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Analysis of the subcellular targeting of the smaller replicase protein of *Pelargonium flower break virus*

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ABSTRACT

Replication of all positive RNA viruses occurs in association with intracellular membranes. In many cases, the mechanism of membrane targeting is unknown and there appears to be no correlation between virus phylogeny and the membrane systems recruited for replication. *Pelargonium flower break virus* (PFBV, genus *Carmovirus*, family *Tombusviridae*) encodes two proteins, p27 and its read-through product p86 (the viral RNA dependent-RNA polymerase), that are essential for replication. Recent reports with other members of the family *Tombusviridae* have shown that the smaller replicase protein is targeted to specific intracellular membranes and it is assumed to determine the subcellular localization of the replication complex. Using *in vivo* expression of green fluorescent protein (GFP) fusions in plant and yeast cells, we show here that PFBV p27 localizes in mitochondria. The same localization pattern was found for p86 that contains the p27 sequence at its N-terminus. Cellular fractionation of p27GFP-expressing cells confirmed the confocal microscopy observations and biochemical treatments suggested a tight association of the protein to membranes. Analysis of deletion mutants allowed identification of two regions required for targeting of p27 to mitochondria. These regions mapped toward the N- and C-terminus of the protein, respectively, and could function independently though with distinct efficiency. In an attempt to search for putative cellular factors involved in p27 localization, the subcellular distribution of the protein was checked in a selected series of knockout yeast strains and the outcome of this approach is discussed.

1. Introduction

The replication of all positive strand RNA viruses of eukaryotes takes place in membrane-associated complexes in the cytoplasm of infected cells. The reasons for membrane association of viral RNA synthesis are not well understood. It is generally believed that the membranes play a structural and/or organizational role during assembly of the replication machinery and permit the increase of the local concentration of replicative enzymes and viral RNAs. In addition, the compartmentalization of the replication process may prevent double-stranded viral replication intermediates from being sensed by antiviral defence systems of the host cell (Denison, 2008; Mackenzie, 2005). Membrane systems that can be compromised in viral replication include plasma membrane, endoplasmic reticulum, Golgi apparatus, vacuoles, chloroplasts, mitochondria, peroxisomes and endo/lysosomes (reviewed in Ahlquist et al., 2003; Salonen et al., 2005). In many cases the replication complexes also induce morphological alterations of the target membranes, which can interfere with their normal functions.

Pelargonium flower break virus is a member of the genus *Carmovirus* in the family *Tombusviridae*. Its genome consists of a monopartite, positive-sense RNA of 3923 nt which is neither capped nor polyadenylated and contains five open reading-frames (ORFs) (Rico and Hernández, 2004). Proteins encoded by the internal and 3′-terminal ORFs are dispensable for PFBV multiplication and are rather involved in viral movement, encapsidation or suppression of RNA silencing (Martínez-Turiño and Hernández, 2009, 2011). In contrast, the translation products of the ORFs located 5′-proximal in the genomic RNA, ORFs 1 and 2, correspond to polypeptides of 27 and 86 kDa, respectively, that are strictly required for viral replication (Martínez-Turiño and Hernández, 2010). The larger replicase protein (p86) is synthesized as a readthrough product of the shorter one (p27) and, due to the low frequency of the stop codon suppression even, the latter is synthesized at 10–20 fold higher amounts than the former (Fernández-Miragall and Hernández, 2011). While p86 encloses the eight motifs conserved in the viral RNA dependent-RNA polymerases (RdRp) of supergroup II of the positive strand RNA viruses (Koonin, 1991; Koonin and Dolja, 1993), p27 has no obvious replication motifs as occurs with equivalent proteins in the family *Tombusviridae*.

