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Identification of a maize chlorotic dwarf virus silencing suppressor protein

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ABSTRACT

Maize chlorotic dwarf virus (MCDV), a member of the genus *Waikavirus,* family *Secoviridae,* has a 11784 nt (+)ssRNA genome that encodes a 389 kDa proteolytically processed polyprotein. We show that the N-terminal 78 kDa polyprotein (R78) of MCDV acts as a suppressor of RNA silencing in a well-established assay system. We further demonstrate that R78 is cleaved by the viral 3C-like protease into 51 and 27 kDa proteins (p51 and p27), and that p51 is responsible for silencing suppressor activity. Silencing suppressor activity of R78 is conserved in three divergent MCDV strains (MCDV-Severe, MCDV-M1, and MCDV-Tennessee), as well as the waikavirus *Bellflower vein chlorosis virus*, but was not detected for orthologous protein of *Rice tungro spherical virus* (RTSV-A) or the similarly-positioned protein from the sequivirus *Parsnip yellow fleck virus* (PYFV). This is the first identification of a virus suppressor of RNA silencing encoded by a waikavirus.

1. Introduction

Maize chlorotic dwarf virus (MCDV) belongs to a unique group of plant viruses, classified as the genus *Waikavirus*, within the family *Secoviridae*. Waikaviruses share an icosahedral particle morphology and positive sense (+) single-stranded (ss) RNA genome with other members of the family, but are monopartite and encode one large polyprotein. This large polyprotein needs to be proteolytically processed into various mature proteins, among them three different capsid protein subunits, in order to function at different steps of the viral multiplication cycle. Despite the enormous progress in our understanding of plant viruses in general, the biology of these viruses remain largely unknown, due to the phloem limitation of species studies so far, and reliance on specific arthropod vectors for efficient transmission. MCDV is a phloem-limited virus transmitted by the blackfaced leafhopper, *Graminella nigrifrons* in a semi-persistent manner (Nault, 1987). Its host range is limited to graminaceous plants (Nault et al., 1976). It was originally identified in southern Ohio in 1969 as part of a destructive maize virus complex (Gingery, 1976; Gordon and Nault, 1977; Rosenkranz, 1969). For many years, only two other members of the genus *Waikavirus* were described: *Rice tungro spherical virus* (RTSV; (Galvez, 1967, 1968; Ling, 1966)), one of two viruses that together cause rice tungro disease (Jones et al., 1991); and *Anthriscus yellows virus* (AYV; (Hemida et al., 1989; Murant et al., 1976)). Deep sequencing technologies continue to bring to light other waikaviruses in a variety of plants, including *Blackcurrant virus A* (GenBank accession nos. KJ572567-KJ572572)), and *Bellflower vein chlorosis virus* (Seo et al., 2015). Within the genus *Waikavirus*, there is considerable variability, with only 30–41% polyprotein amino acid sequence identity between virus species, and 58–73% within-species identity among the described MCDV strains. MCDV strains are MCDV-M1, a mild strain (GenBank accession no. AY829112, (Ammar et al., 1993; Hunt et al., 1988); MCDV-TN, from Tennessee (GenBank accession no. U67839, (Reddick et al., 1997); and MCDV-T, the type strain (Mcmullen et al., 1996) and its almost identical laboratory variant MCDV-S, the severe strain (GenBank accession no. AY36255, (Chaouch et al., 2004). Very little is known about gene function for viruses in the genus *Waikavirus*.

MCDV icosahedral virions are composed of three capsid proteins: the 31 kDa CP1, 23 kDa CP2, and 22 kDa CP3 (Chaouch et al., 2004; Gingery, 1976; Gingery and Nault, 1990; Reddick et al., 1997). The 11.8 kb MCDV-S RNA genome encodes a single large polyprotein (389 kDa; 3457 aa) encoding the capsid and other viral proteins (Fig. 1; (Chaouch et al., 2004). The polyprotein sequence includes a predicted 3C-like protease similar to that of other polyprotein-encoding viruses that infect both plants and animals (Chaouch et al., 2004; Chaouch-Hamada et al., 2004). This virus-encoded protease is expected to postranslationally cleave the polyprotein during virus maturation. Many of the cleavage sites within the polyprotein are not yet experimentally defined, but similar virus proteases cleave at sites following glutamine (Q) residues (Bazan and Fletterick, 1988). Protease cleavage sites identified so far include those of the capsid

Abbreviations: MCDV, *Maize chlorotic dwarf virus*; dpi, days post infiltration

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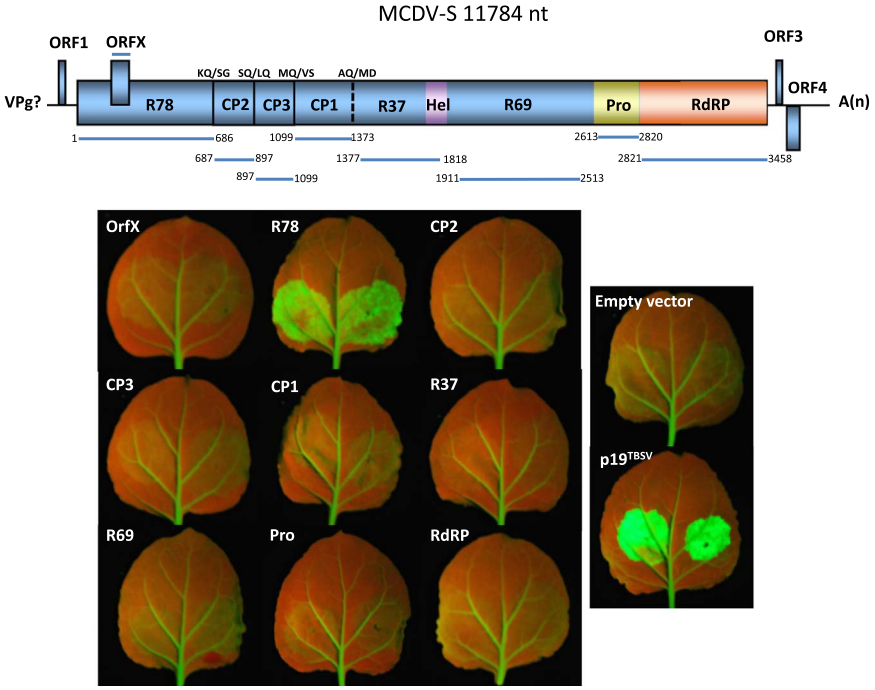

