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Cytorhabdovirus P protein suppresses RISC-mediated cleavage and RNA silencing amplification *in planta*

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ABSTRACT

Plant viruses have evolved to undermine the RNA silencing pathway by expressing suppressor protein (s) that interfere with one or more key components of this antiviral defense. Here we show that the recently identified RNA silencing suppressor (RSS) of lettuce necrotic yellows virus (LNYV), phosphoprotein P, binds to RNA silencing machinery proteins AGO1, AGO2, AGO4, RDR6 and SGS3 in protein–protein interaction assays when transiently expressed. *In planta*, we demonstrate that LNYV P inhibits miRNA-guided AGO1 cleavage and translational repression, and RDR6/SGS3-dependent amplification of silencing. Analysis of LNYV P deletion mutants identified a C-terminal protein domain essential for both local RNA silencing suppression and interaction with AGO1, AGO2, AGO4, RDR6 and SGS3. In contrast to other viral RSS known to disrupt AGO activity, LNYV P sequence does not contain any recognizable GW/WG or F-box motifs. This suggests that LNYV P may represent a new class of AGO binding proteins.

Introduction

The RNA silencing pathway in plants has been shown to function in part as an antiviral defense mechanism (Burgyan and Havelda, 2011; Incarbone and Dunoyer, 2013). Viruses have evolved to encode suppressors of RNA silencing (Kasschau et al., 2003). Plant viral RNA silencing suppressor (RSS) proteins show little sequence conservation among one another, but functionally act on similar key steps in the RNA silencing pathway (Ding and Voinnet, 2007). Viral RSS are typically multifunctional proteins that are involved in other distinct roles such as encapsidation, cell-to-cell movement, vector transmission, replication and transcription initiation (Valli et al., 2009).

The majority of known plant viral RSS function by sequestering primary or secondary small interfering RNAs (siRNAs) and preventing their incorporation into the RNA induced silencing complex (RISC) (Anandalakshmi et al., 1998; Brigneti et al., 1998; Chen et al., 2008; Lakatos et al., 2006; Schnettler et al., 2010; Schott et al., 2012; Silhavy et al., 2002). However, recent insights indicate that viral RSS may compromise multiple rather than single components of the RNA silencing pathway.

Cucumber mosaic virus (CMV) 2b protein has been shown to i) interact with Argonaute (AGO) proteins 1, 4, and 6, ii) bind to siRNAs and microRNAs (miRNAs) *in vivo*, iii) inhibit systemic silencing and iv) suppress RNA-directed DNA methylation (Brigneti et al., 1998; Duan et al., 2012; Guo and Ding, 2002; Hamera et al., 2012; Zhang et al., 2006). Similarly, potato virus X (PVX) P25 protein interacts with *Arabidopsis thaliana* AGO1, AGO2, AGO3 and AGO4 expressed in *Nicotiana benthamiana* (Chiu et al., 2010), and inhibits systemic silencing (Voinnet et al., 2000). In addition, another potexvirus, *Plantago asiatica* mosaic virus (PlAMV) P25, was shown to target host components involved in RNA silencing amplification, namely, RNA-dependent RNA polymerase 6 (RDR6) and suppressor of gene silencing 3 (SGS3) (Okano et al., 2014). However, despite targeting multiple components of the RNA silencing pathway, PVX P25 is considered a weak RSS (Powers et al., 2008; Senshu et al., 2009) and PVX is unable to replicate in the non-host *A. thaliana*, unless the silencing pathway is impaired by introducing null mutations for Dicer-like (DCL) proteins 2 and 4 (Jaubert et al., 2011). Since only a handful of plant viral RSS have been identified to target multiple components of the RNA silencing pathway, this area of study is not well characterized.

Cellular distribution and interactions of viral proteins has been identified using transient expression of fluorescent protein fusions in combination with live cell imaging (Goodin et al., 2007) in the model plant *N. benthamiana* (Goodin et al., 2008). This approach

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involving Bimolecular Fluorescence Complementation (BiFC) (Hu et al., 2005; Morell et al., 2008) has been successfully used to determine the subcellular localization of viruses (Baulcombe et al., 1995), individual viral proteins and protein–protein interactions *in planta* between virus–virus (Anderson et al., 2012; Bandyopadhyay et al., 2010; Bejerman et al., 2015; Dietzgen et al., 2015; Dietzgen et al., 2012; Martin et al., 2012; Tripathi et al., 2015) and virus–host (Guo et al., 2013; Kumakura et al., 2009; Min et al., 2010). These techniques have provided a powerful means to greatly enhance the understanding of virus–host interactions and will be even more useful with the advent of reverse genetic systems, like the one recently developed for sonchus yellow net nucleorhabdovirus (SYNV) (Wang et al., 2015).

A recent study has shown that lettuce necrotic yellows virus (LNYV), genus *Cytorhabdovirus*, family *Rhabdoviridae*, order *Mononegavirales* (Jackson et al., 2005; Mann and Dietzgen, 2014), phosphoprotein (denoted "P") acts as a weak local RSS. LNYV P does not inhibit siRNA accumulation but temporarily delays systemic RNA silencing in *N. benthamiana* plants (Mann et al., 2015), leaving the mechanism of suppression to be elucidated. A mechanism of plant rhabdovirus RSS activity has only been determined for the nucleorhabdovirus rice yellow stunt virus P6 protein and involves suppression of RDR6-dependent transitivity (Guo et al., 2013).

