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The p33 auxiliary replicase protein of Cucumber necrosis virus targets peroxisomes and infection induces *de novo* peroxisome formation from the endoplasmic reticulum

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ABSTRACT

Tombusviruses replicate on pre-existing organelles such as peroxisomes or mitochondria, the membranes of which become extensively reorganized into multivesicular bodies (MVBs) during the infection process. *Cucumber necrosis virus* (CNV) has previously been shown to replicate in association with peroxisomes in yeast. We show that CNV induces MVBs from peroxisomes in infected plants and that GFP-tagged p33 auxiliary replicase protein colocalizes with YFP_{SKL}, a peroxisomal marker. Most remarkably, the ER of CNV infected *Nicotiana benthamiana* 16C plants undergoes a dramatic reorganization producing numerous new peroxisome-like structures that associate with CNV p33, thus likely serving as a new site for viral RNA replication. We also show that plants agroinfiltrated with p33 develop CNV-like necrotic symptoms which are associated with increased levels of peroxide. Since peroxisomes are a site for peroxide catabolism, and peroxide is known to induce plant defense responses, we suggest that dysfunctional peroxisomes contribute to CNV induced necrosis.

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Introduction

Cucumber necrosis virus (CNV) is a member of the *Tombusvirus* genus in the Family *Tombusviridae*. The plus-strand, positive-sense RNA genome of CNV contains five open reading frames (ORFs) (Fig. 1) (Rochon and Tremaine, 1989). ORF1 encodes a 33 kDa auxiliary replicase protein (p33) and the readthrough of this ORF produces a 92 kDa protein containing RNA dependent RNA polymerase motifs. ORF3 encodes the 41 kDa coat protein which assembles with viral RNA to form virions with $T=3$ icosahedral symmetry (Katpally et al., 2007; Li et al., 2013). p21 is the viral cell-to-cell movement protein (Rochon and Johnston, 1991) and p20 is a suppressor of RNA silencing (Angel et al., 2011; Hao et al., 2011). p33 and p92 are translated directly from genomic RNA while p41, p21 and p20 are translated from two subgenomic RNAs generated during infection (Johnston and Rochon, 1990, 1995, 1996; Rochon and Johnston, 1991; Sit et al., 1995). As with the p33 proteins of other tombusviruses, CNV p33, or its homolog in *Tomato bushy stunt virus* (TBSV), is an integral membrane protein that is central to the formation of the viral replicase complex. It contains an RNA binding site for specific recruitment of viral RNA into the replication complex, specific domains that promote p33/p33 and p33/p92 interactions and a transmembrane domain for targeting peroxisomes in yeast. P33 interacts with numerous host proteins involved in the assembly, fidelity and regulation of the replicase complex (Nagy, 2011; Nagy et al., 2012; Nagy and Pogany, 2010, 2012; Panavas et al., 2005; Pogany et al., 2005; Rajendran and Nagy, 2003; Serva and Nagy, 2006).

Tombusviruses are known to induce distinctive multivesicular bodies (MVBs) during infection (Martelli et al., 1988). These can derive from peroxisomes as in TBSV and *Cymbidium ringspot virus* (CymRSV) (Jonczyk et al., 2007; McCartney et al., 2005; Navarro et al., 2004; Panavas et al., 2005; Pathak et al., 2008), or from mitochondria as in *Carnation Italian ringspot virus* (CIRV) (Hwang et al., 2008; Weber-Lotfi et al., 2002). However, the origin of the MVBs can depend on the host (Martelli et al., 1988). MVBs derived from peroxisomes undergo a progressive vesiculation of the boundary membrane and contain numerous spherules that are approximately 80–150 nm in diameter which serve as the site of viral RNA replication (Appiano et al., 1983; Martelli et al., 1988; McCartney et al., 2005; Panavas et al., 2005; Pathak et al., 2008). MVBs have been suggested to contain membranous material derived from the endoplasmic reticulum (ER) since ER strands were often seen adjacent to or protruding from MVBs (Martelli et al., 1988). In TBSV infection, p33 traffics from the peroxisome to the ER (McCartney et al., 2005). It has been shown that the ER is the site of replication for TBSV and CymRSV in yeast lacking peroxisomes, demonstrating flexibility in the sites of replication

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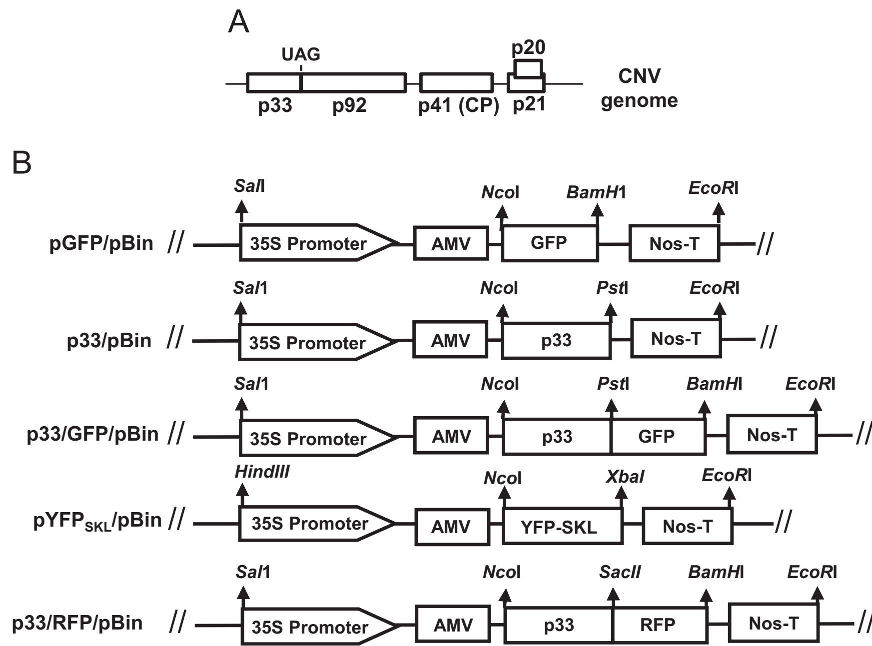

