminus allowed nucleolar entry of both U1-ORF6 and L-ORF6, but hydrophobic residues at the C terminus of L-ORF6 directed this protein to the ER.

INTRODUCTION

Some plant RNA viruses encode small (molecular mass typically <20 kDa) non-structural proteins of unknown function, which are non-essential for virus replication or cell-to-cell movement and are proposed to serve as determinants of virulence or pathogenicity (Lukhovitskaya et al., 2005a, b, 2009). In particular, the genome of tobacco mosaic virus (TMV), one of the best-characterized plant pathogens, has been found to contain, in addition to five well-known genes, a sixth ORF (ORF6), which overlaps the virus movement protein (MP) and coat protein genes and encodes a 4.8 kDa protein (Fig. 1a) (Morozov et al., 1993). ORFs of similar sizes, located in an equivalent position, can be found in all members of the subgroup 1a of the plant virus genus Tobamovirus (Ikeda et al., 1993; Morozov et al., 1993). The amino acid sequences of the ORF6 proteins encoded by different tobamoviruses are only moderately related, raising the question of whether they have a common function in the tobamovirus infection cycle (Fig. 1b). All available experimental data on the properties and functions of the ORF6 protein have come from studies of ORF6 encoded by TMV strain L, known also as tomato mosaic virus (ToMV) (Morozov et al., 1993), and TMV strain U1 (Canto et al., 2004). The properties of ToMV L-ORF6 were studied in in vitro translation experiments, whilst TMV U1-ORF6 was...
analysed in vivo in an attempt to determine its function in the context of virus infection.

In vitro transcription of cloned cDNA of ToMV L-ORF6 and subsequent in vitro translation produces a protein of ~4–5 kDa corresponding to the predicted ORF6 product, as well as three higher-molecular-mass protein complexes of 37, 54 and 66 kDa (Morozov et al., 1993). In immunoprecipitation experiments, the major 54 kDa band was found to represent a complex of ORF6 with the eukaryotic elongation factor 1a (eEF1A) (Fedorkin et al., 1995). This complex is extremely stable, being resistant to treatment with 2-mercaptoethanol and SDS-PAGE conditions, but dissociates in 8 M urea. Mutagenesis of the L-ORF6 gene has revealed that a conserved stretch of 5 aa (aa 10–14) is involved in formation of the ORF6–eEF1A complex. As the ORF6-containing 54 kDa complex was detected after in vitro translation of a transcript equivalent to the ToMV MP subgenomic RNA, this RNA was predicted to be a possible template for ORF6 translation in vivo. The 54 kDa complex was also found to be formed by U1-ORF6 (Morozov et al., 1993).

In vivo experiments using an infectious clone of the complete TMV U1 genome revealed that mutations blocking translation of ORF6 resulted in attenuation of virus symptoms in Nicotiana benthamiana plants, whereas heterologous expression of TMV ORF6 from either potato virus X (PVX) or tobacco rattle virus (TRV) enhanced the virulence of both viruses in N. benthamiana. However, TMV ORF6 had no effect on symptom production in Nicotiana tabacum plants infected with ORF6-expressing TMV or PVX. Therefore, the ORF6 protein can be considered a determinant of viral pathogenicity in N. benthamiana but not in N. tabacum (Canto et al., 2004). Interestingly, U1-ORF6 did not alter the level of accumulation of PVX or TRV in any of these experiments.

In the present study, we performed comparative analyses of the ORF6 proteins of ToMV L and TMV U1. We revealed major differences between both the intracellular distribution and the pathogenicity functions of these two proteins. The L-ORF6 protein accumulated in the ER and was unable to significantly affect the pathogenicity of the virus in infected plants, whereas the U1-ORF6 protein accumulated in the nucleolus with subsequent relocation to mitochondria and strongly enhanced the symptoms of virus infection.

