SHORT COMMUNICATION

mRNA Decapping Factors LSM1 and PAT Paralogs Are Involved in Turnip Mosaic Virus Viral Infection

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Turnip mosaic virus is a devastating potyvirus infecting many economically important brassica crops. In response to this, the plant host engages its RNA silencing machinery, involving AGO proteins, as a prominent strategy to restrain turnip mosaic virus (TuMV) infection. It has also been shown that the mRNA decay components DCP2 and VCS partake in viral infection suppression. Here, we report that the mRNA decapping components LSM1, PAT1, PATH1, and PATH2 are essential for TuMV infection. More specifically, *lsm1a/lsm1b* double mutants and *pat1/path1/path2* triple mutants in *summ2* background exhibit resistance to TuMV. Concurrently, we observed that TuMV interferes with the decapping function of LSM1 and PAT proteins as the mRNA-decay target genes *UGT87A2* and *ASL9* accumulate during TuMV infection. Moreover, as TuMV coat protein can be specifically found in complexes with PAT proteins but not LSM1, this suggests that TuMV “hijacks” decapping components via PAT proteins to support viral infection.

Keywords: LSM1, mRNA decapping, PAT paralogs, *Turnip mosaic virus*

Turnip mosaic virus belongs to the *Potyvirus* genus, which accounts for over 12.5% of all viruses known to infect plant species and causes significant losses in many crops (Fauquet and Fargette 2005; Wylie et al. 2017). The turnip mosaic virus (TuMV) genome is a single positive RNA strand of 9,830 nt covalently linked to VPg (viral genome-linked protein) in its 5' end and contains a 3'-poly(A) tail. The genome encodes two polyproteins processed into 11 mature proteins: P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIa, NIb, and CP (coat protein) (Olsper et al. 2015; Rodamilans et al. 2015; Urcuqui-Inchima et al. 2001; White, 2015).

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Plant RNA silencing is the main antiviral process triggered by endogenous or exogenous double-stranded (ds)RNA, which is processed by DCLs (Dicer-like) into 21- to 24-nt small interfering (si)RNAs (Ding and Voinnet 2007; Pumplin and Voinnet 2013). These siRNAs subsequently associate with AGO proteins to form the RISC (RNA-induced silencing complex), which mediates degradation of viral RNA (Garcia-Ruiz et al. 2010, 2015; Qu et al. 2008). Consequently, large amounts of siRNAs derived from viral RNA can be detected in infected plants (Ding and Voinnet 2007). In response, TuMV has evolved silencing suppressors such as HCPro and VPg to interfere with host antiviral silencing. HCPro suppresses silencing by binding siRNAs and inhibiting DCL2 and DCL4 functions (Lin et al. 2007; Kung et al. 2014; Poque et al. 2018). Deletion of HCPro renders TuMV noninfectious but its infectivity is restored in RNA silencing-deficient mutants such as *dcl2* and *dcl4* (Garcia-Ruiz et al. 2010). On the other hand, VPg promotes autophagic and proteasomal degradation of SGS3 (Suppressor of Gene Silencing 3), a key component of the RNA silencing response (Cheng and Wang 2017).

