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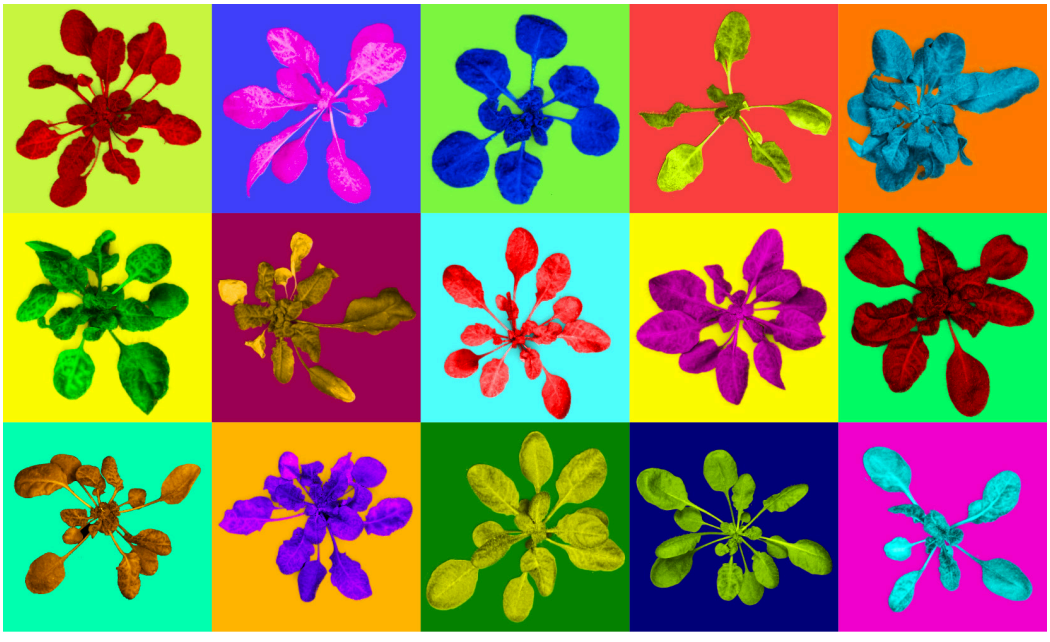
Stress & Granules

Regulation of *Cauliflower mosaic virus* disease in *Arabidopsis thaliana*

Gesa Hoffmann



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SWEDISH UNIVERSITY
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Cover: Arabidopsis accessions photographed 21 days after infection with CaMV, false-coloured.
(Images: Gesa Hoffmann)

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Regulation of *Cauliflower mosaic virus* disease in *Arabidopsis thaliana*

Abstract

Viruses exist in intimate relationships with the organisms they are infecting and are just as dependent on compatibility with their host's cellular components as they are on subverting them. During any virus infection, the cells are flooded with foreign nucleic acids (DNA and/or RNA) and have learned to recognize, disarm, or eliminate them. In turn, viruses have evolved to use the cellular transcription and RNA regulatory machinery for their own benefit. Cytoplasmic RNA granules, namely Processing bodies (PBs) and Stress granules (SGs) are at the forefront of RNA regulation as they contain and store untranslated RNA and are responsive in number, size, and composition to various stresses, including virus infection. For this thesis, we have explored the role of *Arabidopsis thaliana* RNA granules during infection with the pararetrovirus *Cauliflower mosaic virus* (CaMV). We show that PB components aid virus accumulation through shielding of the viral RNA from the antiviral RNA silencing machinery (Paper I). In addition, we find that the cytoplasmic viral replication factory contains several RNA granule proteins during infection, including both, PB components and SG components. Opposite to PBs, SGs are likely antiviral and CaMV subverts their biogenesis through its multifunctional protein P6 (Paper II). In an effort to uncover novel disease determinants, we explore the variation of CaMV disease in naturally occurring populations of *Arabidopsis thaliana* and uncover the importance of the plant hormone abscisic acid and its homeostasis for CaMV infection, as well as a novel CaMV susceptibility factor, the ABA synthesis gene NCED9 (Paper III).

Keywords: CaMV, Arabidopsis, Processing body, Stress granule, viral factory, RNA regulation, natural variation, ABA, NCED9

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Regulierung der Blumenkohl Mosaik Virus Krankheit in *Arabidopsis thaliana*

Zusammenfassung

Viren sind eng verbunden mit ihren Wirtsorganismen und sind ebenso abhängig von der Kompatibilität mit zellulären Komponenten ihrer Wirte, wie davon, eben diese zu untergraben. Bei jeder Virusinfektion werden Zellen mit fremden Nukleinsäuren (DNA und/oder RNA) überschwemmt und haben gelernt, fremde RNAs zu erkennen, unschädlich zu machen oder zu eliminieren. Viren sind evolviert, um die zelluläre RNA-Regulationsmaschinerie zu ihrem eigenen Vorteil nutzen können. Zytoplasmatische RNA-Körperchen, die *Processing bodies* (PBs) und *Stress granules* (SGs), stehen an vorderster Front der RNA-Regulation, da sie nicht-translatierende RNA enthalten und speichern und in Anzahl, Größe und Zusammensetzung auf verschiedene Belastungen, einschließlich Virusinfektionen, reagieren. Für diese Thesis haben wir die Rolle von RNA-Körperchen in *Arabidopsis thaliana* während einer Infektion mit dem Pararetrovirus Blumenkohl Mosaik Virus (CaMV) untersucht. Wir zeigen, dass PB-Komponenten die Virus-Akkumulation unterstützen, indem sie die virale RNA von der antiviralen RNA-Interferenz Maschinerie abschirmen (Papier I). Außerdem beobachten wir, dass die zytoplasmatische virale Replikationsfabrik während der Infektion mehrere RNA-Körperchen Proteine enthält, einschließlich PB-Komponenten und SG-Komponenten. Im Gegensatz zu PBs sind SGs wahrscheinlich antiviral und CaMV untergräbt ihre Biogenese durch sein multifunktionales Protein P6 (Paper II). In dem Bemühen, neue Krankheitsdeterminanten aufzudecken, untersuchen wir die Variation der Blumenkohl Mosaik Krankheit in natürlich vorkommenden Populationen von *Arabidopsis thaliana* und decken die Bedeutung des Pflanzenhormons Abscisin Säure und seiner Homöostase für die CaMV-Infektion auf. Schließlich, beschreiben wir das ABA Synthese Gen *NCED9* als neuen CaMV-Anfälligkeitsfaktor (Papier III).

Schlagwörter: CaMV, *Arabidopsis thaliana*, Processing body, Stress granule, Virus Fabrik, RNA-Regulation, natürliche Varianz, ABA, NCED9

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Reglering av blomkålsmosaikvirussjukdom i *Arabidopsis thaliana*

Abstrakt

Virus är nära knutna till organismer de infekterar, starkt beroende av att vara kompatibla med flera av värdcellens komponenter och av att kunna underminera dessa. Under en virusinfektion översvämmas värdcellen med främmande nukleinsyror och cellen har lärt sig att känna igen, avväpna eller eliminera dessa. I sin tur har virus anpassat sig till att använda cellens RNA-reglerande maskineri till sin egen fördel. Cytoplasmatiska RNA-granuler, så kallade Processing bodies (PBs) och Stress-granules (SGs), ingår i cellens initiala RNA-reglering eftersom de innehåller och lagrar oöversatt RNA och anpassar sig i antal, storlek och sammansättning för olika påfrestningar, som exempelvis en virusinfektion. I denna avhandling har vi utforskat *Arabidopsis thaliana* RNA-granuler roll under en infektion av pararetroviruset Blomkålsmosaikvirus (CaMV). Vi visar att cellens PB-komponenter hjälper viruset att ackumuleras genom att skydda det virala RNA från cellens antivirala RNA-tystnadsmaskineri (Paper I). Dessutom visar vi att den cytoplasmatiska virala replikationsfabriken innehåller flera RNA-granulatproteiner under en infektion, inklusive PB-komponenter och SG-komponenter. I motsats till PBs är SGs troligen antivirala och CaMV undergräver deras biogenes genom sitt multifunktionella protein P6 (Paper II). I ett försök att hitta nya faktorer som påverkar risken för sjukdom utforskar vi variationen av CaMV-sjukdom i naturligt förekommande populationer av *Arabidopsis thaliana.* Vi påvisar också vikten av växthormonet abscisinsyra (ABA) och dess homeostas för CaMV-infektion, och hittar en ny CaMV-mottaglighetsfaktor, ABA-syntesgen NCED9 (Paper III).

Nyckelord: CaMV, *Arabidopsis thaliana*, Processing body, Stress granule, virala replikationsfabriken, RNA-reglering, naturlig variation, ABA, NCED9

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Dedication

For Mimi and Opa, who loved love.

For Amir Taller, who loved science.

"Why sometimes I've believed
as many as six impossible things
before breakfast"

Alice in Wonderland **– *Lewis Caroll***

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Hoffmann, G., Mahboubi, A., Bente, H., Garcia, D., Hanson, J. & Hafrén, A. (2022).** Arabidopsis RNA processing body components LSM1 and DCP5 aid in the evasion of translational repression during Cauliflower mosaic virus infection. *The Plant Cell*

II. **Hoffmann, G., López-González, S., Mahboubi, A., Hanson, J. & Hafrén, A. (2022).** *Cauliflower mosaic virus* protein P6 forms a microenvironment for RNA granule proteins and interferes with stress granule responses. *BioRxiv, in revision*

III. **Hoffmann, G., Shukla, A., López-González, S., Hafrén, A. (2022).** *Cauliflower mosaic virus* disease spectrum uncovers novel susceptibility factor NCED9 in *Arabidopsis thaliana*. *BioRxiv, submitted*

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The contribution of Gesa Hoffmann to the papers included in this thesis was as follows:

I. Designed and performed experiments, analysed data, and generated figures. Wrote the manuscript and completed revisions.

II. Designed and performed experiments, analysed data, and generated figures. Wrote the manuscript.

III. Designed and performed experiments, analysed data, and generated figures. Wrote the manuscript and acquired partial funding.