**RESULTS**

**Mutation of the ORF6 initiator codon has no effect on ToMV pathogenicity**

To determine whether the L-ORF6 protein could influence ToMV pathogenicity in a manner similar to that of U1-ORF6, which has been shown to increase the pathogenicity of TMV in N. benthamiana (Canto et al., 2004), a ToMV full-length infectious clone (Kubota et al., 2003) was modified to introduce an AUG→ACG mutation disabling the ORF6 initiator methionine codon, whilst leaving intact the amino acid sequence of the overlapping MP gene, producing the construct ToMW[−ORF6]. Transcripts of wild-type ToMV and ToMV[−ORF6] were inoculated onto the leaves of N. benthamiana plants. In both cases, a severe systemic necrosis was observed leading to the death of infected plants at 7–10 days post-inoculation (p.i.) (Fig. S1a, b, available in JGV Online). To verify that the introduced mutation was preserved in the ToMW[−ORF6] viral progeny, RT-PCR and sequencing of PCR products was carried out to analyse RNA samples collected from upper, systemically infected leaves. Neither reversion to the wild-type sequence nor a second-site substitution was found (data not shown). Thus, no difference in the symptom severity caused by ToMV and ToMW[−ORF6] was observed in N. benthamiana. The phenotypes of ToMV and ToMW[−ORF6] infections were then compared in other plant hosts. Symptoms induced by the wild-type virus and the mutant in systemically infected leaves were found to be similar in appearance and in timing in Nicotiana clevelandii (leaf malformation) (Fig. S1c), N. tabacum cv. Samsun nn (mosaic symptoms) and tomato

![Image](57x552 to 328x708)

**Fig. 1.** (a) Tobamovirus genome map indicating the position of ORF6 and other proteins. (b) Sequences of the L-ORF6 (unshaded) and U1-ORF6 (shaded) proteins, their recombinants and the mutants used in this study. Identities and similarities between U1 and L sequences are indicated by * and +, respectively.
plants (leaf malformation and mosaic) (data not shown). In addition, RNA samples from *N. benthamiana*, *N. clevelandii* and tomato plants infected with ToMV and ToMV[−ORF6] were analysed by Northern blotting using a ToMV-specific probe. Samples were taken at 2–3-day intervals for 3 weeks or until the plant death (for *N. benthamiana*). In these experiments, no difference in accumulation of virus-specific RNAs was found between ToMV and ToMV[−ORF6] (Fig. S1d) (data not shown). Additionally, to detect a possible effect of ORF6 on virus cell-to-cell movement, the diameter of lesions induced by ToMV and ToMV[−ORF6] in two necrotic-reacting hosts, *N. tabacum* cv. Xanthi NN and *N. tabacum* cv. Samsun NN, was compared. The wild-type and mutant virus transcripts were inoculated onto opposite leaf halves. No significant difference in lesion size was found (data not shown). Thus, ORF6 had no observable effect on ToMV pathogenicity, virus spread or viral RNA accumulation in the plant hosts tested.

**Effects of the L- and U1-ORF6 proteins and their hybrids on the pathogenicity of TRV**

The U1-ORF6 protein has been shown previously to be a pathogenicity determinant, enhancing the virulence of the heterologous viruses PVX and TRV (Canto *et al.*, 2004). To determine whether L-ORF6 could also have a similar effect and to compare the influence of L- and U1-ORF6 on the phenotype of heterologous viral infection, a TRV derivative, TRV[L-ORF6], expressing the L-ORF6, was constructed. *N. benthamiana* plants were inoculated with TRV[L-ORF6] or with the previously described TRV[U1-ORF6] (Canto *et al.*, 2004). As a control, a green fluorescent protein (GFP)-expressing TRV derivative (TRV[GFP]) was used, which induced mild leaf curling in upper, systemically infected leaves (Fig. 2b) compared with healthy plants (Fig. 2a). In addition to the curling, leaves systemically infected with TRV[L-ORF6] and TRV[U1-ORF6] developed necrotic regions, which were milder in the case of the TRV[L-ORF6] construct (Fig. 2c, d). TRV[U1-ORF6] infection also resulted in severe stem necrosis, which developed at 6 days p.i. and subsequently led to death of the apical part of the infected plant (Fig. 2d). In plants infected with TRV[L-ORF6], systemic necrosis of the plant apex did not occur. Therefore, both L- and U1-ORF6 were able to enhance TRV pathogenicity, although to different extents.