The specific role of the smaller replicase protein of members of the family *Tombusviridae* has long been a subject of debate. Recent results with PFBV p27 and previous ones with the homologous product of *Tomato bushy stunt virus* (TBSV), namely p33, have revealed that these proteins bind cognate viral ssRNAs with

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high affinity suggesting that play an essential role in selection and recruitment of replication templates (Martínez-Turiño and Hernández, 2010; Pogany et al., 2005; Rajendran and Nagy, 2003). Other roles, however, cannot be discarded. Indeed, a recent report indicates that TBSV p33 has RNA chaperone activity and likely facilitates proper folding of viral RNAs during replication (Stork et al., 2011). In addition, in the last years distinct studies with species of the genera *Tombusvirus*, *Dianthovirus* and *Panicovirus* have shown that the protein encoded by ORF1 is targeted to specific intracellular membranes and it is assumed to determine the subcellular localization of the replication complex. The specific membrane system recruited varies from one virus to another. Thus, the ORF1 products of *Red clover necrotic mosaic virus* (RCNMV, genus *Dianthovirus*) and of *Panicum mosaic virus* (PMV, genus *Panicovirus*) associate to membranes of the endoplasmic reticulum (Batten et al., 2006; Turner et al., 2004), whereas the ORF1 products of TBSV, *Cymbidium ringspot virus* (CymRSV) and *Cucumber necrosis virus* (CNV) in the genus *Tombusvirus*, are targeted to peroxisomes (McCartney et al., 2005; Navarro et al., 2004; Panavas et al., 2005). Despite *Carnatian Italian ringspot virus* (CIRV) also belongs to genus *Tombusvirus*, its ORF1 product (p36) is sorted to mitochondria (Weber-Lotfi et al., 2002). Most of these proteins induce organelle aggregation and/or proliferation of the membranes they associate with and seem to be truly integrated in the corresponding membranes. In many cases, α-helices that function as transmembrane domains (TMs) play a critical role in both targeting and integration to specific membranes. That is the case of CIRV p36 which has been proposed to associate to the mitochondrial outer membrane through two TMs and multiple recognition signals present at the N-terminus that might function cooperatively as a so-called signal loop-anchor type mitochondrial targeting sequence (Weber-Lotfi et al., 2002; Hwang et al., 2008).

Information on the membrane association of replication proteins from members of the genus *Carmovirus* is relatively scarce. Recently, the interaction of the ORF1 product (p29) of *Melon necrotic spot virus* (MNSV) with mitochondrial membranes has been described and at least one TM has been found to be required for such interaction (Mochizuki et al., 2009). In addition, the presence of N-terminal, classical mitochondrial targeting signals (MTS), that consist of 15–40 amino acid (aa) residues and form positively charged amphipathic α-helices (Chacinska et al., 2009), was suggested for the ORF1 products of other carmoviruses (Ciuffreda et al., 1998) but experimental approaches to study the subcellular sorting of other carmoviral replicase proteins have not yet been made.

Here we show that PFBV p27 is able to target the green fluorescent protein (GFP) reporter to mitochondria *in vivo* upon transient expression of a fusion protein in plant and yeast cells. Similar results were obtained with the complete replicase p86 which contains the p27 sequence at its N-terminal region. Analysis of deletion mutants indicated that two regions toward the N- and C-terminus, respectively, of p27 contain signals for mitochondrial targeting. Biochemical fractionation experiments revealed that p27 sedimented mainly with mitochondrial enriched fractions, in agreement with the confocal microscopy observations, and that associates tightly to membranes. Finally, the subcellular distribution of the protein was checked in a selected series of knockout yeast strains in an attempt to search for putative cellular factors involved in p27 localization.