The following papers were written during my doctoral thesis but are not part of the present dissertation:

IV. **Shukla, A., López-González, S., Hoffmann, G., & Hafrén, A. (2019)**. Diverse plant viruses: a toolbox for dissection of cellular pathways. *Journal of experimental botany*, *70*(12), 3029-3034. https://doi.org/10.1093/jxb/erz122

V. **Bruessow, F., Bautor, J., Hoffmann, G., Yildiz, I., Zeier, J., & Parker, J. E. (2021).** Natural variation in temperature-modulated immunity uncovers transcription factor bHLH059 as a thermoresponsive regulator in Arabidopsis thaliana. *PLoS genetics*, *17*(1), e1009290 https://doi.org/10.1371/journal.pgen.1009290

VI. **Shukla, A., Hoffmann, G., Hofius, D., & Hafrén, A. (2021).** Turnip crinkle virus targets host ATG8 proteins to attenuate antiviral autophagy. *BioRxiv* https://doi.org/10.1101/2021.03.28.437395

VII. **Shukla, A., Hoffmann, G., Kushwaha, N. K., López-González, S., Hofius, D., & Hafrén, A. (2022).** Salicylic acid and the viral virulence factor 2b regulate the divergent roles of autophagy during cucumber mosaic virus infection. *Autophagy*, *18*(6), 1450-1462. https://doi.org/10.1080/15548627.2021.1987674

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1. Introduction

Nothing in biology makes sense except in the light of interactions. This dogma holds true from the largest ecosystems to the smallest molecules. Every organism is intricately nested within a web of interactors, systems and (a)biotic factors influencing its existence. In biology, every interaction is a give-and-take, and the application of economic game theory and social evolution can explain many interaction outcomes and how they became established (McNamara & Leimar, 2020). Yet, on all scales, there are organisms that seemingly take a lot more than they give (that cheat the game): parasites and pathogens (Friesen, 2020).

Viruses are among the smallest pathogens known to date and were the last of the pathogenic microbes to be described in the end of the 19th century independently by Dmitri Ivanovsky and Martinus Beijerinck (Ivanovsky, 1902; Beijerinck, 1898; Ivanovsky, 1892), but they are fundamentally different from bacteria, fungi, or oomycetes. Viruses are non-cellular, consisting of a genomic nucleic acid (DNA or RNA) wrapped in a proteinaceous shell. Unique in the total reliance on their hosts' cellular machinery for their replication, they are not recognized as living organisms by all. Viruses are ubiquitous and can be found wherever there are suitable hosts (i.e., any cellular organism). With an estimated 10^{31} viral particles on earth, they exceed the number of stars in the universe (Microbiology by numbers, 2011) and are ten times more numerous than all cells combined (Mushegian, 2020). To date, about 12.000 virus species have been classified, more than 2000 of which are plant viruses, but the reservoir of undiscovered virus diversity is likely to be several magnitudes higher (Koonin *et al.,* 2022). As viruses can virtually infect any cellular organism, they have profound impacts on life history and ecosystems.

They are perhaps the most naturalized pathogens in our everyday life. From computer viruses to "going viral", from viral-geometry inspired architecture to arts and literature – viruses are omnipresent, albeit mostly associated with disease and destruction. Viral pandemics are among the deadliest disasters in human history, with the ongoing Coronavirus pandemic being a powerful example of how devastating newly emerging viruses can be (Piret & Boivin, 2021). But viruses have shaped our world and our history beyond disease. In the oceans, they are the major mortality factor for microorganisms, including harmful cyanobacteria, and as such reduce the risk of phytoplankton blooms, essentially keeping our oceans blue (Danovaro *et al*., 2011). On land, some of the most sought-after ornamental plant varieties were once caused by plant virus infections (Valverde et al., 2012). In the golden age of the Netherlands several beautiful, variegated varieties of tulips were created, which caused the Tulipomania, a speculation bubble around these unpredictable tulips. Unbeknownst to the people then, the beautiful flower patterns were caused by *tulip breaking virus* (TBV) which also led to the demise of the germline in the long run and the bursting of the bubble, throwing the Netherlands into a national economic crisis (Dash, 2011).
Despite being the first viruses to be discovered, sequenced, and crystallized, our knowledge about plant viruses compared to their animal- and bacteria infecting counterparts is limited.

1.1 Plant viruses – agricultural threats and evolutionary drivers

Plant viruses are ubiquitous in wild and cultivated environments and are a major cause for disease in staple crops and ornamental plants. In fact, they represent 47% of pathogens causing emerging or re-emerging plant disease epidemics worldwide, tendency rising (Anderson *et al.,* 2004). Viral disease outbreaks occur more frequently, when ecosystems are simplified (e.g. in monocultures of crop plants) and when naïve plants are introduced into new environments (Figure 1A, Roossinck & Garcia-Arenal, 2015). An especially dire example is the introduction of Cocoa trees to West Africa, where they encountered *Cocoa swollen shoot virus* (CSSV), a virus that is now the leading cause for crop losses in cocoa trees within that region. CCSV causes mild symptoms in native Malvaceae plant populations of the West African rain forests, but kills the introduced cocoa trees (Owusu *et al.,* 2010). The

only way of virus management is to eradicate diseased trees, which led to the cutting of over 200 million cocoa trees since 1946 in Ghana (Andres *et al.,* 2017). On the other hand, multinational trade and the production of crops outside their domestication centers have spread plant viruses across the globe. Historical evidence of virus spread along trade-routes is provided by *Turnip mosaic virus* (TuMV). Nowadays, TuMV is one of the most damaging viruses to Brassicaceae plants worldwide (Walsh & Jenner, 2002). It likely originated in Europe from an orchid infecting ancestral strain about 1000 years ago and subsequently spread via the silk road trade into Asia (Kawakubo *et al*., 2021). A more recent example of trade-related virus spread is the emergence of *Plum pox virus* (PPV) in North- and South America. "Sharka" disease caused by PPV infection in the genus *Prunus* has been reported in Europe and the Mediterranean region since 1917, but was first introduced to Pennsylvania in 1999, by a single shipment of infected cuttings of peach trees (Cambra *et al.*, 2006; Levy *et al.,* 2000). Drastic diagnostic, quarantine and eradication measures have prevented the spread of PPV within the US, but such measures are not always successful, as the spread of *Banana bunchy top virus* (BBTV) among banana producing countries shows (Qazi, 2016). The propagation and distribution of virus infected plant material has made humans into the "superspreaders" of plant viruses (Ranawaka *et al*., 2020), a title that was previously reserved for their native vectors. The most common plant virus transmission vectors are phloem-sucking insects like aphids, whiteflies and thrips, but nematodes, mites, fungi and zoosporic endoparasites have also been described to transmit viruses (Dietzgen *et al.,* 2016, Figure 1B). The spread of *African Cassava mosaic virus* (ACMV) for example, is closely linked with the distribution of its whitefly vector *Bemisia tabaci* and severe outbreaks of Cassava mosaic disease are often preceded by unusually large populations of whiteflies (Legg *et al.*, 2011). Apart from their plant host occurrence, the geographic range of their vectors is a major limitation for plant virus occurrence. The increasing temperatures and changing climates will likely lead to an expansion of insect vector distribution, along with an increase in their generation numbers and enhanced overwintering survival rates (Skendžić *et al*., 2021). This, together with human interference and global trade, will lead to more frequent virus disease outbreaks in the future.

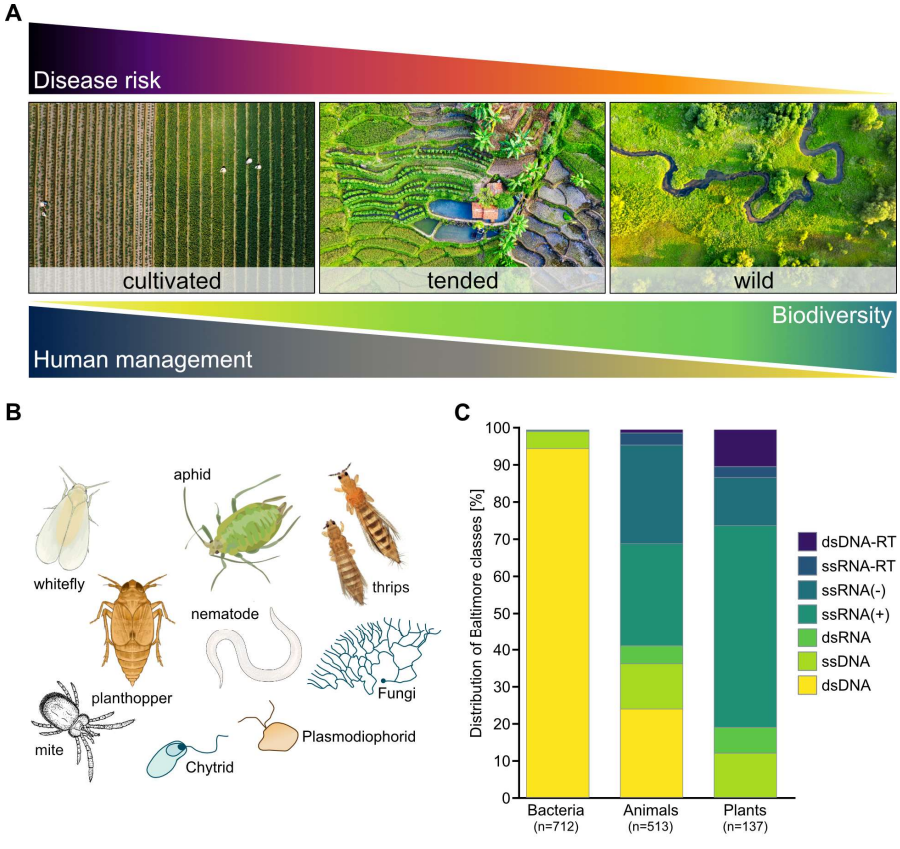

Figure 1: Plant virus risks, vectors, and genomes [A] Schematic of the relationship between habitat heterogeneity and disease risk by viruses and other pathogens. Arable lands, sown with crop plants in monoculture are highly managed landscapes with very little biodiversity, which greatly increases the risk of plant disease. Wild ecosystems, not regulated by human presence, harbour a strong biodiversity, which slows the spread of pathogens. Adapted from Roossinck & Garcia-Arenal, 2015. Images obtained from Pexels.com (CC BY 4.0). [B] Drawings of common plant virus vectors indispensable for plant virus lifecycles. Images acquired from DataBase Center for Life Science (CC BY 4.0). [C] Distribution of Baltimore classes of virus genera in bacteria, animals, and plants. Adapted from Koonin *et al.,* 2021.