Fig. 1. Diagrammatic representation of constructs used in this study. (A) Boxes in the CNV genome correspond to the five open reading frames which encode the p33 auxiliary replicase protein, the p92 RNA dependent RNA polymerase, the p41 coat protein (CP), the p20 silencing suppressor and the p21 cell-to-cell movement protein. p33 is expressed directly from genomic RNA and p92 is expressed by translational read-through of the p33 amber (UAG) stop codon. (B) Schematic representation of the pBin(+) fusion protein expression constructs used in this study. Clones were constructed as described in Materials and methods section. The desired ORFs were placed downstream of the dual 35S promoter of Cauliflower mosaic virus and the Alfalfa mosaic virus (AMV) translational enhancer. Nos-T corresponds to the nopaline synthase transcription termination site. Restriction enzyme sites used for cloning purposes are shown. pGFP/pBin was previously described (Hui et al., 2010).

of these viruses. In addition, TBSV can replicate efficiently on ER membranes *in vitro* (Jonczyk et al., 2007; Navarro et al., 2004; Xu et al., 2012). Specific sequence elements in p33 have been shown to be involved in peroxisomal or mitochondrial membrane targeting (Burgyan et al., 1996; Hwang et al., 2008; McCartney et al., 2005; Navarro et al., 2004; Panavas et al., 2005; Rubino and Russo, 1998)

CNV p33 has been shown to target peroxisomes in *Saccharomyces cerevisiae* however its targeting site in plants has not been determined. An N-proximal transmembrane domain in CNV p33 is required for peroxisomal targeting in yeast as is a p33:p33/p92 interaction domain (Panavas et al., 2005). Mutations in the interacting domain result in ER rather than peroxisomal targeting of CNV p33.

In this manuscript we show that CNV p33 targets peroxisomes in *N. benthamiana* as well as *N. clevelandii*. The peroxisomes develop into typical MVBs and contain numerous spherules with necks open to the cytoplasm. Some peroxisomes are donut-shaped containing cytoplasm in the center. In addition, we show that the endoplasmic reticulum of CNV infected plants becomes highly remodeled during infection rendering *de novo* production of peroxisomes. Since peroxisomes play an important role in H_2O_2 breakdown we investigated whether CNV infected plants show defects in peroxide metabolism. Using DAB staining, we show that H_2O_2 accumulates in both CNV infected and p33 agroinfiltrated leaves. Since high levels of H_2O_2 are known to induce necrosis in plants, we speculate that infection induced alteration of peroxisomes results in inefficient H_2O_2 breakdown and subsequent necrosis.

Results and discussion

p33 targets peroxisomes in agroinfiltrated plants

To determine the subcellular targeting site of p33, the p33 ORF was fused to the N-terminus of GFP in pBin(+) to produce the construct p33/GFP/pBin (Fig. 1) and this construct was used to agroinfiltrate leaves of *N. benthamiana*. Confocal microscopy showed that at early times post-agroinfiltration (~24 h) cells of leaves contained small, highly mobile fluorescent spherical bodies approximately 0.9–1.6 um in diameter (average = 1.22 um) (Fig. 2A) reminiscent of peroxisomes. GFP fluorescence was found to be predominantly associated with the boundary membrane giving a “haloed” appearance to the peroxisome. In addition, a single punctate foci of brighter fluorescence was often found associated with the boundary membrane (Fig. 2A). At later times post-agroinfiltration (2 days) large aggregates of spheres predominated (Fig. 2B). The peroxisomal nature of the spherical bodies seemed likely since as described above it has previously been shown that CNV p33 associates with peroxisomes in yeast (Panavas et al., 2005) and the p33 proteins of two other tombusviruses are also known to associate with peroxisomes in *N. benthamiana* (McCartney et al., 2005; Navarro et al., 2004). To confirm that the spherical structures associated with CNV p33 correspond to peroxisomes the construct $pYFP_{SKL}$/pBin (Fig. 1) was used in co-agroinfiltration experiments. This construct contains the YFP gene with a C-terminal peroxisomal targeting tripeptide SKL (Gould et al., 1989; Serviene et al., 2005) which results in peroxisomal targeting of YFP. Fig. 2C shows that YFP_{SKL} targets small spherical bodies approximately 1.0–1.8 um in diameter (average = 1.34 um). These bodies were highly mobile as expected (not shown). Note, however, that in this case, the peroxisomal spheres are “filled” since the SKL motif targets YFP to the peroxisomal matrix (Gould et al., 1989; Serviene et al., 2005). It is noted that peroxisomes in $pYFP_{SKL}$/pBin infiltrated plants did not show a high level of aggregation as was observed in p33/GFP/pBin infiltrated leaves indicating that p33/GFP/pBin induces peroxisomal aggregation. This might result from high levels of p33 expression and consequent p33/p33 interaction between peroxisomes since as stated earlier p33 inherently forms dimers. To assess colocalization of p33 and the peroxisomal marker $pYFP_{SKL}$/pBin, p33/GFP/pBin and $pYFP_{SKL}$/pBin were coinfiltrated and leaves were examined by confocal microscopy. It can be seen in Fig. 2E

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