Fig. 1. Genome organization of *Maize chlorotic dwarf virus*, severe strain (MCDV-S) and agroinfiltration silencing suppressor assay results in *Nicotiana benthamiana*. Predicted open reading frames (ORFs) are indicated by boxes and proteins expressed for agroinfiltration are indicated by lines below with polyprotein amino acid coordinates indicated. Small ORFs were predicted using MacVector (Apex, NC, USA). ORFX was predicted for waikaviruses by (Atkins and Firth, 2009). Coat proteins 1, 2, and 3 (CP1, CP2, and CP3) are indicated within the polyprotein, along with predicted helicase (Hel), 3C-like protease (Pro), and RNA-dependent RNA polymerase (RdRP) domains. Other polyprotein regions are named as by Chaouch-Hamada et al. (2004) based on approximate predicted molecular weight: R78, R37, and R69. Known polyprotein cleavage sites are indicated by solid lines, and a predicted cleavage site by a dashed line. 3′ polyadenylation and predicted 5′ genome linked protein are indicate by A(n) and VPg. Images shown are of leaves infiltrated with 35S-expressed MCDV-S predicted ORFs (Fig. 1). Samples are shown at 4 days post-inoculation (dpi) with GFP and test construct, with empty vector negative control and the known silencing suppressor, p19 from *Tobacco bushy stunt virus* (TBSV) as a positive control.

proteins, determined by N-terminal sequencing of the coat proteins in MCDV and RTSV from virion preparations (Mcmullen et al., 1996; Reddick et al., 1997; Shen et al., 1993) and with some conservation across waikaviruses. Cleavage sites releasing the protease from RTSV (Thole and Hull, 1998, 2002), and internal to the predicted protease (Sekiguchi et al., 2005) have also been reported, but these sites are not conserved in MCDV.

Although the waikavirus genome organization is similar to that of other viruses in the family *Secoviridae* (Sanfacon et al., 2009; Thompson et al., 2014, 2013), the functions of few waikavirus proteins have been experimentally determined. In the absence of an efficient inoculation system and infectious clones, many virus functions such as replication, movement, and transmission determinants do not have facile assays. However, most plant viruses encode silencing suppressor proteins that interfere with host defense mechanisms and allow the virus to establish infection in host plants. For the family *Secoviridae*, previously reported silencing suppressors include the small coat protein of the comovirus *Cowpea mosaic virus* (CPMV) (Liu et al., 2004); Vp20, one of the three coat proteins of the cheravirus *Apple latent spherical virus* (ALSV) which suppresses systemic but not local silencing (Yaegashi et al., 2007); and the coat protein of the nepovirus *Tomato ringspot virus* (ToRSV) (Karran and Sanfacon, 2014). We assessed proteins encoded by MCDV-S for silencing suppressor activity and identified a protein with this function, mapped its proteolytic cleavage site, and compared activity of waikavirus orthologs.

2. Results

2.1. MCDV-S R78 is a suppressor of RNA silencing

To counteract the potent RNA silencing-based antiviral defense in plants, most plant viruses are known to encode a suppressor of RNAi. To determine which of the putative MCDV proteins and polyproteins acts as the suppressor of RNA silencing, we selected regions of the MCDV polyprotein corresponding to known mature proteins (CP2, CP1, and CP3), as well as protein regions that are probably cleaved to mature proteins at currently unknown or unconfirmed proteolytic sites. Silencing suppression activity of proteins or predicted polyproteins of MCDV-S was evaluated using an agroinfiltration assay in 16C *Nicotiana benthamiana* plants (Voinnet et al., 2000). MCDV-S proteins tested were based on modifications of predicted proteins and polyproteins named previously (Chaouch-Hamada et al., 2004; Firth and Atkins, 2008).

Nine MCDV-S proteins or polyprotein segments were tested for silencing suppression activity corresponding to the predicted OrfX protein, R78: the N-terminal protein region upstream of the coat proteins, CP2, CP3, CP1, R73+Hel (a region spanning R37 and the predicted helicase/NTP-binding domain), R69, the predicted protease (Pro), and the RNA-depended RNA polymerase (RdRP; Fig. 1; see Tables S1 and S3 for nucleotide coordinates and primer sequences used for cloning). Protein expression was verified by western blot detection in at least three replicated experiments for each MCDV protein except for the protease for which no fragment was detected (data not shown). Of the tested fragments, only R78 showed silencing suppression activity in these assays, both at 4 dpi (Fig. 1) and at 6 dpi (data not shown).

2.2. MCDV-S R78 is cleaved by the viral 3C-like protease into p51 and p27

Since previous work indicated that MCDV-S R78 is a polyprotein (Chaouch-Hamada et al., 2004), we tested R78 and R78 mutants in potential cleavage sites for proteolysis. Cleavage of R78 by the virus-encoded protease was confirmed using a wheat germ extract *in vitro* transcription/translation system (Promega, Madison, WI, USA) to co-express the predicted virus protease with the MCDV-S R78 coding