In addition to RNA silencing, mRNA decay also regulates mRNA turnover. mRNA decay and RNA silencing are thought to be antagonistic processes that may compete for RNA substrates (Li and Wang 2018). mRNA decay is initiated by deadenylation, removal of the 3'-poly(A) tail, and degradation either via 3'-5' exosomal exonucleases or via the 5'-3' exoribonuclease activity of the decapping complex (Garneau et al. 2007). mRNA decapping and 5'-3' decay occur in distinct cytoplasmic foci, termed processing bodies (PB). The decapping complex includes the decapping enzymes DCP1 (Decapping 1) and DCP2, which form a heterodimer and remove the cap along with other decapping components, including DCP5, DHH1 (DEXD/H-box RNA helicase), VCS (Varicose), LSM1-7 (Sm-Like 1-7) heptameric complex, PAT1 (Protein Associated with Topoisomerase II 1) and exoribonuclease XRN4 (Garneau et al. 2007; Xu and Chua 2011). The well-conserved protein PAT1 promotes PB assembly and activates decapping by binding mRNA and recruiting other factors including DHH1, the LSM1-7 complex, DCP5, VCS, DCP1/2, and XRN1 (Charenton et al. 2017; Chowdhury et al. 2014; Ozgur et al. 2010; Pilkington and Parker 2008). It has been shown that *dcp2* mutants exhibit an enhanced RNA-silencing phenotype and this can be restored by TuMV infection; this indicates that mRNA decay affects TuMV infection (Thran et al. 2012). A recent study found that VPg interferes with the interaction between DCP1 and DCP2 by switching DCP2 localization from cytoplasmic to nuclear and that HCPro interacts with XRN4 and suppresses its activity (Li and Wang 2018). While *dcp2* mutants exhibit susceptibility to TuMV (Li and Wang 2018), *xrn4* mutants exhibit either resistance or susceptibility to TuMV in different conditions (Li and Wang 2018; Vogel et al. 2011). VCS was found to be an essential component of potyvirus potato

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virus A (PVA)-induced RNA granules, such that silencing *VCS* in host plants leads to resistance to PVA (Hafren et al. 2015). All these findings indicate that mRNA decay components play different roles during TuMV infection. Nothing is known about the role of other mRNA decapping components, such as PAT proteins and LSM1 during TuMV infection. Intriguingly, yeast PAT1, LSM1-7, and DHH1 are required for brome mosaic virus (BMV) protein translation and RNA replication (Jungfleisch et al. 2015) and Ty1 retrotransposition during assembly of retrotransposition-competent virus-like particles (Checkley et al. 2010). Depletion of human PATL1 and LSM1 directly and specifically inhibits hepatitis C virus (HCV) RNA translation and replication (Scheller et al. 2009). This suggests that mRNA decapping components PAT and LSM1 may have a similar function during potyviral infection in *Arabidopsis*.

In *Arabidopsis*, *LSM1* is duplicated and codes for the pair of functionally redundant proteins LSM1A and LSM1B. *Arabidopsis* also encodes three PAT paralogs, PAT1, PATH1, and PATH2. PAT1 is under surveillance by the resistance protein SUMM2 (Suppressor of MKK1 MKK2 2), and loss of PAT1 function activates SUMM2 and triggers autoimmunity (Roux et al. 2015; Zhang et al. 2012). Thus, PAT paralog functions are best studied in a *summ2* mutant background (Roux et al. 2015; Zhang et al. 2012). In this context, we recently reported that all three PAT paralogs target *ASL9* to enable apical hook and lateral root formation (Zuo et al. 2019) and that PAT1 also functions in response to the phytohormone abscisic acid (ABA) and osmotic stress, being involved in the decapping of the canonical ABA responsive gene *COR15A* (Zuo et al. 2021). Here, we report that all three PAT paralogs and LSM1 are needed for TuMV infection. TuMV triggers accumulation of PAT1, LSM1 and their decapping target mRNAs, which indicates that TuMV alters or inhibits the function of mRNA decapping components. We also detected PAT proteins, but not LSM1, in complex with TuMV CP protein. These data indicates that PAT proteins are hijacked by CP to interfere with PAT and LSM1 processes to support viral replication.