Comparison of the L- and U1-ORF6 amino acid sequences revealed a moderate level of similarity in their N-terminal and central parts, whereas the C-terminal regions were found to be less conserved (Fig. 1b). The C-terminal part of U1-ORF6 is 6 aa longer than L-ORF6 but is less hydrophobic (Kyte & Doolittle, 1982). To determine whether the differences in their C-terminal regions could account for the different observed biological effects of L- and U1-ORF6, domain-swap recombinants were constructed with exchanged C-terminal regions. The resulting hybrid protein genes were cloned into TRV producing TRV[U1::L-ORF6], with the N terminus derived from U1 and the C terminus derived from L, and TRV[L::U1-ORF6], with the N terminus derived from L and the C terminus derived from U1. Transcripts of these constructs were inoculated onto *N. benthamiana* plants along with TRV[GFP], TRV[L-ORF6] and TRV[U1-ORF6]. In these experiments, TRV[U1::L-ORF6] induced leaf necrosis symptoms similar to those of TRV[L-ORF6] (Fig. 2f),...
whereas TRV[L::U1-ORF6] produced symptoms resembling the phenotype of TRV[U1-ORF6] leading to death of the plant apex (Fig. 2e). Thus, inclusion of the C-terminal region of U1-ORF6 correlated with severe leaf curling and death of the apical parts of infected plants, whereas the L-ORF6 C-terminal region was associated with milder symptoms. To estimate the levels of viral RNA accumulation, RNA samples were taken from inoculated and systemically infected leaves at 4 and 6 days p.i. and analysed by Northern blotting with a TRV-specific probe. No correlation was found between the degree of symptom severity and the accumulated levels of TRV genomic RNA (data not shown). These data demonstrated that the pathogenicity of the U1-ORF6 protein is associated with its C-terminal region, which is longer and predicted to be significantly less hydrophobic than the respective region of the L-ORF6 protein (Fig. 1b).

To examine further the potential pathogenicity determinants in the ORF6 proteins, we prepared an L-ORF6-HYDR mutant with changes in the C-terminal region that replaced the hydrophobic amino acid residues with more hydrophilic residues, and a U1-ORF6-RK construct in which the two positively charged amino acid residues in the N-terminal region were replaced with neutral amino acids (Fig. 1b). No change in pathogenicity was observed with U1-ORF6-RK (Fig. 2h), confirming the previous findings that the U1-ORF6 C-terminal sequence is important for the extreme pathogenicity of this ORF6. TRV expressing the L-ORF6-HYDR mutant protein produced mild symptoms similar to those seen with TRV[GFP] (Fig. 2g), indicating, perhaps, that increased hydrophilicity is not the sole reason for the severe pathogenicity of the U1-ORF6 C terminus but that specific amino acid residues in this region play a role in this phenotype.

Subcellular localization of U1- and L-ORF6 proteins and their hybrids

To analyse the subcellular localizations of the L- and U1-ORF6, yellow fluorescent protein (YFP) fusions of both proteins were expressed in N. benthamiana by agroinfiltration and visualized at different time points after infiltration by confocal laser-scanning microscopy (CLSM). To visualize subcellular structures, the tagged ORF6 proteins were expressed in transgenic plants expressing monomeric red fluorescent protein (mRFP) or GFP fusions of the nuclear marker protein histone H2B (Martin et al., 2009), the nucleolar marker fibrillarin (Goodin et al., 2007), the Golgi marker sialyltransferase, the plasmodesmata (PD) marker TMV-30K, or GFP with an endoplasmic reticulum (ER) retention signal (HDEL). We also used non-fused YFP as a control, which was distributed as a dispersed fluorescence in the cytoplasm and in the nucleus but not in the nucleolus (Fig. 3). In our experiments, at 1–2 days p.i., the YFP-fused U1-ORF6 was found to localize predominantly in the nucleus, being concentrated in the nucleolus, often predominantly at its periphery (Fig. 3a). Later, at 3–4 days p.i., the protein localized mostly in the cytoplasm in numerous small (0.8–1 μm diameter) bodies of unknown nature (Figs 3b and S2). A small number of such cytoplasmic bodies were already visible at 1–2 days p.i. when most of the protein was present in the nucleolus, and later a major increase in their population occurred simultaneously with the disappearance of the U1-ORF6 protein from the nucleolus (Fig. 3b). Cytoplasmic bodies formed by U1-ORF6 did not co-localize with a Golgi marker (Fig. S2). To provide clues as to the nature of the cytoplasmic bodies, the U1-ORF6 amino acid sequence was analysed using motif prediction software (TargetP and WOLF PSORT). Both programs predicted that the U1-ORF6 protein would associate with mitochondria. To test this prediction, the U1-ORF6–YFP fusion was expressed in plants that were also infiltrated with the mitochondrial marker MitoTracker Red CMXRos. When examined at 3 days p.i., as predicted, the U1-ORF6–YFP fusion protein co-localized perfectly with the MitoTracker marker (Fig. 3c).