We have very little knowledge and data about virus occurrence and selection pressures in wild populations and ecosystems. It can be assumed that virtually all plants encounter viruses during their lifetime and that 60-70% of plants in an environment house one or several viruses at any given time (Roossinck *et al.,* 2015). Most virus infections do not cause disease symptoms in diverse environments, but they could exert a selection pressure on their hosts (Montes *et al.,* 2019; Brosseau *et al.,* 2019). Like other pathogens, plant viruses have a defined host-range, categorizing them in specialists and generalists. Specialist viruses are generally confined to one plant family or genus, like the economically important *Citrus tristeza virus* (CTV) which is only known to infect members of the genus *Citrus*. Generalists on the other hand have likely become established in species rich environments and can infect a variety of hosts. *Cucumber mosaic virus* (CMV) is the virus with the largest host range found so far, including 1200 host species belonging to 100 plant families and likely more (Jacquemond, 2012; Watterson, 1993). Whether a virus evolves to be a generalist or specialist involves the range of its transmission vector, its interactions within the host communities and the ecological niches it can adapt to, but remains poorly understood (Lefeuvre *et al.,* 2019). The "*jack of all trades is the master of none*" maxim states that there cannot be a single genotype with the highest fitness in all environments; that generalists would bear a cost in each environment they could inhabit and would eventually be outcompeted by specialist within each (Remold, 2012). However, several animal viruses, as well as the *Tobacco etch virus* (TEV) were shown to become no-cost generalists through serial passages of different hosts, providing the first evidence that (plant) viruses can indeed evolve to become "*master of all*" in the tested conditions (Lefreuvre *et al.,* 2019; Bedhomme *et al.,* 2012).

Viruses can be classified based on their morphology, host range, genome organization or replication mechanism. But viruses with different genomes and replication strategies can adapt the same particle morphology (see Appendix, Table 3) and different species within the same family can infect humans, insects and plants, making classification based on host range unreliable (Hogenhout *et al*., 2013). A commonly used system is the Baltimore classification that categorizes viruses into seven groups based on their mode of mRNA synthesis and genome organization (Baltimore, 1971). Large differences in the distribution of Baltimore classes occur between

procaryote and eukaryote infecting viruses, as well as between animal and plant viruses (Figure 1C). While most plant virus genomes are composed of single stranded (ss)RNA, the overrepresentation of dsDNA-RT genomes compared to bacteria and animal viruses is notable (Koonin *et al.,* 2021). The *Caulimoviridae* family is the only family of plant viruses with a double-stranded genome transcribed through reverse transcription and likely shares a common ancestry with mammalian retroviruses and retrotransposons (Krupovic & Koonin, 2017). Several members of the *Caulimoviridae* cause great economic losses in the tropics, including *Banana streak virus* (BSV), *Rice tungro bacilliform virus* (RTBV) *(*in co-infections with the Waikivirus *Rice tungro spherical virus* [RTSV]), as well as the aforementioned CCSV (Geering, 2019). The typemember of this class of dsDNA-RT viruses, however, is the Caulimovirus *Cauliflower mosaic virus* (CaMV) which is the main study object of this thesis.

1.1.1 *Cauliflower mosaic virus* – a blessing in disguise

A transmissible disease in cauliflower and other cabbages was first described in the 1930s in the San Francisco Bay Area (Tompkins, 1937). Yet, CaMV likely originated in the eastern Mediterranean area 400-500 years ago and was spread through the emerging trade of broccoli and cauliflower to Japan and the US in the 19th century (Yasaka *et al.,* 2014). Since the isolation of viral particles in 1960 by the group of R. J. Shepherd (Pirone *et al.,* 1960) CaMV was found worldwide in temperate regions. It is transmitted by at least 27 aphid species (Kennedy *et al.,* 1962), but has a restricted host range to cruciferous plants and exceptionally *Nicotiana clevelandii* (Hills & Campbell, 1968) and *Datura stramomium* (Lung & Pirone, 1972). CaMV particles are icosahedral, ~50 nm in diameter and composed of 420 subunits of coat protein (Cheng *et al.,* 1992, Figure 2A).

CaMV was the first plant virus to be sequenced (Franck *et al.,* 1980). The circular genome is comprised of ~ 8000 bp and encodes for seven proteins, six of which have been detected *in planta* (Figure 2B). Upon entry into a cell's cytoplasm, the CaMV particle is targeted to the nucleus, where it releases its genome to be transcribed by the host transcriptional machinery. The CaMV genome harbors discontinuities from the reverse transcription process that first lead to the massive production of small, non-coding *8S* RNAs, which act as a decoy for the RNA silencing machinery (Blevins *et al*., 2011).

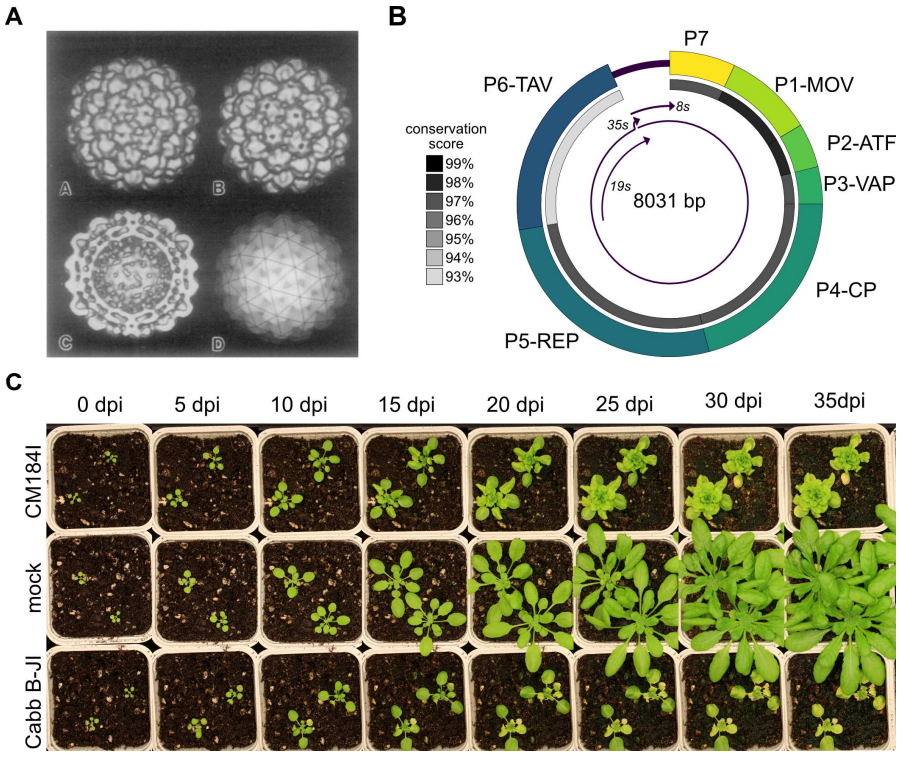

Figure 2: ***Cauliflower mosaic virus.*** [A] Surface-shaded representations of CaMV reconstructed from single particles [upper panel]. Cutaway view with the large cavity for nucleic acids is shown in the left lower panel. An icosahedral lattice net with a T7 symmetry of the coat protein is overlaid on the particle representation in the right lower panel. Reproduced from Cheng *et al.,* 1992 Figure 4. [B] CaMV genome organization, open reading frames giving rise to the seven CaMV proteins (P1-P7) are depicted as thick coloured boxes on the circular CaMV genome (based on strain CM1841, 8031 bp). DNA sequence conservation of each open reading frame was calculated from MUSCLE multiple sequence alignment (Edgar, 2004) with 100 CaMV strains and depicted within the genome organisation. The three RNA species (*8S*, *19S* and *35S*) produced from PolII transcription are shown inside the genome. Adapted from Shoelz *et al.,* 2016 [C] 35-day time course photography of *Arabidopsis thaliana* Col-0 infected with CaMV strains CM184I (upper panel) and Cabb B-JI (lower panel) or treated phosphate buffer (mock) in five-day intervals in the growth conditions used throughout this study. Dpi = days post infection. Photos: Gesa Hoffmann

The discontinuities are repaired during the transcription process, giving rise to histone-associated minichromosomes. In contrast to true retroviruses, pararetroviruses, like CaMV, do not incorporate into their hosts genome, but are solely transcribed from these numerous minichromosomes. Integration events have been described for several members though and CaMV infectious particles can be produced from a transgenic *Arabidopsis thaliana* plant containing a monomeric copy of the viral genome (Squires *et al.,* 2011; Staginnus & Richert-Pöggeler, 2006).
During infection, CaMV closed minichromosomes are transcribed by RNA polymerase II (PolII) into two terminally redundant transcripts. The *19S* RNA only encodes for the P6 protein, while the *35S* RNA serves as a polycistronic RNA for the translation of all viral proteins, as well as a template for reverse transcription of the viral genome.
CaMV has been listed among the ten most important viruses in plant molecular pathology and has shaped molecular biology research far beyond the field of virology (Scholthof *et al.,* 2011). Importantly, CaMV can infect the cruciferous model plant *Arabidopsis thaliana* (hereafter referred to as Arabidopsis), which together form a powerful tool for cell biologists and geneticists. Several strains of CaMV are being used in laboratories worldwide, including the milder strain CM1841 and the severe strain Cabb B-JI. Both strains induce visible symptoms after about 10-14 days post infection (dpi) and lead to vein clearing, stunting and early senescence (Figure 2C). CaMV research has led to many seminal discoveries, e.g., the widely used *35S* promoter, translational transactivation and ribosome shunting (Pooggin & Ryabova et al., 2018). Yet perhaps the most striking feature of CaMV biology is the CaMV P6 protein with its numerous functions, including the generation of electron-dense inclusion bodies (hereafter termed viral factories).

1.1.2 P6 – the Swiss army knife of CaMV

Plant viruses have limited coding capacity due to size restrictions in encapsidation and transport and often encode for very few proteins (Wang *et al.,* 2022). Consequently, viral proteins are often multifunctional. CaMV P6 was initially described as the most abundant protein during CaMV infection (Odell & Howell, 1980) and four decades of research on this protein have continuously expanded its known functional repertoire (Figure 3A).