While *dcp2* mutants exhibit increased susceptibility and *vcs* mutants increased resistance upon TuMV infection, *xrn4* susceptibility seems conditional (Hafren et al. 2015; Li and Wang 2018; Vogel et al. 2011). To evaluate if other decapping components are also involved in potyvirus infection, we infected *lsm1a/lsm1b*, *dhh1*, and *pat1/path1/path2/summ2* mutants with the infectious clone pCB-TuMV-GFP (Garcia-Ruiz et al. 2010). Since PAT1 is under SUMM2 surveillance, we generated *pat* single, double, and triple mutants in *summ2* backgrounds for further studies (Roux et al. 2015; Zuo et al. 2019). We used *elf4e* as a negative control since eIF4E is required for potyvirus translation initiation (Duprat et al. 2002). Detection of TuMV CP using anti-TuMV CP in protein isolated from systemic infected leaves revealed less TuMV CP in *lsm1a/lsm1b* compared with *Arabidopsis thaliana* ecotype Columbia (Col-0) and *dhh1* 14 days postinfection (dpi) (Fig. 1A). Mutants with all three PAT genes knocked out also had much less TuMV CP compared with single and double *pat* mutants, which had TuMV CP levels similar to Col-0 and *summ2* (Fig. 1B). To better visualize the viral infection, we also examined and quantified the green fluorescent protein (GFP) fluorescence in *lsm1a/lsm1b* and the complementation line *c-lsm1a* (Perea-Resa et al. 2016) (Supplementary Fig. S1A and B). While GFP could be detected in local infected leaves, we barely observed any GFP signal in *lsm1a/lsm1b* systemic leaves. However, massive GFP signal was detected in *c-lsm1a* systemic leaves, similar to levels observed in Col-0 leaves (Supplementary Fig. S1A and B). Unlike TuMV CP immunoblotting analysis in which *pat* single and double mutants showed no difference from *summ2*, *pat1* single and *pat*

double mutants exhibited less GFP signal in systemic leaves than *summ2*, while the *pat* triple mutant only exhibited GFP signal in local leaves (Supplementary Fig. S1C and D). This indicates that the three PAT proteins are functionally redundant and additive during TuMV infection. Together, these findings suggest that decapping components LSM1 and PAT paralogs function differently from DCP2 and DHH1 but similarly to VCS, which is needed for viral infection.

To evaluate if *LSM1* and *PAT* genes are quantitative determinants of TuMV infection severity over time, we infected *lsm1a/lsm1b*, *pat* single, double, and triple mutants with TuMV and checked viral accumulation at 28 dpi. We observed lighter disease symptoms, including less stunting and decelerated leaf senescence, in *pat* single and double mutants than in Col-0 and *summ2*, while *pat* triple mutants appeared almost unaffected (Fig. 2A and B; Supplementary Fig. S2A and B). Double mutant *lsm1a/lsm1b* also showed mild symptoms, while *c-lsm1a* exhibited disease symptoms similar to Col-0 (Fig. 2A and C; Supplementary Fig. S2C). TuMV CP accumulation revealed that *pat* single, double, and triple mutants all had increased resistance to TuMV compared with Col-0 and *summ2* (Supplementary Fig. S2A). These data confirms that the PAT proteins function in TuMV propagation in an additive way and the quantity of PAT proteins affects viral accumulation (Fig. 2; Supplementary Fig. S2).

Since LSM1 and PAT are needed for TuMV infection, we examined whether their protein levels were affected by TuMV.

Fig. 1. mRNA decay mutants *lsm1a/lsm1b* and *pat1/path1/path2/summ2* exhibit resistance to turnip mosaic virus (TuMV). **A**, Quantification of viral coat protein (CP) levels in Col-0, *elf4e*, *lsm1a/lsm1b*, *dhh1*, and *c-lsm1a* and **B**, *summ2*, *pat1/summ2*, *path1/summ2*, *path2/summ2*, *pat1/path1/summ2*, *pat1/path2/summ2*, *path1/path2/summ2*, and *pat1/path1/path2/summ2* at 14 days postinfection (dpi). All values represent means ± standard error, *n* = 9. The experiments were repeated three times. The amido black staining (A) and Ponceau S staining (B) of Rubisco serve as a loading control (LC).