In a previous study, it was shown that U1-ORF6 is distributed in spots at the cell periphery and that these spots co-localize with PD (Canto et al., 2004). In our experiments, by scanning cells in the Z plane and reconstructing stacked images, we observed YFP-tagged U1-ORF6 to be distributed across the entire cell periphery (Figs 3a, b and S3a, left), whereas, when we analysed only a single optical section, the same PD-like localization as that observed by Canto et al. (2004) was seen (Fig. S3a, right). To examine further whether some of the U1-ORF6 bodies co-localized with PD, we expressed the U1-ORF6 fusion in transgenic plants expressing the TMV MP fused with GFP (Fig. S3b). To separate the plasma membrane from the cell wall, the cells were plasmolysed by infiltration of the leaves with sorbitol (Fig. S3c, d). In these experiments, U1-ORF6–YFP remained associated with the plasma membrane but not with the cell wall, leading us to conclude that the ORF6 protein was not localized to PD.

Unlike U1-ORF6, L-ORF6 was localized in the cytoplasm in association with the polygonal network of the ER and nuclear envelope (Fig. 3e), with a clear co-localization of L-ORF6–YFP with the ER–GFP marker (Fig. S4). Importantly, the localization of L-ORF6 did not change over time.

As the C-terminal regions of the two ORF6 proteins contained sequences that were shown (see above) to affect the pathogenicity of TRV differently, the subcellular localization of the chimaeric proteins U1::L-ORF6 and L::U1-ORF6 was analysed. The recombinant proteins were fused to YFP and expressed by agroinfiltration in leaves of N. benthamiana plants transgenic for H2B–mRFP or fibrillarin–mRFP. The L::U1-ORF6 recombinant was found to be concentrated mostly in the nucleolus and also to localize diffusely in the nucleoplasm and cytoplasm; however, no mitochondrial localization was observed (Fig. 3f). This localization pattern persisted for 1–4 days p.i. In contrast, the U1::L-ORF6 recombinant was found in association

Subcellular localization of tobamovirus ORF6 protein
with the ER but not with the nucleus or nucleolus (Fig. 3g), and this localization did not change over time. Thus, either the entire U1-ORF6 sequence (N- and C-terminal parts) is required for mitochondrial localization of the protein or, as with the U1::L-ORF6 recombinant, an N-terminal U1-derived mitochondrial targeting domain is overridden by the ER-targeting activity of the C-terminal part of L-ORF6.

**Determinants of subcellular localization of U1-ORF6 and L-ORF6**

As described above, inspection of the L-ORF6 amino acid sequence revealed that its C terminus was more hydrophobic than that of U1-ORF6 (Fig. 1b), and, when fused to the N terminus of U1-ORF6, was able to direct the chimaeric protein to the ER. To test the hypothesis that it is
the hydrophobicity of this sequence that governs its subcellular localization, an L-ORF6-HYDR mutant was constructed with changes in the C-terminal region designed to replace the hydrophobic amino acid residues with more hydrophilic residues (Fig. 1b). The YFP-fused L-ORF6-HYDR was expressed in N. benthamiana leaves by agroinfiltration and analysed by CLSM. As revealed by co-localization with the transgenic fibrillarin–mRFP marker, at 1–2 days p.i., L-ORF6-HYDR was located predominantly in the nucleolus, often being concentrated at its periphery (Fig. 4a). At 3–4 days p.i., the protein was found both in the nucleus, where it had a diffuse localization, and within the nucleolus, predominantly in the familiar Christmas tree pattern formed by fibrillarin (Chen et al., 2002; Raska, 2003) (Fig. 4b). Additionally, at all time points, a small fraction of L-ORF6-HYDR was found diffusely distributed in the cytoplasm (Fig. 4a, b). These results demonstrated, therefore, that the C-terminal hydrophobic region is required for L-ORF6 co-localization with the ER.