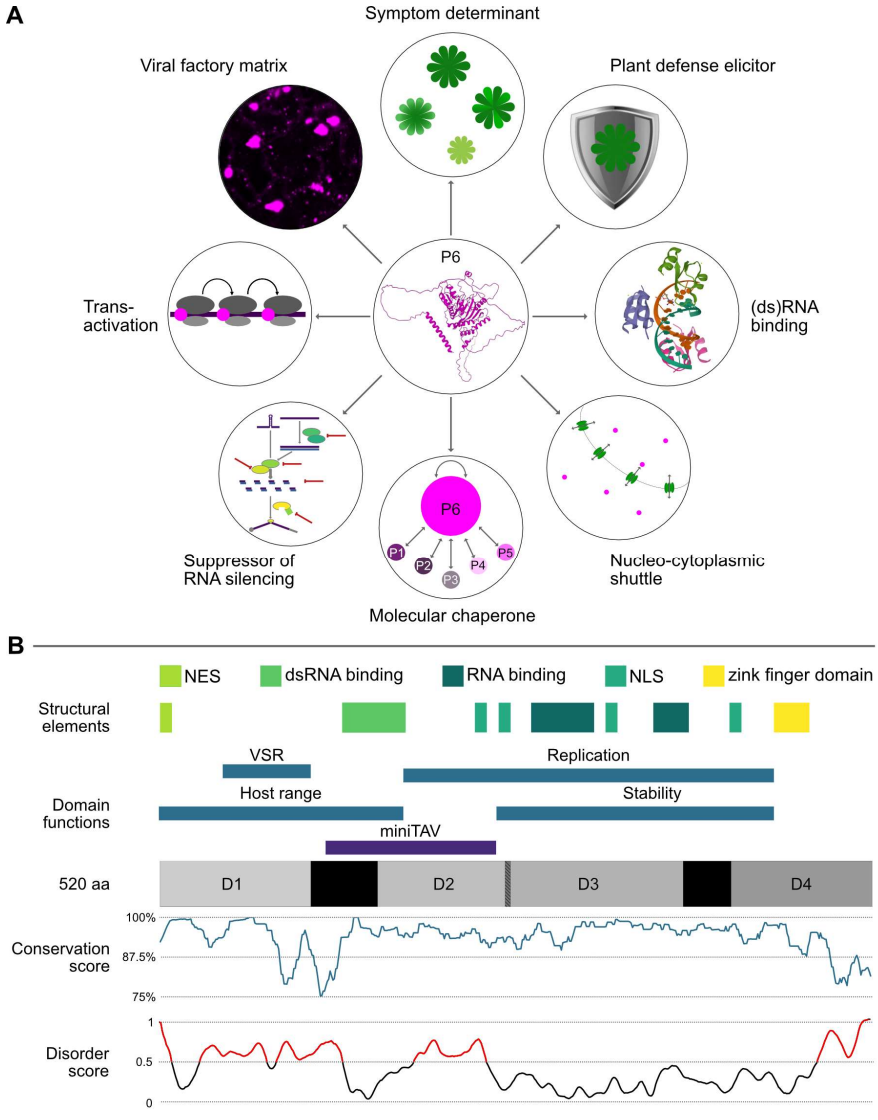

Figure 3: The CaMV P6 protein functions and structure. [A] The main functions of P6 known to date are depicted. P6 structure was modelled with Alphafold2 by Aimer Gutierrez Diaz. [B] Functional, as well as structural domains are depicted above the 520 aa of P6 with its four self-association domains (D1-D4). Protein sequence conservation of P6 was calculated from MUSCLE multiple sequence alignment (Edgar et al., 2004) with 100 CaMV P6 protein sequences from BLASTp search and depicted over the 520-aa-length with a sliding window of 15 aa below the structure. Protein disorder prediction was performed with the PrDOS server (Ishida *et al.,* 2007). predicted disordered regions are coloured in red. NES = Nuclear export signal; NLS = Nuclear localization signal. Adapted from Schoelz & Leisner, 2017.

P6 is the main symptom determinant of CaMV infections (Hull, 2002; Daubert *et al.,* 1984) and transgenic plants expressing P6 without infection can develop virus-like symptoms, like chlorosis and stunting (Yu *et al.,* 2003; Cecchini *et al.,* 1997). P6 protein is present in the nucleus and the cytoplasm and through its RNA binding capacity is proposed to act as a molecular shuttle during nuclear export of viral RNA (Haas *et al.,* 2005). P6 self-associates in an unknown process that involves at least four regions (Figure 3B, D1-D4) and in addition can bind to all other viral proteins, possibly acting as a molecular chaperone (Hapiak *et al.,* 2008; Himmelbach *et al.,* 1996). The P6 coding region is the least conserved among all ORFs on the CaMV genome (Figure 2B) and the protein harbours several regions of low conservation among the different isolate (Figure 3B), which in part accounts for the variation in disease caused by CaMV isolates (Cecchini *et al.,* 1998). It is possible that P6 is the youngest protein of CaMV (Yasaka *et al.,* 2014), a feature that P6 shares with many other viral suppressors of RNA silencing, another function of P6 that will be discussed in detail in section 1.4.2 (Laird *et al.,* 2013).

One essential task of P6 is the translational transactivation (TAV), allowing for the translation of consecutive long open reading frames (ORFs) on the viral mRNA. Usually, eukaryotic ribosomes release the small ribosomal subunit 40S upon STOP codon encounter and the loss of initiation-promoting factors during the elongation process prohibits the recruitment of a new subunit, essentially aborting the translation processes. CaMV P6 binds the translation initiating factor eIF3 via its g subunit (eIF3g) and through eIF3g binds the 40s ribosome subunit (Park *et al.,* 2001), as well as components of the large ribosomal subunit, namely eL24, eL18 and eL13 and the reinitiation supporting protein (RISP) (Thiébeauld *et al.,* 2009; Bureau *et al.,* 2004; Park *et al.,* 2001; Leh *et al.,* 2000). This P6 induced network of translation enhancing proteins leads to the stabilization of polysomes on mRNA and enables the translation reinitiation after stop codon encounter, ultimately allowing the translation of polycistronic *35S* RNA (Pooggin & Ryabova, 2018). The TAV function is essential for CaMV replication and governed by the translational regulator Target of Rapamycin (TOR), which is also bound by P6 through a domain located within the mini-TAV domain (the minimal P6 portion to retain transactivation activity; de Tapia *et al.,* 1993) and activates the RISP through phosphorylation. TOR-deficient plants are

resistant to CaMV infection, underscoring the importance of TOR for this process (Schepetilnikov *et al.,* 2011).
In addition, P6 established large cytoplasmic inclusions, the viral factories (VFs). The VFs of Caulimoviruses are unique in the plant virus realm. While most viruses induce the formation of compartments to facilitate their replication, these are mainly composed of host membranes and host proteins (de Castro *et al.,* 2021). CaMV P6 protein builds a proteinaceous matrix in the cytoplasm that harbours all viral proteins, viral RNA and DNA, as well as mature viral particles (Schoelz & Leisner, 2017). In fact, most viral particles reside inside VFs, some in proximity to plasmodesmata and very few freely in the cell. CaMV VFs are amorphous and grow during the infection time course through fusion (Schoelz & Leisner, 2017). They are surrounded by host ribosomes and are the sites of viral translation, replication, and particle packaging. VFs are essential for infection and disruption of P6 protein through mutations often results in loss of infectivity. VF shapes vary in shape and size, depending on the CaMV strain, as well as the host (Shalla *et al.,* 1980), pointing towards direct interactions within the VFs of viral and host components.

1.2 Genome-wide association studies – finding the hidden modulators of complex traits

Pathogen infections are highly complex traits influenced by a myriad of factors, including the genetic architectures of both, the pathogen and the host. It is all but impossible to predict which genes can influence a disease outcome without unbiased screening methods. With the advances in sequencing approaches and subsequently the availability of high-quality genomes, genome-wide association studies (GWAS) have arisen as a powerful approach to identify genetic loci influencing complex traits, like disease in humans and plants (Bartoli & Roux, 2017; Chapman & Hill, 2012). In fact, the first GWA study in Arabidopsis included the disease resistance against the pathogenic bacterium *Pseudomonas syringae* (Aranzana *et al.,* 2005).
In short, GWA algorithms scan for associations between a phenotype of interest and variations in the DNA sequence of an individual line within a population (Nordborg & Weigel, 2008). Single nucleotide polymorphisms (SNPs) are commonly used as genetic markers for genome mapping, due to

their high frequency across genomes. Naturally inbreeding plants, like Arabidopsis are especially suited for these studies, as their genomes have been homogenized over thousands of generations and researchers can work on identical material. The population structure and relatedness of individual plants can be strong confounding elements in GWA studies, and the number of genotypes involved will impact the chance to find meaningful associations. In Arabidopsis, impactful alleles for several phenotypes have been found with as little as 96 natural populations (hereafter referred to as accessions) (Atwell *et al.,* 2010; Aranzana *et al.,* 2005).

The intimate relationship of a virus with its host makes these pathogens especially dependent on host compatibility. A virus needs the interactions of viral and host proteins to work efficiently without triggering a strong antiviral response. It can be assumed that virtually all plants will encounter viruses throughout their lifecycle, with most virus infections not triggering notable symptoms in natural populations, but the potential to become pathogenic in monocultures of susceptible genotypes (Prendeville *et al.,* 2012).
Several GWA studies have been conducted in plant-virus systems, mostly for crop and fruit species and RNA viruses, that identified novel disease genes and re-mapped known resistance factors (Monnot *et al.,* 2021). Interestingly, Arabidopsis was seldomly used as the host for viral GWA studies, despite the superb genomic resources available for this species. The Arabidopsis-virus GWA studies that have been conducted, focused on RNA viruses, most notably potyviruses (Table 1). The MATH-domain protein RESTRICTED TEV MOVEMENT 3 (RTM3) restricts the long-distance movement of several potyviruses (Cosson *et al.,* 2010; Whitham *et al.,* 1999) and was identified in two independent studies for the potyviruses TuMV and PPV (Rubio *et al.,* 2019; Pagny *et al.,* 2012). The identification of *RTM3* strengthens the applicability of GWA to validate known candidates, while the discovery of the RNA silencing modulators *REDUCED DORMANCY 5* (*RDO5*) and *ANTIVIRAL RNAi REGULATOR 1* (*VIR1*) for CMV (Liu *et al*., 2022) exemplify the potential to find novel candidates for Arabidopsis viruses, despite the polygenic nature of virus infections. All six Arabidopsis-virus studies identified candidate genes influencing virus disease through GWA mapping, although several of the candidates still await functional validation. Arabidopsis has evolved in the presence of viruses and exhibits a

broad range of symptoms after infection. Notably, the GWAS studies have all but one focused on either virus accumulation (Liu *et al.,* 2022; Pagny *et al.,* 2012) as the tested trait or plant disease (Butković *et al.,* 2022; Butković *et al.,* 2021; Montes *et al.,* 2021) but only Rubio *et al.,* 2019 correlated both.

Table 1: GWA studies performed in Arabidopsis-virus system.
Virus: PPV = *Plum pox virus*, TuMV = *Turnip mosaic virus*, CMV = *Cucumber mosaic virus*, viral family is stated in (); Acc. #: Number of Arabidopsis accessions used in this study; Identified gene(s): Red color indicates that candidate genes were not validated.