We infected transgenic plants expressing LSM1-GFP and Venus-PAT1 driven by their own promoters in *lsm1a/lsm1b* or *pat1* mutant backgrounds. We detected more LSM1 and PAT1 accumulation in TuMV-infected plants (Fig. 3A). Given the function of PAT and LSM1 proteins in mRNA decay, it is possible that viral RNA degradation is affected in *lsm1a/lsm1b* and

pat1/path1/path2/summ2 mutants. To test this, we blocked transcription using cordycepin and examined the degradation rate of viral RNA in these lines, but only small differences were observed in respect to Col-0 (Supplementary Fig. S3). To study the possibility that the degradation of the mRNA decay targets is disrupted upon TuMV infection, we checked the expression

Fig. 2. The *pat* single, double, and triple mutants and *lsm1a/lsm1b* are resistant to turnip mosaic virus (TuMV) in longer terms. **A**, Virus-induced symptoms and **B**, plant growth rate in *pat* single double and triple mutants and **C**, *lsm1a/lsm1b*, *c-lsm1a* and control plants at 28 days postinfection (dpi). Scale bar in **A** = 2 cm. All values represent means $\pm$ standard deviation, $n = 3$. Bars marked with the same letter are not significantly different from each other (P value > 0.05).

Fig. 3. Turnip mosaic virus (TuMV) interferes with the mRNA decay function of LSM1 and PAT1. **A**, Immunoblot analysis of LSM1 and PAT1 accumulation in *c-lsm1a* and *pat1/Venus-PAT1* plants at 14 days postinfection (dpi) with TuMV. Anti-GFP (green fluorescent protein) bands indicate accumulation of Venus-PAT1 (upper panel) and LSM1-GFP (lower panel), the amido black staining of Rubisco serves as a loading control (LC). Venus-PAT1 and LSM1-GFP signals in negative control (NC) plants are normalized to 1, respectively. **B**, *UTG87A2* and *ASL9* accumulated in Col-0 at 14 dpi and **C**, 28 dpi with TuMV. Quantitative PCR was used to check transcript levels; error bars indicate standard error ($n = 3$). Bars marked with two asterisks (**) are statistically significant (P value < 0.01) and bars marked with three asterisks (***) are statistically extremely significant (P value < 0.001). **D**, Viral RNA accumulation serves as infection marker 14 dpi and **E**, 28 dpi separately.

level of mRNA decapping target genes *UGT87A2* and *ASL9* at 14 and 28 dpi in TuMV-infected plants. Surprisingly, both *UGT87A2* and *ASL9* transcripts accumulated in TuMV infected plants at these two timepoints (Fig. 3B, C, D, and E), indicating that the function of PAT and LSM1 in mRNA decay were altered or inhibited by TuMV infection.

PAT1 and LSM1-7 are required for BMV and HCV RNA replication and translation in yeast and human cells and Ty1 retrotransposition during assembly of retrotransposition-competent virus-like particles (Checkley et al. 2010; Jungfleisch et al. 2015; Scheller et al. 2009). To investigate if LSM1 and PAT paralogs are involved in processes related to CP, such as viral assembly, we transiently expressed hemagglutinin (HA)-tagged PAT proteins PAT1-HA, PATH1-HA, PATH2-HA, and LSM1-HA with a TuMV infectious clone and performed coimmunoprecipitation assays. TuMV CP could be detected in all three PAT-HA immunoprecipitates but not the LSM1-HA or SUMM2-HA (negative control) immunoprecipitates (Fig. 4), indicating that PATs are directly targeted by TuMV CP (e.g., for processes such as viral encapsidation) while LSM1 is targeted indirectly, possibly through PAT paralogs.

LSM1-7 and PAT1 form complexes and promote mRNA decapping together with other decapping components (Tharun and Parker 2001; Tharun et al. 2000). As *Arabidopsis* PAT paralogs PAT1, PATH1, and PATH2 have all been shown to form complexes with LSM1, it is not surprising that *lsm1a/lsm1b* and *pat* triple mutants exhibit similar resistance phenotypes to TuMV infection (Figs. 1 and 2; Supplementary Figs. S1 and S2). Since TuMV interferes with mRNA decay (Fig. 3) and we detected

PATs in complexes with TuMV CP (Fig. 4), it is likely that TuMV hijacks PAT proteins for processes such as viral assembly.