These data also suggest that both the U1- and L-ORF6 proteins contain a nuclear/nucleolar localization signal (NLS/NoLS), which is usually composed of positively charged residues, targeting them to the nucleus and nucleolus (Musinova et al., 2011). However, the region of hydrophobic residues at the C terminus of the L-ORF6 protein probably contains a hydrophobic sequence that either directs its transport out of the nucleus/nucleolus (if the ORF6 protein is a nuclear shuttling protein) to the ER or retains it tightly at the ER immediately after translation, preventing nuclear import. Nevertheless, regardless of the precise mechanism, replacement of hydrophobic amino acid residues in the C terminus of the L-ORF6-HYDR protein changes accumulation of the protein from the ER to nucleus/nucleolus.

To identify the L-ORF6 NoLS, two approaches were used. Firstly, mutagenesis of L-ORF6-HYDR was carried out. As signals of nuclear and nucleolar localization are often represented by basic amino acid residues (reviewed by Hiscox, 2007; Taliansky et al., 2010), the mutant L-ORF6-HYDR-RK was constructed carrying, in addition to the HYDR mutation, other changes affecting five positively charged residues, targeting them to the nucleus and nucleolus (Musinova et al., 2011). However, the region of hydrophobic residues at the C terminus of the L-ORF6 protein probably contains a hydrophobic sequence that either directs its transport out of the nucleus/nucleolus (if the ORF6 protein is a nuclear shuttling protein) to the ER or retains it tightly at the ER immediately after translation, preventing nuclear import. Nevertheless, regardless of the precise mechanism, replacement of hydrophobic amino acid residues in the C terminus of the L-ORF6-HYDR protein changes accumulation of the protein from the ER to nucleus/nucleolus.

To analyse whether the TMV-U1 ORF6 protein also contained a similarly located NoLS, a mutant of U1-ORF6 (U1-ORF6-RK) was constructed with mutations targeting the two positively charged amino acid residues in the N-terminal region of the protein (Fig. 1b). Subcellular localization of the YFP-fused U1-ORF6-RK in infiltrated N. benthamiana leaves revealed that it had lost the ability to be targeted to the nucleolus (Fig. 4e), showing that both the L- and U1-ORF6 proteins have a similarly positioned NoLS. However, the U1-ORF6-RK did exhibit a diffuse nuclear localization similar to that of unfused YFP. The RK mutations were predicted not to affect the mitochondrial localization of the U1-ORF6 protein, and the YFP-tagged U1-ORF6-RK protein retained the pattern of cytoplasmic distribution in mitochondria shown by the wild-type U1-ORF6 protein (Fig. 4e).

DISCUSSION

The data presented in this paper revealed that the ORF6 proteins of TMV U1 and ToMV L, in spite of being moderately related in sequence and encoded by genes occupying equivalent positions in the respective genomes (Morozov et al., 1993), have a number of functional differences. Firstly, unlike U1-ORF6, the L-ORF6 protein was not associated with an increased ToMV pathogenicity. Secondly, L-ORF6 caused only a moderate increase in viral pathogenicity (sporadic areas of leaf necrosis) when expressed from TRV, whilst similarly expressed U1-ORF6 caused a very severe TRV infection leading to the death of the plant. Thirdly, the subcellular localization of the two proteins appeared to be very distinct: U1-ORF6 was detected initially in the nucleolus and relocated to the mitochondria at later time points, whereas L-ORF6 was...
constantly associated with the ER network. It is known that some eukaryotic proteins that localize to both mitochondria and the nucleus can act as gene-expression regulators. For example, PNM1, a plant pentatricopeptide repeat protein, localizes to both mitochondria and the nucleus (Hammani et al., 2011). In mitochondria, PNM1 is associated with ribosomes in a RNA-dependent manner. In the nucleus, it can interact with nucleosome assembly proteins and transcription factors. Analysis of mutants where PNM1 is unable to localize to the nucleus suggests that PNM1 is a negative regulator of nuclear genes encoding mitochondrial proteins (Hammani et al., 2011). In addition, mitochondrial transcription factor A, a member of the high-mobility-group protein family, localizes to the nucleus in some human cancer cells where it can regulate the expression of nuclear genes and enhance the growth of cancer cell lines (Han et al., 2011).