Virus	Acc. #	Identified gene(s)	Reference
PPV (potyvirus)	147	*RTM3*	Pagny *et al.,* 2012
TuMV (potyvirus)	317	*RTM3, Dead box helicase 1, eIF3b*	Rubio *et al.,* 2019
TuMV (potyvirus)	450	Several	Butković *et al.,* 2021
TuMV (potyvirus)	1050	Several	Butković *et al.,* 2022
CMV (Cucumovirus)	154	Several	Montes *et al.,* 2021
CMV; CMVΔ2b (Cucumovirus)	500	RDO5, VIR1	Liu *et al.,* 2022

CaMV infects Arabidopsis in the wild (Pagán *et al.,* 2010), which makes this host-pathogen pair suitable for GWA studies, as CaMV could be a selective pressure on Arabidopsis evolution. The dynamics of CaMV disease in Arabidopsis in response to drought have been studied in a limited number of accessions (Bergès *et al.,* 2021; Bergès *et al.,* 2020; Bergès *et al.,* 2018), but no systemic analyses of CaMV disease in Arabidopsis or an examination of the underlying genetics had been performed before this thesis.

1.3 Tolerate or resist - plant defences against viruses

Plants are not defenceless against invading viruses. A host can actively hinder viral replication or degrade viral components to reduce the amount of virus within a plant ("resistance"). But every action taken comes at an energy cost and defence against biotic stresses is especially costly, often leading to reduced growth and fecundity (Huot *et al.*, 2014). Another possibility for the plant is to tolerate the pathogen within a margin and to not invest resources

into fighting the infection, but rather to invest in growth and reproduction, while the pathogen accumulates to high levels ("tolerance"). The visible symptoms plants develop during a viral infection are one outcome of the competition for limited molecular resources. It has become evident that viral accumulation in the host and the development of symptoms are not linked linearly for many infection pairs and studies in natural populations proved that symptom severity is not or weakly correlated with virus accumulation (Rubio *et al.,* 2019; Pagán *et al.,* 2007; Paper III). This is in part because symptoms are not caused by the presence of the virus itself, but by the compatibility of molecular interactions and the reaction of the host towards the invader, which manifests in tolerance and resistance.

1.3.1 Tolerance – the main driver in natural settings?

Plant pathogen interactions are commonly referred to as an evolutionary arms-race with an oscillating outcome of either the pathogen or the plant winning the upper hand during their co-evolution. Yet, viral infections often do not cause symptoms in natural ecosystems, can protect their host against other biotic and abiotic stresses and viruses can even become so intimately linked that they become persistent, i.e. solely transmitted vertically through seeds (Pagán, 2022; Bradamante *et al.,* 2021; Paudel & Sanfaçon, 2018; Roossinck, 2013). In fact, persistent viruses of the families Partitiviridae and Endornaviridae are the most common in wild plant populations (Roossinck, 2012; Roossinck, 2015) and *Lotus japonicus*, a host of the Alphapartitivirus *White clover cryptic virus* (WCCV), was found to use the viral coat protein for its own benefit (Nakatsukasa-Akune *et al.,* 2005), indicating that some persistent viruses have become symbiotic over time (Roossinck, 2011). A recent report found that a substantial fraction of Arabidopsis lines used in laboratories throughout the world are infected with the non-symptomatic *Arabidopsis latent virus 1* (ALV), exemplifying that viruses can go undetected for a long time even in the best studied plants (Verhoeven *et al*., 2022).

For these, as well as other more pathogenic viruses, the host tolerates the virus replication without strong triggering of defence pathways. Thus, plant tolerance to virus infection was defined as the mitigation of virus infection irrespective of the pathogen load, with minimal or absent cost for plant growth, yield or reproduction (Cooper and Jones, 1983). The mechanisms of tolerance are not well understood and understudied compared to resistance

pathways, which can surely be attributed to the focus of research on damaging viral diseases in agricultural settings that heavily rely on the eradication of the virus, rather than its co-existence. But virus infections in wild ecosystems suggest that tolerance is a stable defence against viruses and evidence emerges that tolerance can also be used in agricultural settings to protect broadly against plant fitness costs of viral infections (Korbecka-Glinka *et al.,* 2017). Most agricultural breeding programs, however, are still aimed to prevent the multiplication of the virus within the plant to not establish a virus source for re-infection of susceptible plants. Many virus-resistant cultivars used in agriculture today thus rely on resistance (*R*) genes or recessive resistance (de Ronde *et al.,* 2014).

1.3.2 Resistance – from incompatibility to elimination

The total reliance of viruses on their hosts machinery to complete their replication cycle makes viruses vulnerable to incompatibilities. As a result, recessive resistance of plants due to loss of compatibility in critical host succeptibility genes is a common feature of virus infections (Sanfaçon, 2015). Well studied examples are the translation initiation factors eIF4E indispensable for potyvirus translation (Duprat *et al.,* 2002; Lellis *et al.,* 2002) and eIF4G for Cucumo- and Betacarmoviruses in Arabidopsis (Yoshii *et al.,* 2004). For CaMV, TOR-deficient plants cannot promote the P6 dependent translational transactivation and fail to support virus multiplication (Schepetilnikov *et al.,* 2011).

In addition, dominant resistance entails the recognition of pathogen signatures, like the viral coat protein, by *R*-genes, which subsequently can initiate a signalling cascade inducing a hypersensitive response (HR), in turn leads to programmed cell death and confines the invading virus in the local lesion (Marathe *et al*., 2002). As a second layer, the HR induces systemically acquired resistance (SAR) which is defined by an activation of immune pathways in distant tissues of the plant. This immune activation can last for weeks and primes the tissue against invading pathogens (Soosaar *et al.,* 2005).

R-gene mediated resistance is relying on the detection of a viral effector by an R-gene, which can lead to a narrow window of recognition, as exemplified by Arabidopsis *RCY1* gene that only confers resistance to CMV strain Y, but not strain O (Takahashi *et al.,* 2002). While *R*-gene mediated resistance is an effective method to protect plants against pathogens, it averts a selection

pressure on the pathogen, leading to the emergence of new strains that cannot be recognized by the *R*-gene and thus overcoming the resistance. One worrying example is the emergence of the Tobamovirus *Tomato brown rugose fruit virus* (ToBRFV), first reported in Israel and Jordan in 2016 (Salem *et al.,* 2016), now found in cultivated tomato around the globe. Tomato resistance against Tobamoviruses has relied on the R-allele *Tm-2*^{2} from wild tomato species for the past 60 years (Lanfermeijer *et al.,* 2003). A resistance that ToBRFV has now overcome, with devastating consequences for affected tomato growers (Hak & Spiegelmann, 2021; Luria *et al.,* 2017). Finding resistance loci can be tedious, and several resistance phenotypes still await mapping. For CaMV, four Arabidopsis accessions, namely En-2, Wil-2, Sv-0, Tsu-0 (Leisner & Howell, 1992) were found to be resistant to infection. The En-2 resistance locus is linked to the microsatellite marker nga128 on chromosome 1 and referred to as CAR1 (Callaway *et al.,* 1996), but the causal gene remains unknown. Resistance in En-2 can be overcome by the CaMV strain NY8153 mediated by the movement protein P1 (Adhab *et al.,* 2018). Interestingly, resistance in the ecotype Tsu-0 is broken by the viral P6 protein, indicating that Arabidopsis has evolved different resistance mechanisms, possibly enacted by different genes against CaMV (Hapiak *et al.,* 2008). Systemic *R*-gene mediated resistance requires fast long-distance signalling to prime all parts of the plant for the invading infection. A key role in this signalling is played by endogenous chemical regulators, the plant hormones.

1.3.3 Plant hormones - the dilemma of replication and transmission

Plant hormones are a crucial, yet often overlooked, players in plant-virus and plant-virus-vector interactions. Hormones are small molecules, occurring in low concentrations that can easily be transported throughout the plant and act as fast and mobile long-distance signalling molecules, inducing antiviral SAR (Verberne *et al.,* 2003). Salicylic acid (SA), jasmonic acid (JA) and ethylene are among the classical well-established hormones for plant immune regulations, but auxins, gibberellins, cytokinins, brassinosteroids and abscisic acid (ABA) have been shown to also regulate plant immune responses to pathogen attack (Pieterse *et al.,* 2009). Through specific variation within the hormone cocktail, plants can sense “disease signatures” of the invading pathogen and in turn tailor the immune response to reduce cost. The developmental abnormalities plants exhibit upon virus infection,

can in part be explained by a miss-regulation of auxin and auxin responsive genes, causing growth deformities (Zhang *et al.,* 2020a; Padmanabhan *et al.,* 2006; Padmanabhan *et al.,* 2005). Viruses can cause disruption of hormone homeostasis by simultaneously inducing several synergistic and antagonistic hormones (Xie *et al.,* 2018). ABA and gibberellins are emerging as nodes for viral manipulation due to their role as potent modulators for the classical defence hormones SA and JA (Li *et al.,* 2022; Xie *et al.,* 2018; Zhu *et al.,* 2005). Generally, SA confers resistance against biotrophic pathogens, like viruses, while its antagonists JA and ethylene modulate responses against necrotrophs and herbivores (Robert-Seilaniantz *et al.,* 2011). Vector transmitted viruses must manoeuvre between their need for efficient replication by inhibiting SA mediated SAR and efficient transmission, by reducing insect deterring hormones, like JA. Evidence from several virus infections suggests that viruses cope with elevated SA levels to reduce insecticidal compound levels, increase vector feeding time and frequency and thus increase transmission rates (Wu *et al.,* 2017; Yang *et al.,* 2016; Casteel *et al.,* 2015). The inducible systemic defence response against viruses elicited by SA links several defence pathways together and it is becoming apparent that apart from their role in gene expression regulation and growth vs. immunity trade-offs, plant hormones also influence and cooperate with the most important defence plants employ against viruses: the antiviral RNA silencing (Alazem *et al.,* 2017; Goméz-Muños *et al.,* 2016; Alamillo *et al.,* 2006).

1.4 RNA silencing – the archetype of antiviral plant defences

In the late 1980s agrobacterium mediated T-DNA transformation of plants became widely used, spawning a number of studies to engineer specific plant traits through overexpression. Initial observations included that the plants expressing a viral coat protein became resistant to subsequent infections (Abel *et al.,* 1986), which heralded the development of several virus-resistant transgenic plants, some of which are still grown for food production today (Fuchs & Gonsalves, 2007). Another example is the overexpression of Chalcone synthase (*CHS)* in *Petunia x hybrida* plants, initially introduced to create intense purple flowers. What the researchers observed though, was a variety of pigmentation ranging from deep purple, over spotted to completely

white flowers. They found that the introduction of a second *CHS* gene sometimes led to the shut off (silencing) of the endogenous gene (Napoli *et al.,* 1990; van der Krol *et al.,* 1990). Early work on the plant viruses TEV and *Tomato black ring virus* (ToBRV) suggested that gene silencing occurs upon perception of new nucleic acids as invading viruses, thus triggering plant defences (Ratcliff *et al.,* 1997; Lindbo *et al.,* 1993). Subsequent work in rice calli and *Potato virus Y* (PVY) constructs in tobacco identified double stranded (ds)RNA as the initiator of gene silencing in plants and originated the field of RNA silencing research (Wang & Waterhouse, 2000; Waterhouse *et al.,* 1998).

