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Membrane association of a nonconserved viral protein confers virus ability to extend its host range

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ABSTRACT

Citrus tristeza virus (CTV), the largest and most complex member of the family *Closteroviridae*, encodes a unique protein, p33, which shows no homology with other known proteins, however, plays an important role in virus pathogenesis. In this study, we examined some of the characteristics of p33. We show that p33 is a membrane-associated protein that is inserted into the membrane via a transmembrane helix formed by hydrophobic amino acid residues at the C-terminal end of the protein. Removal of this transmembrane domain (TMD) dramatically altered the intracellular localization of p33. Moreover, the TMD alone was sufficient to confer membrane localization of an unrelated protein. Finally, a CTV variant that produced a truncated p33 lacking the TMD was unable to infect sour orange, one of the selected virus hosts, which infection requires p33, suggesting that membrane association of p33 is important for the ability of CTV to extend its host range.

Introduction

Citrus tristeza virus (CTV), the largest and most complex member of the family *Closteroviridae*, is a phloem-limited virus, which infects *Citrus* spp. and close relatives (Bar-Joseph et al., 1979; Dolja et al., 1994, 2006; Agranovsky, 1996; Karasev, 2000). CTV has significantly impacted citrus industries in many different countries all over the world by causing two types of disease—quick decline and stem pitting, which led to dramatic losses of fruit yield and death of millions of trees (reviewed in Moreno et al., 2008). CTV has a 19.3 kb positive-sense RNA genome organized into 12 open reading frames (ORFs; Fig. 1), which encode proteins that function at different stages of the virus life cycle (Pappu et al., 1994; Karasev et al., 1995; Karasev, 2000). ORFs 1a and 1b are expressed from the genomic RNA and encode polyproteins involved in virus replication. The other ten genes in the 3′ half of the genome are expressed by 3′ co-terminal subgenomic RNAs (sgRNA; Hilf et al., 1995; Karasev et al., 1997). Five of these genes represent a signature gene block conserved among the members of the *Closteroviridae* and encode a hydrophobic protein p6, p65 (or HSP70h), which is a homolog of the cellular heat shock proteins, p61, and two coat proteins, the major and the minor coat proteins (CP and CPm, respectively). The latter four proteins are involved in virion assembly and also are needed for virus movement along with p6 (Alzhanova et al., 2000; Satyanarayana et al., 2000, 2004; Peremyslov et al., 2004; Tatineni et al., 2010). The remaining five genes that encode p33, p18, p13, p20, and p23 proteins are not found in other members of the *Closteroviridae* (Dawson et al., 2013). Among those, p20 and p23 proteins are absolutely required for infection of plants and have been shown to be involved in suppression of host RNA silencing along with CP (Lu et al., 2004; Tatineni et al., 2008). Three nonconserved proteins, p33, p18, and p13, are dispensable for systemic infection of most *Citrus* spp. CTV mutants with deletions of the corresponding genes are able to infect, multiply, and spread systemically throughout plants of several citrus varieties similarly to the wild type virus (Tatineni et al., 2008). On the other hand, the presence of these genes, and p33 in particular, in the virus genome is required for infection of a few other varieties (Tatineni et al., 2011). Interestingly, the p33 gene product appears to have a major role in extending the virus host range. Thus, acquisition of the p33 gene allowed systemic infection of sour orange, lemon, grapefruit, and calamondin. The products of the other two genes appear to carry out some redundant functions in the latter two hosts: in the absence of p33, p18 permits infection of grapefruit, while p13 allows the virus to infect calamondin (Tatineni et al., 2011).

In addition to extending the ability of CTV to interact with multiple hosts, p33 plays a crucial role in virus superinfection exclusion, a phenomenon in which an initially established viral infection blocks a secondary infection with the same or closely related virus. As we showed recently, mutations within the p33 ORF, which prevented production of the functional protein, resulted in a loss of virus ability to exclude superinfection by the wild type

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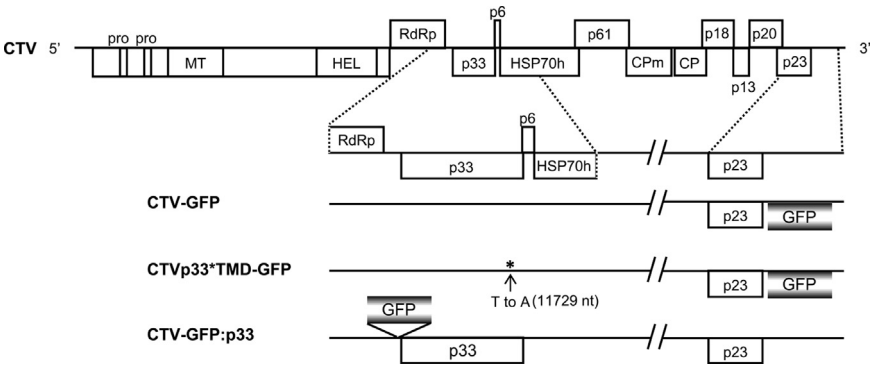

Fig. 1. Schematic representation of the genome of CTV and its derivatives generated in this study. The boxes represent ORFs and their translated products. Pro, papain-like protease domain; MT, methyltransferase; HEL, helicase; RdRp, RNA-dependent RNA polymerase; HSP70h, HSP70 homolog; CPm, minor coat protein; CP, coat protein. The derivatives are shown under the enlarged genome segment. CTV-GFP has GFP ORF inserted between the p23 gene and 3′ NTR of the CTV genome under the native promoter of the CTV CP sgRNA. CTVp33*TMD-GFP has a single nucleotide substitution (T to A) at the position 11,729, which converts the cysteine (C280) codon to a stop codon. CTV-GFP:p33 has the GFP ORF fused to the 5′-terminal end of the p33 gene.