2. Materials and methods

2.1. Generation of constructs

For protein expression in *Saccharomyces cerevisiae*, the GFP gene was cut out from pBin19-s*gfp* (Peña et al., 2003) with *Bam*HI/*Eco*RI digestion and subcloned into the *Bam*HI/*Eco*RI sites of plasmid pYES 2.0 (Invitrogen) containing the galactose-activated GAL1 promoter. The resulting recombinant plasmid was named pYES-GFP. In addition, the PFBV p27 coding sequence was amplified with Expand High Fidelity PCR System (Roche) using the PFBV infectious clone pSP18-IC (Rico and Hernández, 2006) as template, and primers CH67 and CH70 which harbored an *Nco*I restriction site at their 5′ end (primers listed in Supplemental Table S1). After *Nco*I digestion, the PCR-generated fragment was cloned in the *Nco*I site which precedes the start codon of the GFP gene in construct pYES-GFP to yield pYES-p27GFP that contained the p27 cDNA fused in frame to the GFP gene. A similar construct, signed as pYES-p86GFP, was prepared with the p86 gene which was PCR amplified with primers CH67 and CH182 (Supplementary Table S1) from plasmid p27tyr, a full-length PFBV clone in which the amber stop codon of ORF1 was mutated to a tyrosine codon (Martínez-Turiño and Hernández, 2010). To study possible co-localization of p27 and p86, the GFP gene of plasmid pYES-p86GFP was replaced by the gene encoding the monomeric red fluorescent protein (mRFP) yielding construct pYES-p86mRFP. The complete expression cassette of this construct (the p86mRFP fusion gene flanked by the GAL1 promoter and the terminator sequence) was PCR amplified with primers CH222 and CH223, encompassing a *Spe*I restriction site at their 5′ end (Supplementary Table S1), subsequently digested with *Spe*I and ligated into plasmid pYES-p27GFP through the *Spe*I site present in the vector sequence. The resulting construct with two expression cassettes in tandem was named pYES-p27GFP/p86mRFP.

Different regions of the p27 gene were also PCR amplified with specific primers (Supplementary Table S1) and fused in frame with the GFP gene of construct pYES-GFP using appropriate restriction sites (introduced by PCR into the p27 derived cDNAs). Following this approach, a total of thirteen p27 deletion mutant constructs were generated: pYES-p27(21–243)GFP (mutant 1, created with primers CH115 and CH70), pYES-p27(34–243)GFP (mutant 2, primers CH113/CH70), pYES-p27(73–243)GFP (mutant 3, primers CH162/CH70), pYES-p27(1–215)GFP (mutant 4, primers CH67/CH114), pYES-p27(1–180)GFP (mutant 5, primers CH150/CH215), pYES-p27(1–162)GFP (mutant 6, primers CH150/CH228), pYES-p27(1–155)GFP (mutant 7, primers CH150/CH163), pYES-p27(21–155)GFP (mutant 8, primers CH115/CH163), pYES-p27(73–155)GFP (mutant 9, primers CH162/CH163), pYES-p27(51–155)GFP (mutant 10, primers CH318/CH163), pYES-p27(34–155)GFP (mutant 11, primers CH113/CH163), pYES-p27(73–162)GFP (mutant 12, primers CH162/CH228), and pYES-p27(73–215)GFP (mutant 13, primers CH162/CH114).

For transient expression of proteins in *Nicotiana benthamiana* protoplasts, the GFP gene, the cDNA encoding the p27GFP fusion and GFP fusions with the p27 derivatives of mutants 3, 8, 10, and 13 were recovered from the corresponding yeast constructs with *Bam*HI/*Pst*I digestion and subcloned into the *Bam*HI/*Pst*I sites of a pBluescript KS+ derived-plasmid containing the *Cauliflower mosaic virus* (CaMV) 35S promoter upstream of the *Bam*HI site and the terminator sequence of the *Solanum tuberosum* proteinase inhibitor II gene downstream of the *Pst*I site. All constructs were routinely sequenced to avoid unwanted modifications.

2.2. Expression of gene constructs in yeast and plant cells

For expression in yeast cells, the pYES 2.0 derived constructs were employed to transform *S. cerevisiae* strain W303-1A (*MATα*, *his3-11/15*, *leu2-3/112*, *trp1-1*, *ura3-1*, *ade2-1*, *can1-100*, Wallis et al., 1989). The plasmid p36K-GFP, allowing expression of protein p36 of *Carnation Italian ringspot virus* (Rubino et al., 2000), was also included for comparison purposes. Transformation of plasmids was done with the lithium acetate–polyethylene glycol method (Ito et al., 1983). Transformed cells were spread on minimal selective