1.4.1 dsRNA – replication intermediate and silencing trigger

Endogenous plant genes can give rise to dsRNA either by amplification through RNA-depended RNA polymerases (RDRs) or through the hybridization of complementary sequences. Repetitive DNA sequences, like transposons, are commonly silenced through a pathway termed RNA-directed DNA methylation (RdDM). Here, ssRNA synthesized by PolIV is processed into dsRNA by RDR2. The dsRNAs are then cleaved into small, 24 nt long RNAs which propagate the deposition of repressive methylation marks on the DNA (Matzke & Mosher, 2014). Viruses that transcribe their genome in the nucleus, like geminiviruses and caulimoviruses, are targeted by the RdDM pathway and their viral minichromosomes are highly methylated (Omae *et al.,* 2020; Raja *et al.,* 2008).

dsRNA structures also commonly occur during virus replication in the cytoplasm and independent of their origin, dsRNAs are commonly recognized by RNAse-III-type endonucleases, the dicer-like proteins (DCLs), which additionally require DOUBLE-STRANDED RNA BINDING (DRB) proteins for their action (Hiraguri *et al.,* 2005). The DCLs cut dsRNA into small (s)RNA fragments (hereafter termed siRNA for small interfering RNAs) of 21-24 nt in length from which one strand is subsequently loaded into Argonaute (AGO) proteins, forming the RNA-induced silencing complex (RISC). The siRNA then guides the AGO nuclease to their target RNA, where AGO either induces cleavage of the target or inhibits its translation (Figure 4). The RNA silencing machinery has greatly diversified in plants with Arabidopsis encoding four DCLs with five DRBs (Bologna & Voinnet, 2014; Hiraguri *et al.,* 2005), ten AGOs (Mallory & Vaucheret, 2010) and six RDRs (Wassenegger & Krczal, 2006), together

providing an efficient and adaptive machinery in development and immunity.

In viral infections DCL2 plays an especially important role. DCL2 produces 22nt siRNAs which serve as a primer for amplification of dsRNA from the target through the cytoplasmic RDR6, which in turn generates the dsRNA substrate for DCL4 to produce secondary siRNAs, amplifying the silencing signal (Lopez-Gomollon & Baulcombe, 2022; Wu *et al.,* 2020). 22nt siRNA protrude one nucleotide outside of the AGO nucleoprotein, recruiting dsRNA binding proteins like SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Iwakawa *et al.,* 2021). The AGO-SGS3 complex stably binds the target RNA, stalling ribosomes, which again leads to the activation of RDRs and enhances the production of secondary siRNAs, thus increasing the siRNA pressure on the invading virus (Baulcombe, 2022; Wang *et al.,* 2018).

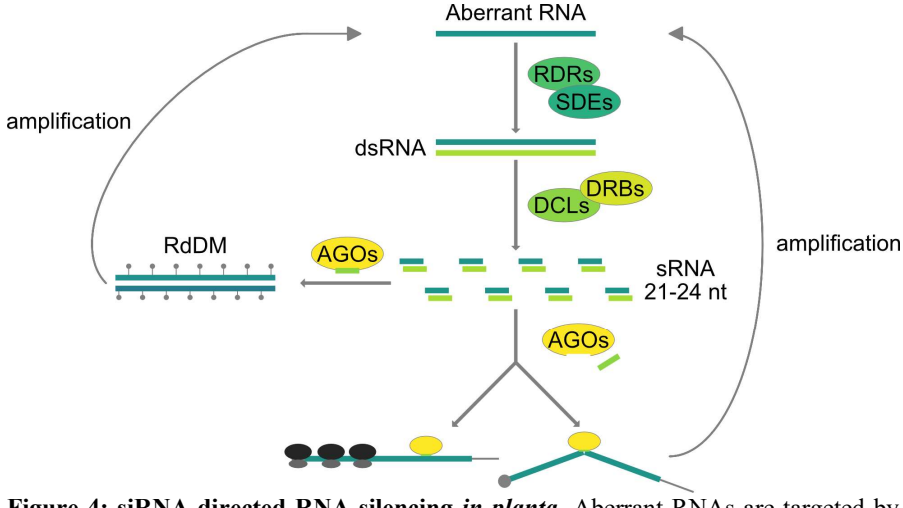

Figure 4: siRNA directed RNA silencing *in planta.* Aberrant RNAs are targeted by RDRs and their helper proteins (SDEs) and turned into dsRNA, which are then processed into 21-24 nt siRNAs by DCLs, guided and aided by DRBs. siRNAs are then loaded into AGO proteins and guide these either to nuclear transcripts where they interact with PolV transcripts to induce de novo RNA-directed DNA methylation (RdDM, depicted as grey lollipops on the double stranded DNA) or to cytoplasmic target RNAs leading to transcript degradation or ribosome stalling. siRNAs are cell-to-cell mobile and can spread the silencing signal throughout the plant, while the targeting of RNAs produces more siRNAs, leading to strong amplification of the silencing signal. Adapted from Pooggin, 2018.

All 26 families of land plant viruses give rise to virus derived small RNAs during infection (Pooggin, 2018). The amount of siRNA produced from viral RNA during infection often ranges between 30%-70% of the total (viral and plant) siRNA count (Annacondia & Martinez, 2021; Blevins *et al.,* 2011; Garcia-Ruiz *et al.,* 2010; Paper I), but extreme cases can reach up to 90% viral siRNA, for example during asymptomatic infection of *Nicothiana benthamiana* with *Pelargonium line pattern virus* (PLPV, Pérez-Cañamás et al., 2017). Yet despite the massive defence plants build, viruses can overcome RNA silencing in compatible infections, by outrunning the mobile antiviral siRNAs or by deploying a strong counter defence – the viral suppressors of RNA silencing.

1.4.2 Viral suppressors of RNA silencing – the SWAT team

Every viral protein exerts one or several functions within the host cell and interacts with a multitude of host proteins to facilitate replication and subvert immune responses. Especially important are the viral suppressors of RNA silencing (VSR) proteins that, as their name suggests, inhibit the antiviral RNA silencing. All viruses express one or more VSRs (Li & Ding, 2006) and the extreme diversification of VSR functions and structures indicates that they have evolved independently, frequently and recently (Csorba *et al.,* 2015). In their variety, VSRs target every node of the RNA silencing machinery (Figure 5). A large body of work has been published on VSRs and to name it all would exceed the measures of this thesis, but the most common modes will be shortly described in the following paragraph.

A common way for viruses to subvert RNA silencing is by sequestering its dsRNA away from the host machinery, either by establishing inaccessible replication compartments, like *Brome mosaic virus* (BMV) (Schwartz *et al.,* 2002) or by blocking access to the dsRNA itself through binding by viral proteins like the dsRNA binding proteins P38 of Betacarmoviruses and 2b of Cucumoviruses. The 2b protein of CMV is one of the most versatile and best studied VSRs to date. In addition to dsRNA binding, it also sequesters siRNAs and the methyltransferase HUA ENHANCER 1 (HEN1), inhibits the expression of *RDR6* and *AGO1* and interferes with the host miRNA pathway (Csorba *et al.,* 2015). 2b exemplifies that not only do different viruses attack different parts of the RNA silencing, but one virus and even one viral protein can interfere with several processes.

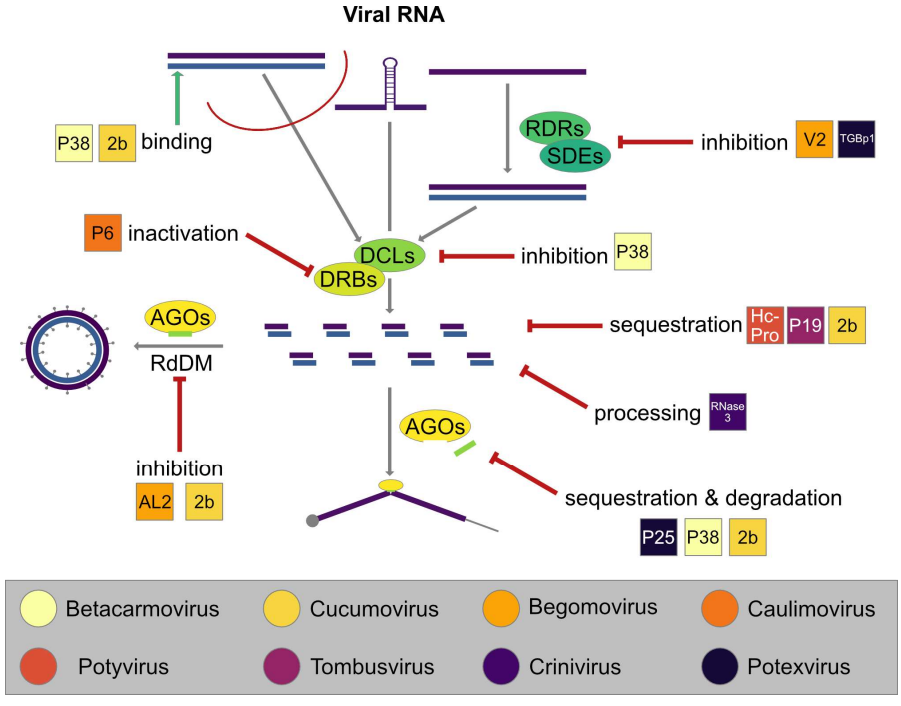

Figure 5: Viral suppressors of RNA silencing target every step of the pathway. Viruses have evolved diverse suppressor strategies to escape or combat antiviral RNA silencing. Virtually every node of the pathway (see Figure 4) is targeted by a VSR, sometimes one VSR can interfere with several processes within the mechanism. Protein identifiers are indicted in boxes, colour corresponds to virus family depicted below. Adapted from Csorba *et al.,* 2015.

The second common mode of RNA silencing suppression is the sequestration of double stranded siRNAs (Silhavy *et al.,* 2002). The proteins P19 of tombusviruses and HC-Pro of potyviruses are so efficient in binding siRNAs that both have been used outside the viral context to elucidate the endogenous siRNA pathways and develop methods for highly sensitive siRNA capture (Anandalakshmi *et al.,* 2000; Qavi *et al.,* 2010). A different route with similar outcomes is taken by the Crinivirus *Sweet potato chlorotic stunt virus* (SPCSV) that encodes for RNAse3, an enzyme that further degrades siRNAs into 14 nt long fragments, rendering them useless for the plant (Cuellar *et al.,* 2009; Kreuze *et al.,* 2005).