CTV (Folimonova et al., 2010). Moreover, the p33 protein appeared to function in a homology-dependent manner such that its substitution with a cognate protein from a heterologous strain did not confer exclusion, suggesting the existence of precise interactions of p33 with other viral factors involved in this phenomenon (Folimonova, 2012). In spite of an apparent role of p33 in CTV pathogenesis, the protein, however, has not been characterized nor the mechanism of its action is not yet elucidated. The p33 protein appears to be unique and does not share a significant homology with other known proteins, which complicates further understanding of how it functions.

In this study, we examined some of the biochemical properties of the p33 protein along with its subcellular localization. We show that p33 is a membrane protein and is inserted into the membrane via a transmembrane (TM) helix formed by a highly conserved array of hydrophobic amino acid (aa) residues at the C-terminal end. Removal of this TM domain (TMD) dramatically altered the subcellular localization of the protein and also affected virus ability to infect an extended host range.

Results

The p33 protein of CTV contains a putative TMD

Analysis of the aa sequence of the p33 protein encoded in the genome of the T36 isolate of CTV using computer programs MPEx and TMpred (see Materials and methods) revealed presence of a highly hydrophobic region at the C-terminal end of the protein spanning aa residues 279 to 299 (Fig. 2(A)). The protein structure prediction programs, which utilized different algorithms (see Materials and methods), consistently predicted a single α-helical TMD within this hydrophobic region. The free energy for membrane insertion of the predicted TMD based on a biological hydrophobicity scale (Hessa et al., 2005) appeared to be negative enough ($\Delta G_{app} = -1.807$ kcal mol^{-1}) for the protein to be efficiently integrated into the endoplasmic reticulum (ER) membrane by the translocon complex (Woolhead et al., 2004; Elofsson and von Heijne, 2007). Further examination of the aa array of the predicted TMD region by the helix structure analysis tool (http://heliquest.ipmc.cnrs.fr/) showed that all faces of the surface of the TMD helix are uniformly hydrophobic, which suggested that p33 could be tightly inserted into the membrane (data not shown). Interestingly, one of the aromatic residues, tyrosine (Y282), was found toward the N-terminal end of the p33 TMD. Such residues are presumed to be positioned preferably near the end of the membrane-integrated helix and to interact with lipid-water interfacial layer of the membrane (Ulmschneider and Sansom, 2001; Beuming and Weinstein, 2004). No proline residues that could greatly reduce the efficiency of membrane insertion (von Heijne, 1991; Hessa et al., 2005) were found in the p33 TMD. Alignment of the aa sequences of p33 proteins encoded in the genomes of isolates from different CTV strains showed that the TMD region is highly conserved among the cognate proteins of those isolates, with almost identical aa composition (Fig. 2(B)), indicating that membrane insertion of the p33 protein has been preserved in all CTV variants, which could suggest the importance of this region for the protein function.

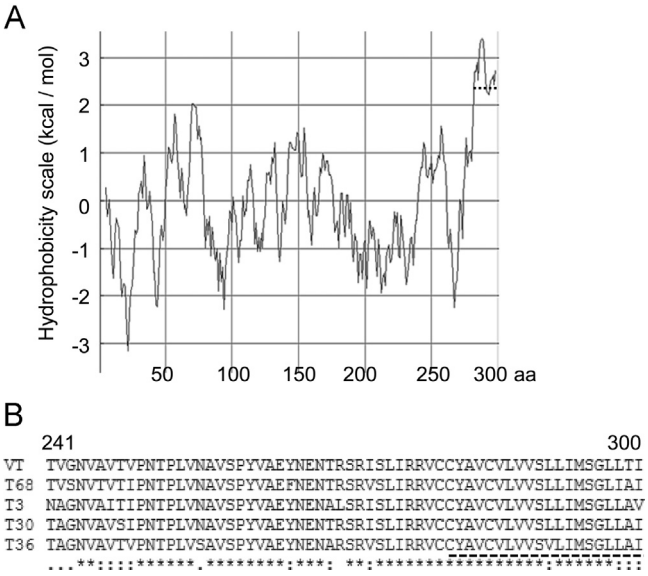

Fig. 2. Analysis of the aa sequence of the p33 protein of CTV. (A) Hydrophobicity plot. Predicted TMD region, which corresponds to the most hydrophobic region, is shown as a dotted line. (B) Alignment of the p33 aa sequences from various strains of CTV. The conserved region matching to the predicted TMD is shown as a dotted line.

p33 is an integral membrane protein

To examine membrane association of p33, we first analyzed protein extracts obtained from *Nicotiana benthamiana* plants, which were infiltrated with *Agrobacterium tumefaciens* culture transformed with a binary vector carrying a fusion of the green fluorescent protein (GFP) gene to the p33 ORF (pGFP:p33) under the 35S