Although *lsm1a/lsm1b* and *pat* triple mutants accumulate some TuMV CP compared with *eif4e*, all three genotypes exhibit resistance to TuMV (Fig. 1). Since PAT1 has been found to interact with eIF4E (Drummond et al. 2011; Wyers et al. 2000), which is targeted by TuMV for viral protein translation, it is possible that PATs and LSM1 function through eIF4E in viral infection. In any event, our results show that LSM1 and the PAT proteins, similarly to eIF4, are required for TuMV infection.

Given the function of PAT proteins and LSM1 in mRNA decay, the accumulation of target genes due to a nonfunctional mRNA decay machinery in *pat* triple and *lsm1a/lsm1b* mutants could affect TuMV infection. However, accumulation of *UGT87A2* and *ASL9* transcripts upon TuMV infection in Col-0 indicates mRNA decay is compromised by TuMV (Fig. 3). Importantly, viral TuMV RNA is not degraded via the mRNA decay machinery, since the viral RNA degradation rate in *pat* triple and *lsm1a/lsm1b* mutants was similar to the one observed for Col-0 (Supplementary Fig. S3). This indicates that PAT proteins and LSM1 are recruited for other processes during viral infection. Human PATL1 and LSM1 relocate from PBs to replication complexes upon HCV infection and are essential for translation and RNA replication (Scheller et al. 2009). Plant PAT and LSM1 proteins might also play similar roles during potyvirus infection. Nevertheless, only PAT proteins but not LSM1 are detected together with TuMV CP (Fig. 4), this suggests that LSM1 recruitment by TuMV might be indirect and possibly mediated by the PAT proteins.

In summary, we found that the mRNA decapping components LSM1 and PATs are essential for TuMV infection, which is in marked contrast to previously described functions for DCP2 and DHH1. Finally, we show that TuMV hijacks the host decapping machinery by using its CP to recruit PATs, probably to assist with viral assembly.

Col-0 was used as a control. T-DNA insertion lines At1g79090 (PAT1) *pat1* (Salk_040660), At1g12280 (SUMM2) *summ2-8* (SAIL_1152A06), double mutants of PAT1 and SUMM2 *pat1/summ2* and of LSM1A and LSM1B *lsm1a/lsm1b*, CRISPR mutants *path1/summ2*, *pat1/path2/summ2*, *path1/path2/summ2*, and *pat1/path1/path2/summ2*, and the transgenic plants *c-lsm1a* (*lsm1a/lsm1b*/LSM1A-GFP) and *pat1/Venus-PAT1* have been described (Perea-Resea et al. 2016; Roux et al. 2015; Zhang et al. 2012; Zuo et al. 2019). The T-DNA insertion line for At3g61240 (DHH1) *dhh1* (Salk_016921) is from Nottingham *Arabidopsis* Stock Centre (U.K.), and primers for genotyping are listed in Supplementary Table S1. Plants were grown in 9 × 9-cm pots at 21°C with 8 h of light and 16 h of dark.

The infectious pCB-TuMV-GFP clone in *Agrobacterium tumefaciens* used in this study has been described (Garcia-Ruiz et al. 2010). Viral particles purified from TuMV-GFP (Garcia-Ruiz et al. 2010) infected *Nicotiana benthamiana* plants were mechanically rubbed on the first true leaves of 3-week-old *Arabidopsis* plants using a sponge and spatula with Carborundum. Infected plants were identified by visual inspection of local leaf GFP signal, typical disease symptoms, and by quantification of TuMV CP from new emerging leaves with anti-CP antibodies.

Viral RNA transcription was blocked by cordycepin treatment as previously described (Xu and Chua 2009). The 14-dpi TuMV-infected plants were incubated in liquid phosphate buffered saline (PBS) for 2 h, then, the same amount of PBS or PBS + 1 mM cordycepin (Sigma-Aldrich) were added and incubated for 1 hr or 3 hr before collected for RNA extraction.