Apart from the RNA and siRNA, proteins involved in RNA silencing are often directly bound, sequestered, and inhibited by viral proteins. Several

VSRs destabilize AGO proteins and mark them for degradation either through autophagy (Karran and Sanfacon, 2014; Derrien *et al.,* 2012) or the proteasome pathway (Chiu *et al.,* 2010), while others, like the *Tomato yellow leaf curl virus* (TYLCV, Begomovirus) V2 protein and *Potato virus X* (PVX, Potexvirus) TGBp1, inhibit the RDR6/SGS3 dsRNA generation from viral RNA to block silencing amplification (Okano *et al.,* 2014; Glick *et al.,* 2008). The P6 protein of CaMV, though harbouring a dsRNA binding domain, inhibits RNA silencing through inhibition of the nuclear DCL4 cofactor DRB4 (Haas *et al.,* 2008). Since most P6 protein is present in the cytoplasm, this limits the efficiency of P6 as a VSR. Self-attenuation of VSR strength can be seen in many viral infections either through sub-pooling of the VSR proteins or unfavourable protein translation. A decrease rather than a complete shutdown of RNA silencing allows for viral usage of this pathway on endogenous genes and may help balance between viral replication and plant fitness. Work on several VSRs suggests that these viral effectors, while necessary in facilitating viral infection, evolved to an intermediate strength (Torres-Barcelo *et al.,* 2008).

1.4.3 Endogenous inhibition of RNA silencing - the spirits that I called

Just like viruses subvert RNA silencing during infection, the plant itself tightly regulates these processes to not fall victim to its own creation. The main endogenous competitor of RNA silencing is a conglomerate of processes termed RNA quality control (RQC).

From the moment of transcription, an RNA is monitored and scrutinized at every step of its maturation process. It is imperative for a cell to produce error-free templates for protein production, yet mistakes occur routinely and these aberrant RNAs then need to be removed from the pool. RNA-polymerases produce errors in about 1 in 10.000 copied nucleotides (Gordon *et al.,* 2015), which might not sound like much, but with up to 10 000 polymerase proteins in a cell (Bremer & Dennis, 1996) and an average elongation speed of 1000 to 4000 nucleotides per minute (Maiuri *et al.,* 2011), 4000 mistakes per minute are made by the polymerases alone. In addition, errors occur during the splicing of pre-mRNA, during posttranscriptional changes like capping and polyadenylation and the export from the nucleus.

In the cytoplasm, a plethora of proteins monitor mRNAs for error-marks, like pre-mature termination codons (PTC), the absence of stop codons and other

features like translatability, 7-guanosine cap (cap) or polyA-tail length. Recognition of error-marks then leads to the removal of this RNA molecule. In contrast to RNA silencing, RQC processes are not self-amplifying as they only lead to the destruction of the detected aberrant RNA molecule. Both pathways compete for the same RNA clients, but data suggests that RQC degradation is preferentially used and only when the RQC is oversaturated, the RNA silencing machinery is activated (Liu & Chen, 2016). In line with this several components of the RQC pathway are suppressors the RNA silencing pathway (Martinez de Alba *et al.,* 2015; Thran *et al.,* 2012).
As there is no cellular organelle dedicated for RQC, cells regulate these processes through transient accumulations of RNA and RNA-binding proteins, the biomolecular condensates.

1.5 Biomolecular condensates – phase to phase with the ancient RNA world

The interior of cells is an extremely crowded space with 30-40% of the volume occupied by RNA and proteins in eukaryotes (Sharp, 2016). Cells thus need to compartmentalize their processes in an adaptable manner to ensure molecular interactions and enhance efficiency. While membrane-enclosed organelles are the most obvious and stable compartments in a eucaryotic cell, several processes are enabled through the transient condensation of proteins and nucleic acids into biomolecular condensates (BioMCs).
The nucleolus was the first BioMC to be described (Valentin, 1837; Wagner, 1835), only three years after the discovery of the nucleus (Brown, 1833) and before description of other membrane-bound organelles such as mitochondria or the Golgi apparatus (Mullock & Luzio, 2005). In the broadest sense, BioMCs dynamically self-assemble through several weak, multivalent interactions between nucleic acids and low-complexity domain harbouring proteins in a process termed liquid-liquid phase-separation (LLPS) (Yoshizawa *et al.,* 2020). A catalogue of defining criteria has been established for BioMCs, including, but not limited to, a spherical shape and the possibility to fuse independent of their localization and function (Hyman *et al.,* 2014). Several were found to form from a substructure (the “core”) that serves as a scaffold for accessory proteins and is the most dense and stable region within the BioMC (Xing *et al.,* 2020; Brangwynne *et al.,* 2011;

Souquere *et al.,* 2009). Presently, over 20 types of BioMCs, or phase-separated compartments are known in eukaryotic cells, many of them in the nucleus (Lin & Fang, 2021; Fare *et al.,* 2021). Their functions range from fine-tuning of transcriptional and posttranscriptional processes, over mRNA maturation and ribosome biogenesis in the nucleus over protein and mRNA storage, degradation and sequestration in the cytoplasm, to protein translocation in chloroplasts (Xu *et al.,* 2021; Lin & Fang, 2021; Figure 6). A common feature of BioMCs is their responsiveness to environmental cues and it's thus no wonder that they are mainly implicated in developmental and defence pathways that need to be quickly adjustable.

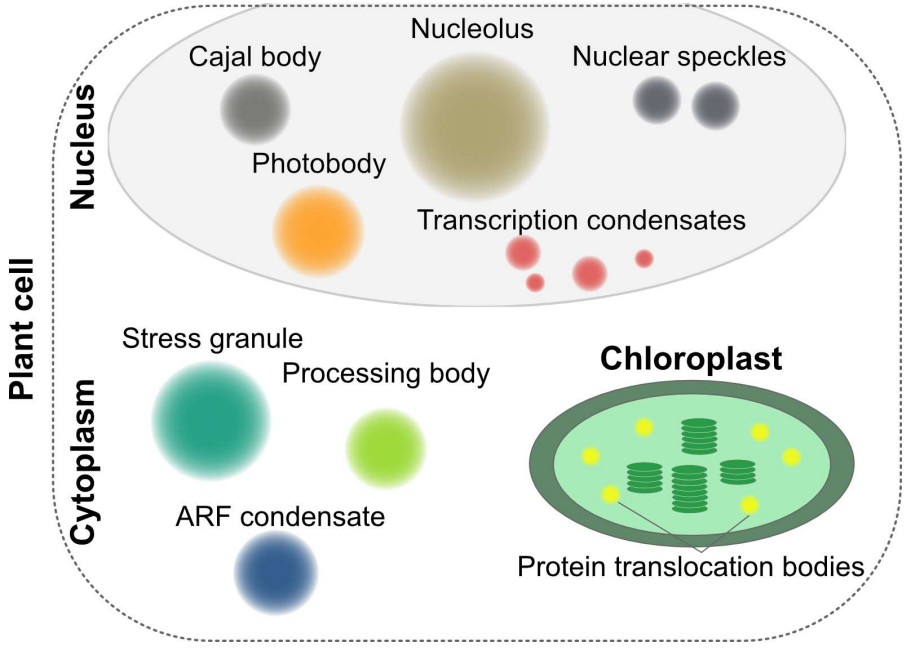

Figure 6: Overview of major types of BioMCs in plants. BioMCs can form in the cyto- as well as nucleoplasm and even within organelles, like the chloroplasts. They mostly contain RNA and proteins, except for transcription condensates that can form around DNA (Wang & Liu, 2019) and protein translocation bodies that form through multivalent interactions with a signal peptide (Ouyang *et al.,* 2020).

1.6 Cytoplasmic RNA granules – keep it or toss it?

Cytoplasmic BioMCs (hereafter referred to as RNA granules) are involved in the posttranscriptional regulation of mRNAs. RNA granules sequester mRNAs not undergoing translation and sequester them from polysome access (Chantarachot & Bailey-Serres, 2018). They are highly conserved across species and have been described in plants, animals and yeast, where they are transported via the cytoskeleton and can move quickly throughout the cell (Steffens *et al.,* 2014).

RNA granule assembly processes take place in multiple steps. First, the abundance of non-ribosome associated RNA leads to an increased formation of RNA- Protein complexes (RNPs), as well as RNA-RNA interactions. High concentrations of RNPs then trigger nucleation of the RNA granule core facilitated by promiscuous interactions between RNA and intrinsically disordered domains (IDRs) of the RNA-binding proteins (Van Treeck *et al.,* 2018). Once the RNA granule core is established, additional proteins are recruited through local concentrations of IDRs that promote LLPS and allow the RNA granule to grow into the detectable microscale. It is important to note that interactions between the core-components can exist on the sub-microscopic scale prior to the assembly into granules (Gutierrez-Beltran *et al.,* 2021).

The condensation into RNA granules can broadly speaking influence biology in one of two ways: First, Proteins and RNA are sequestered from the bulk cytosol, limiting their interactions, and spatially restricting them. Or second, high local concentration of proteins and RNA will favour interactions within the RNA granule and thus lead to increased activity within the confined space. The two major types of RNA granules, Stress granules (SG) and Processing bodies (PBs) are generally seen as the antagonists to polysomes, as mRNAs are triaged between translation at polysomes and storage and degradation in RNA granules (Figure 7). This mechanism was termed the "mRNA cycle" (Buchan & Parker, 2009) and the balance between the three corners of the triangle can shift greatly during stress. Any condition affecting translation rates or even poorly translating RNAs can trigger the condensation of RNA granules and the translational repression of their associated RNAs (Chantarachot & Bailey-Serres, 2018). Although PBs and SG can share mRNA clients and are known to fuse, they serve distinct functions and are each defined by core set of proteins. The following paragraphs will describe each separately.