Regardless of their differences, the U1- and L-ORF6 proteins do both possess NoLSs. The ER localization of L-ORF6 was determined by its C-terminal hydrophobic

Fig. 4. Nucleolar localization signals in the L- and U1-ORF6 proteins. YFP-fused proteins were expressed by agroinfiltration in transgenic N. benthamiana plants expressing either the nucleolar marker fibrillarin (AtFib2) fused with mRFP (a, b, e) or the nuclear marker H2B fused with mRFP (c, d). ORF6 proteins are L-ORF6-HYDR (a, b), ORF6-HYDR-RK (c), L-ORF6-RK (d) and U1-RK (e). As well as whole-cell images (left), single optical sections are shown (right). Blue bodies are autofluorescent chloroplasts. Bars, 20 μm (whole-cell images and cell periphery); 5 μm (nuclear images).
However, when this region was mutated or replaced with the respective sequence from U1-ORF6, the protein was targeted to the nucleolus by a signal located in the N-terminal 10 aa. A similarly located NoLS was mapped in U1-ORF6. This could suggest that the wild-type L-ORF6 is permanently associated with the ER and that its signals for nucleus/nucleolus localization are functionally overridden by the C-terminal ER-directing sequence. Alternatively, L-ORF6 may shuttle between the nucleus/nucleolus and the ER, but, as the C-terminal region contains a strong signal for export from the nucleus/nucleolus to the ER, L-ORF6 cannot be detected in the nucleus due to its fast trafficking back to the ER. Prediction software did not identify any known nuclear export signal in L-ORF6, but non-canonical sequences may still exist, as occurs in some other proteins.

In addition to the well-characterized role of the nucleolus in transcription and processing of rRNA genes as well as the subsequent assembly of ribosome subunits, many other functions are now described for the nucleolus, including those associated with plant host antiviral defence and pathogenicity of viruses (Taliansky et al., 2010; Shaw & Brown, 2012). The ability of plant viruses to infect and move systemically in the host plant is determined largely by virus-encoded suppressors of RNA silencing that inhibit plant defence responses (Csorba et al., 2009). The potyvirus protein VPg, one of the functions of which is RNA silencing suppression, can localize to the nucleus and nucleolus, interacting with the nucleolar protein fibrillarin. A VPg amino acid motif directing nuclear/nucleolar localization was also found to be essential for the suppression function of the protein (Rajamäki & Valkonen, 2009). Similarly, the well-studied silencing suppressor cucumber mosaic virus (CMV) 2b protein has been shown to be localized in the nucleus and nucleolus where it interacts with AGO1, the core component of the RNA-induced silencing complex; binding of AGO1 by the 2b protein is sufficient to inhibit the AGO1 slicer activity in vitro (González et al., 2010; Duan et al., 2012). Interestingly, high-level expression of the 2b protein from a heterologous virus vector resulted in necrosis in the systemically infected leaves and stem, leading to death of the plants (Brigneti et al., 1998). Possibly, the tobamovirus ORF6 protein, when localized to the nucleolus, might perturb the host RNA silencing machinery, resulting in phenotypes similar to those observed upon CMV 2b overexpression. In a previous study, U1-ORF6 did not show RNA silencing suppression activity (Canto et al., 2004). In other experiments, L-ORF6 did not show RNA silencing suppression activity when transiently co-expressed with GFP and a double-stranded GFP hairpin sequence (N. I.