A. tumefaciens strains carrying HA-tagged PAT proteins, LSM1 and SUMM2 fusions, and pCB-TuMV-GFP were grown in yeast-extract peptone medium supplemented with appropriate antibiotics overnight. Cells were centrifuged and were resuspended in

Fig. 4. PATs are in complexes with turnip mosaic virus (TuMV) coat protein (CP). Coimmunoprecipitation between the hemagglutinin (HA)-tagged PAT proteins LSM1, SUMM2, and TuMV CP. *Agrobacteria* carrying PAT1-HA, PATH1-HA, PATH2-HA, LSM1-HA, and SUMM2-HA were coinfiltrated with the infectious pCB-TuMV-GFP clone in *Nicotiana benthamiana* and tissue was harvested 3 days postinfiltration. Immunoblots of inputs (lower panels) and HA immunoprecipitates (upper panels) were probed with anti-HA antibodies and anti-TuMV CP antibodies.

buffer (10 mM MgCl₂, 10 mM MES-K (pH 5.6), and 100 μM acetosyringone) to an optical density at 600 nm = 0.8. *A. tumefaciens* strains carrying pCB-TuMV-GFP and PAT1-HA, PATH1-HA, and PATH2-HA were mixed 1:1 and were infiltrated into 3-week-old *N. benthamiana* leaves. Leaf samples for protein extraction and immunoprecipitation were collected 3 days post-infiltration. Tissues for extraction were ground in liquid nitrogen and immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% [vol/vol] NP40, 10 mM dithiothreitol, 5% [vol/vol] glycerol, 1 mM EDTA, protease inhibitor cocktail [Roche], Phosstop [Roche]) was added at 2 ml per gram of tissue powder. Following a 20-min centrifugation at 4°C and 13,000 rpm using an Eppendorf Centrifuge 5417R, sample supernatants were adjusted to 2 mg of protein per milliliter and were incubated 4 h at 4°C with monoclonal anti-HA agarose antibody (Sigma). Beads were washed four times with wash buffer (20 mM Tris, pH 7.5, 150 M NaCl, 0.1% [vol/vol] NP40 before adding 4× sodium dodecyl sulfate [SDS] buffer [Novex]) and were denatured by heating at 95°C for 5 min.

Tissue samples were ground in liquid nitrogen and 4× SDS buffer was then added and samples were heated at 95°C for 5 min, were cooled to room temperature for 10 min, were centrifuged 5 min at 13,000 rpm using the Eppendorf Centrifuge 5417R, and supernatants were separated on 10% SDS-polyacrylamide gel electrophoresis gels, were electroblotted onto polyvinylidene difluoride membrane (GE Healthcare), were blocked in 5% (wt/vol) bovine serum albumin in TBS-Tween 20 (TBS-T; 0.1%, vol/vol), and were incubated for 1 h to overnight with primary antibody (anti-TuMV CP 1:1,000, anti-HA 1:1,000). Membranes were washed 3× for 10 min in TBS-T (0.1%) before 1 h of incubation in secondary antibodies (anti-rabbit or -mouse horseradish peroxidase or ammonium phosphate [AP] conjugate [Promega], 1:5,000). Chemiluminescent substrate (ECL Plus [Pierce]) was applied before camera detection. For AP-conjugated primary antibodies, membranes were incubated in NBT/BCIP (Roche) until bands were visible.

Total RNA was extracted with TRIzol Reagent (Thermo Scientific), 1 μg of total RNA were treated with DNase I (Thermo Scientific) and were reverse-transcribed into cDNA using a RevertAid first strand cDNA synthesis kit (Thermo Scientific). The *ACT2* gene was used as an internal control. Quantitative PCR assays with SYBR Green master mix were performed on a Bio-RAD CFX96 system (Thermo Scientific). Primers are listed in Supplementary Table S1. All experiments were performed at least three times each in technical triplicates.

Statistical details of experiments are reported in the figures and captions. Statistical significance between groups was determined by one-way analysis of variance followed by Duncan's new multiple range test and Tukey's honest significance test with Systat software.

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