In this study, we provide several lines of evidence that LNYV P protein inhibits the activity of multiple proteins of the RNA silencing pathway, in particular, those involved in RISC and double-stranded RNA amplification. We also examine functional domains of LNYV P and the relationship between LNYV P and nucleoprotein (N) with respect to RNA silencing and interactions for viral core formation.

Results

LNYV P protein C-terminal domain is essential for local RNA silencing suppression

To determine the minimum region required for RSS activity of LNYV P protein (Mann et al., 2015), a series of progressive N- and C-terminal deletion mutants were generated (Fig. 1A). The positions of these deletions were chosen based on a recent structural analysis indicating that LNYV P has a modular organization consisting of an N-terminal domain (N_T: 1–63 amino acids), central domain (C_D: 64–184 a.a.), disordered linker (DL: 185–229 a.a.) and C-terminal domain (C_T: 230–300 a.a.) (Martinez et al., 2013). We generated six P protein deletion mutants spanning various portions of these proposed domains (Fig. 1A) by PCR amplification and cloning into the binary plant expression vector pSITE-Flag-C1. For simplicity, these P protein deletions were classified broadly into N- or C-proximal mutants whereby individual N-terminal mutant constructs were denoted $N_T\Delta20$ (first 20 amino acids of the N_T deleted), $N_T\Delta63$ (entire N_T deleted), $N_T\Delta184$ (entire N_T and C_D deleted) while the C-terminal mutant constructs were denoted $C_T\Delta20$ (last 20 amino acids of C_T deleted), $C_T\Delta70$ (entire C_T deleted) and $C_T\Delta115$ (C_T and DL deleted) (Fig. 1A). Transient expression of mutant flag-tagged P proteins was confirmed by anti-Flag antibody western blot analysis of protein extracts from

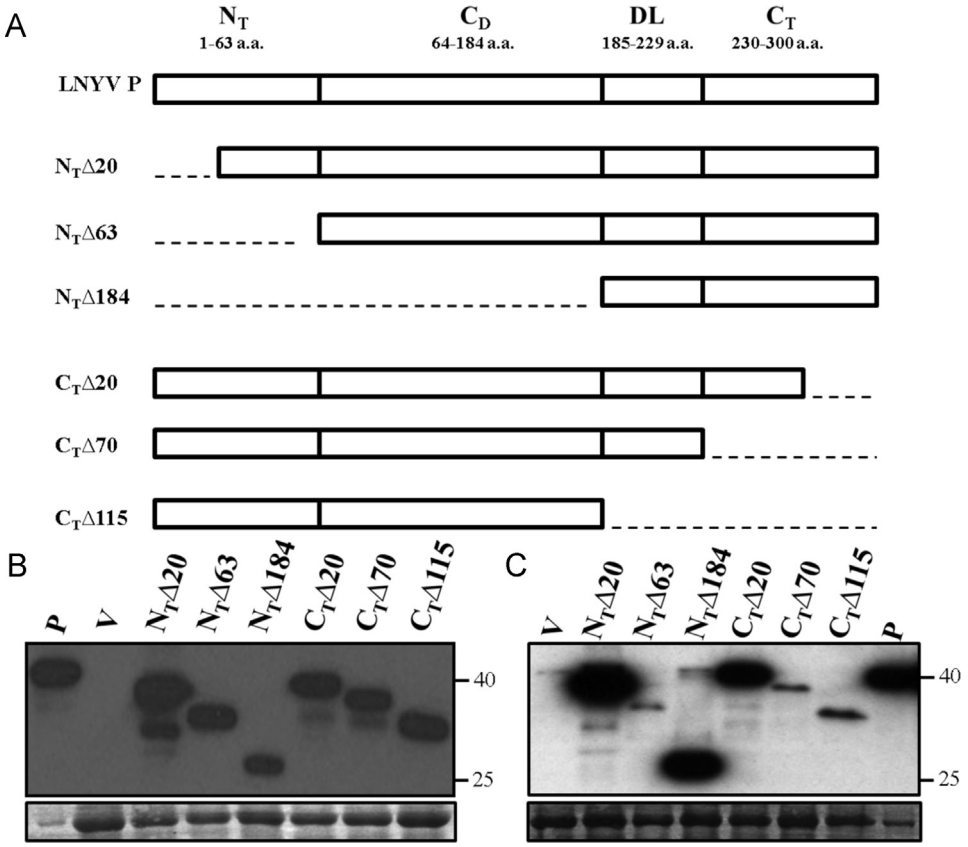

Fig. 1. Schematic diagrams and transient expression of LNYV P protein deletion mutants in *Nicotiana benthamiana*. (A) Domain organization of full-length LNYV P protein and diagrams of P deletion mutant constructs. (B and C) Western blot analysis of agroinfiltrated leaf tissue to validate transient expression of P protein constructs. Flag-tagged viral proteins were detected in leaf extracts of *N. benthamiana* plants collected at 3 (B) and 6 (C) days postinfiltration. All protein lysates were loaded in equal amounts with the exception of full-length LNYV P, of which 5 times less protein was loaded to reduce high signal intensity. The migration positions of selected molecular weight markers (kD) are indicated in the right margins and protein loading control (Rubisco) is shown below the immunoblots. N_T: N-terminal domain; C_D: central domain; DL: disordered linker region; and C_T: C-terminal domain. V indicates empty pSITE-Flag vector.