**Fig. 5.** Identification of a NoLS in the L-ORF6 protein. Four fragments of L-ORF6-HYDR fused at the N terminus to YFP were imaged at 2 days p.i. N-terminal fragment FR1 (MKPRRRSRIL) was co-expressed with the nucleolar marker fibrillarin (AtFib) fused with mRFP (a) and the nuclear marker H2B fused with mRFP (b). Internal fragments FR2 (MILIRIKYVLL) (c) and FR3 (MLNHFSIAIC) (d), C-terminal fragment FR4 (MISASATRTG) (e) and unfused YFP (f) were co-expressed with the nuclear marker H2B fused with mRFP. The pictures are single optical sections. Bars, 5 μm.
Lukhovitskaya, unpublished data). Nevertheless, the possible interaction of ORF6 with specific components of the host RNA silencing machinery remains to be examined.

The majority of the ORF6 constructs carrying the C-terminal region of U1-ORF6 caused a large increase in the severity of TRV infection symptoms and localized to the nucleus and nucleolus. From these results, we conclude that nuclear/nucleolar localization of the U1-ORF6 protein may be responsible for the strong enhancement of viral pathogenicity, perhaps by interfering with some nuclear/nucleolar function(s) involved in host defence.

Previous work has demonstrated that U1-ORF6 plays a direct role in enhancing the pathogenicity of TMV, whereas our experiments did not find a similar role for L-ORF6 in ToMV infection, although both the U1- and L-ORF6 proteins could influence the pathogenicity of TRV. One explanation could be that there is a host specificity in the functionality of L-ORF6 as a natural component of ToMV. An example of this type of specificity is the inclusion in the citrus tristeza virus genome of three different genes encoding proteins that function separately to allow the virus to infect a range of different citrus species (Tatineni et al., 2011). Further work is required to understand the precise role of ORF6 in the life cycle of tobamoviruses, as well as the significance of the nucleolar, ER and mitochondrial localization of ORF6.

**METHODS**

**Viral constructs and plasmids.** All manipulations with DNA were carried out using standard procedures (Sambrook et al., 1989). The plasmid pTLW3 containing the full-length ToMV CDNA, with a T7 RNA polymerase promoter and an M13 restriction site for linearization at the 3’ terminus (Kubota et al., 2003), was obtained from Masayuki Ishikawa (National Institute of Agrobiological Sciences, Tsukuba, Japan). The ToMV–ORF6 clone was constructed from pTLW3 by subcloning of an Ncol–EcoRI fragment containing the ORF6 gene into pGEM-T and subsequent mutagenesis of the AUG codon using overlapping primers (all primer sequences are available from authors on request) and a QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s protocol. A mutated Ncol–BstEII subfragment was then cloned back into pTLW3.

Infectious clones of TRV RNA1 and RNA2 have been prepared previously (MacFarlane & Popovich, 2000). A clone of TRV RNA2 containing the GFP gene flanked by Ncol and KpnI sites (TRV[GFP]) was modified to express a series of ORF6 constructs using a previously described procedure to express U1-ORF6 (Canto et al., 2004). The L-ORF6 gene was amplified by PCR from pTLW3, and hybrids of L-ORF6 and U1-ORF6 were constructed by PCR with primers introducing a hybrid 3’ end. The different ORF6 constructs were flanked by BspHI and KpnI sites, and were inserted in place of the GFP gene in TRV[GFP]. The constructs were linearized with Smal prior to in vitro transcription and capping using a commercial kit (Ambion MEGAscript T7; Epicentre ScriptCap).

The genes encoding L-ORF6, U1-ORF6 and their mutants were amplified to add a BamHI site at the translation initiation codon and a XhoI site downstream of the C-terminal codon, and were cloned in a three-fragment ligation together with a XhoI/XhoI-flanked YFP gene into the binary vector pGY-A4T4/1 (Paape et al., 2006), producing ORF6–YFP fusions. Expression cassettes were excised with EcoRI and cloned into pLH7000. All variants of ORF6 in the binary vector had the same linker sequence (SRGP) between the ORF6 and YFP proteins.

**Plants and transgenic lines.** Tobacco (N. tabacum cv. Samsun nn or cv. Samsun NN and cv. Xanthi nn or cv. Xanthi NN), transgenic tobacco line CB137 (Golgi-targeted mRFP) (kindly provided by Chris Haves, Oxford Brookes University, Oxford, UK), non-transgenic N. benthamiana and transgenic N. benthamiana line CB003 (expressing the TMV MP–GFP) (kindly provided by Christophe Reichel, The Scripps Research Institute, CA, USA), CB132 (expressing ER-targeted GFP) (kindly provided by Olivier Voinnet, Institute of Agricultural Sciences, Zurich, Switzerland), CB157 (expressing H2B–mRFP for nuclear labelling) and CB161 (expressing a fibrillarin–mRFP fusion) (both kindly provided by Michael Goodin, University of Kentucky, KY, USA), tomato (Solanum lycopersicum cv. Micro Tom and cv. Craigella) and N. clevelandii plants were grown from seed and maintained under glasshouse conditions with a day length of 16 h, a minimum daytime temperature of 28 °C and minimum night-time temperature of 22 °C. Supplementary lighting was provided below a daytime threshold of 250 W m⁻² and sun screening was used above 400 W m⁻².

**Plant inoculation and analysis.** ToMV infection used capped transcripts that were ribo-inoculated onto carbormundum-dusted leaves of small N. benthamiana plants. TRV infection used capped transcripts of TRV RNA2 (expressing the different ORF6 constructions) in combination with total plant RNA extracted from RNA1-only-infected plants (Mueller et al., 1997) or rubbed directly onto leaves that had been previously (24 h) infiltrated with Agrobacterium NoLS containing a binary plasmid carrying full-length TRV RNA1 (Liu et al., 2002). For transient protein expression by agroinfiltration, Agrobacterium tumefaciens strain C58C1 was used. Bacteria carrying pLH700-based vectors were cultured overnight at 28 °C with 10 mM MES (pH 5.85) and 20 mM acetoxyrinceone, resuspended in 10 mM MES, 10 mM MgCl₂, 150 μM acetoxyricineone to a final optical density of 600 nm of 0.1–1.0, incubated for 3 h at room temperature and infiltrated onto the lower leaf surfaces of plants with a syringe. The plants were incubated for 24 h in a growth chamber at 24 °C before microscopy was performed. For studies of mitochondrial localization, pre-agroinfiltrated areas of leaf were reinfiltated with MitoTracker Red CMXRos (Invitrogen/Molecular Probes) at a concentration of 100 nM. The tissue was incubated at room temperature in the dark for 30 min prior to imaging. To induce plasmolysis a detached leaf was infiltrated with 0.75 M sorbitol and incubated at room temperature for 5–15 min.

Cells expressing fusions of fluorescent proteins were imaged on a Leica TCS SP2 confocal microscope. Images were obtained using a Leica HCX APO × 63/0.9 W water-dipping lens and whole lesions using a HCX PL Fluotar × 1.6/0.05 lens. GFP and YFP were imaged sequentially at excitation 488 nm and emission 490–510 nm for GFP, and excitation 514 nm and emission 535–545 nm for YFP. YFP and mRFP were imaged sequentially at excitation 514 nm, emission 525–555 nm for YFP, and excitation 561 nm, emission 580–610 nm for mRFP. Unless otherwise stated, images are maximum-intensity projections of multiple-layered stacks. Images were assembled and edited using Adobe Photoshop CS.

For Northern blotting, leaf tissue was collected in 1.5 ml tubes and frozen with liquid nitrogen followed by storage at −70 °C before RNA extraction (Verwoerd et al., 1989). The RNA was separated in a denaturing agarose gel and transferred to Hybond-N + membrane (Amersham), followed by subsequent processing using an AlkPhos

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labelling and detection kit (GE Healthcare) according to the manufacturer’s instructions. For ToMV detection, a negative-strand, 3’-untranslated region-specific RNA probe was prepared from the Ncol/EcoRI pTLW3 fragment that was subcloned into pGEM-T and transcribed with a MEGAScript SP6 kit (Ambion). For TRV detection, a negative-stranded probe was prepared for both TRV RNA1 and RNA2 using similar methods.

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