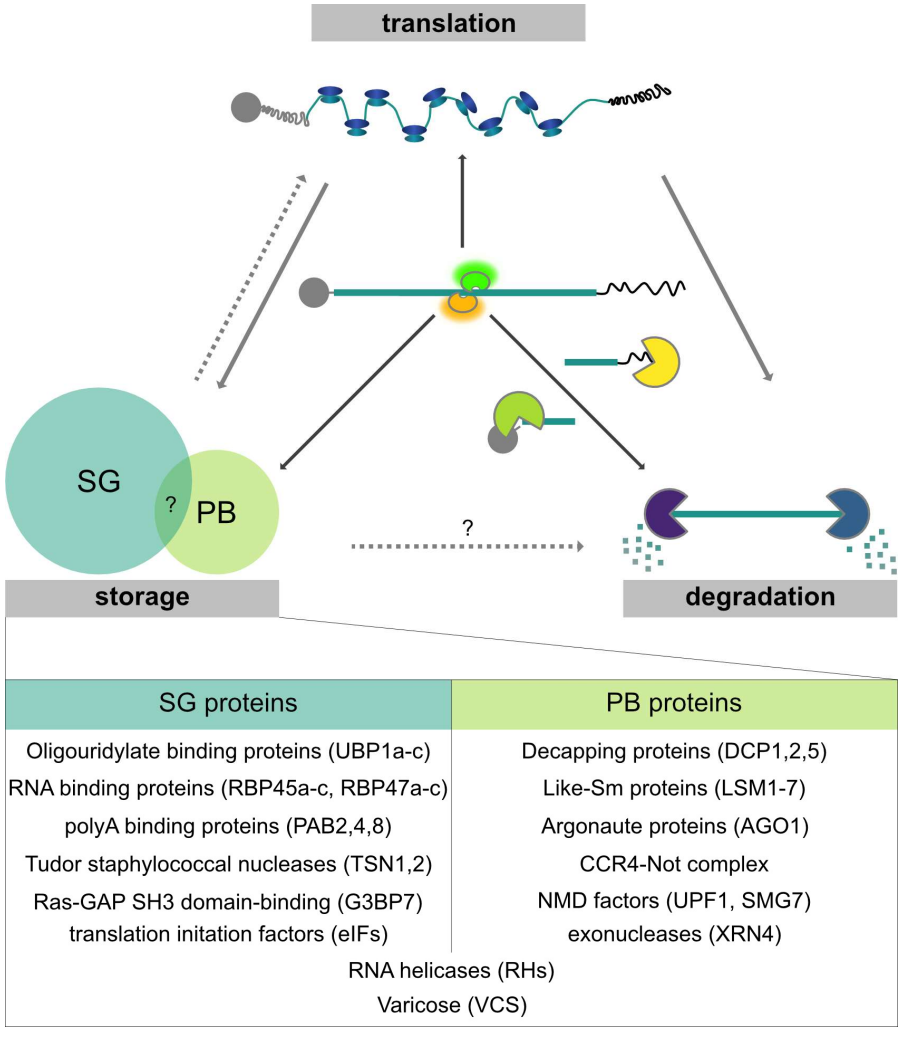

Figure 7 The mRNA cycle. From the moment of transcription, mRNA integrity is constantly monitored. If greenlighted, an RNA will undergo translation until it is not used anymore and targeted for degradation. If error marks are detected within an RNA, or the translation of said mRNA is not favourable at the moment, cells will store it in RNA granules, the SGs and PBs. mRNAs can be released from the granules to return to the translating pool of RNA or be targeted for degradation by exo- and endonucleases. Prominent components of SGs and PBs and Arabidopsis are listed below the figure.

1.6.1 Stress granules –reservoirs of non-translating RNA

SG formation is an immediate response to acute stress and heat stress granules (HSGs) in tomato cells were the first cytoplasmic BioMCs observed through electron microscopy (Nover *et al.,* 1983). SGs are dependent on the release of RNA from polysomes which can be chemically induced by sodium arsenite (arsenite) treatment (Kedersha *et al.,* 1999) and radiation (Kedersha *et al.,* 2013) or triggered by several abiotic and biotic factors, including temperature (heat; Weber *et al.,* 2008) and cold (Hofmann *et al.,* 2012)), salt and osmotic stress (Bhasin & Hülskamp, 2017; Yan *et al.,* 2014), hypoxia (Chantarachot *et al.,* 2020; Sorenson & Bailey-Serres, 2014) and pathogen attacks (Reuper *et al.,* 2021). Dependent on the type of stress and observed protein, SGs can assemble in a matter of minutes or hours and disperse again after re-entry of translation-favouring conditions (Weber *et al.,* 2008).
While generally considered a storage space for transiently unused mRNAs, SGs are diverse in their protein and RNA composition and consequentially their functions. To this day, over 500 proteins have been annotated in the mammalian SG proteome database (Nunes *et al.,* 2019). Proteomic analyses in mammalian cells revealed that about 50% of SG core proteins are RNA binding proteins (Jain *et al.,* 2016) with the notable components T-cell Intracellular Antigen (TIA-1), Ras-GAP SH3 domain-binding protein (G3BP1 and G3BP2), poly(A)-binding protein cytoplasmic 1 (PAB1) and eukaryotic translation initiation factors eIF3 and eIF4G. In plants, many of the core SG genes have undergone gene duplication, adding another layer of complexity in the composition of these RNA granules. The nine-member RBP45/RBP47/UBP1 family is the closest homologue to the SG initiation factor TIA-1 (Lorković & Barta, 2002). PABs are encoded by two genes in humans, while the Arabidopsis genome harbors eight genes, three of which are highly expressed throughout various tissues (Belostotsky, 2003). Especially RBP47b and UBP1b/c are used to monitor SG dynamics and elucidate the SG proteome *in planta* (Gutierrez-Beltran *et al.,* 2021; Chantarachot *et al.,* 2020; Weber *et al.,* 2008). An overview of important SG proteins is depicted in Figure 7.

1.6.2 Processing bodies – the hubs of RNA quality control

PBs are the second class of prominent BioMCs in the cytoplasm conserved throughout eukaryotes. Unlike SGs, proteinaceous PB components are present in phase-separated foci under non-stressed conditions, although

number and size of these foci can be drastically increased upon stress induced translational arrest. Canonical PB proteins include the decapping machinery, the exosome, RNA silencing components, as well as proteins involved in nonsense-mediated decay (Xing *et al.,* 2020; Anderson & Kedersha, 2009; Xu *et al.,* 2006; Figure 7).

This led to the hypothesis that PBs are site of RNA degradation in the cytoplasm, however several works in yeast and mammals have shown that PB-associated RNAs can be stabilized and return to the translating pool, questioning the degradation function of these proteins within PBs (Courel *et al.,* 2019; Wang *et al.,* 2018a; Hubstenberger *et al.,* 2017). In any case PBs are condensation points for RNA quality control (RQC) pathways and as such have profound impacts on RNA homeostasis. Disruption of the major PB proteins DECAPPING PROTEIN 1 (DCP1), DCP2 and VARICOSE (VCS) are embryo lethal, while mutations of the de-capping activator genes DCP5 and LSM1 exhibit growth phenotypes, underscoring the importance of these proteins and PBs during plant development (Perea-Resa *et al.,* 2012; Jang *et al.,* 2009; Xu & Chua, 2009; Xu *et al.,* 2006, Figure 8).

Several PB marker lines have been established in Arabidopsis, most notably all components of the decapping complex (Figure 8) were used to monitor PB dynamics (Chicois *et al.,* 2018; Perea-Resa *et al.,* 2016; Roux *et al.,* 2015; Motomura *et al.,* 2012).

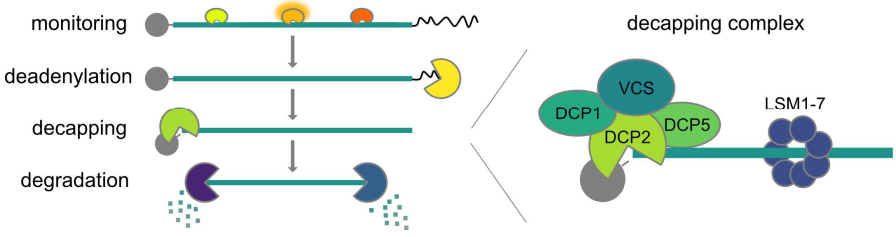

Figure 8: The decapping complex in Arabidopsis. In the cytoplasm, mature mRNAs are protected from degradation by a 5'-cap and a 3'-polyA tail. Before an mRNA can be degraded, both need to be removed. While the CCR4-Not complex cleaves of the polyA tail, the decapping complex consistent of the NUDIX hydrolase DCP2, the decapping activator DCP1, the helper protein DCP5 which are held together by the scaffold (VCS), remove the 5'-cap structure. The decapping complex is assembled through the presence of the LSM1-7 ring on the mRNAs. After 3' and 5' stripping, the mRNA is vulnerable to exonucleolytic digest by the exonuclease XRN4 and the exosome complex.

1.7 BioMCs – boon and bane of plant viruses

Due to their key-roles in RNA biology, BioMCs of all kinds are prime targets for viruses (Etibor *et al*., 2021). In the nucleus, three major compartments are conserved between plants and animals: the nucleolus, the Cajal bodies (CBs) and nuclear speckles (Emenecker *et al.,* 2020). The nucleolus functions in rRNA biosynthesis and ribosome biogenesis (Kalinina *et al.,* 2018) and several viral proteins from diverse virus classes are enriched within (Taliansky *et al.,* 2010; Figure 9). Some viral proteins, like CMV 2b and the VPg of *Potato virus A* (PVA) contain nucleolar localization signals, indicating a targeted translocation to this compartment (González *et al.,* 2010; Rajamäki & Valkonen, 2009). CBs and nuclear speckles concentrate mRNA metabolism- and splicing factors (Ohtani, 2017; Reddy *et al.,* 2012; Spector and Lamond, 2011) and serve as additional sites of viral protein localization and manipulation (Ding & Lozano-Duran, 2020). Interestingly, it is often the viral movement proteins that are targeted to the nuclear phase-separated compartments, and Kim *et al.,* 2007 found that the interaction with the nucleolar/CB protein Fibrillarin is required for the long-distance movement of the Umbravirus *Groundnut rosette virus* (GRV). Compared to the large number of viral proteins targeting nuclear bodies, fewer viral proteins are known to interact with the cytoplasmic SGs and PBs (Figure 9). The nuclear shuttle proteins of the Begomovirus *Abutilon mosaic virus* (AMV) and the Nanovirus *Pea necrotic yellow dwarf virus* (PNYDV) both localize to SGs *in planta*, possibly to interfere with SG assembly (Krapp *et al.,* 2017). The potyviral VSR HC-Pro induces the formation of cytoplasmic granules that contain both SG and PB proteins (Hafrén *et al.,* 2015) and the Begomovirus *Cabbage leaf curl virus* (CaLCuV) uses its nuclear shuttle protein to enhance decapping activity in PBs (Ye *et al.,* 2015). Apart from the nucleolar localization of P6 (Haas *et al.,* 2005), nothing was known about the role of cytoplasmic RNA granules during CaMV infection before this thesis. But overwhelming evidence from the animal field suggest that these BioMCs have profound impacts on both RNA and DNA virus infections (Poblete-Duran *et al.,* 2016; Tsai & Ljoyd, 2014; Lloyd, 2013).

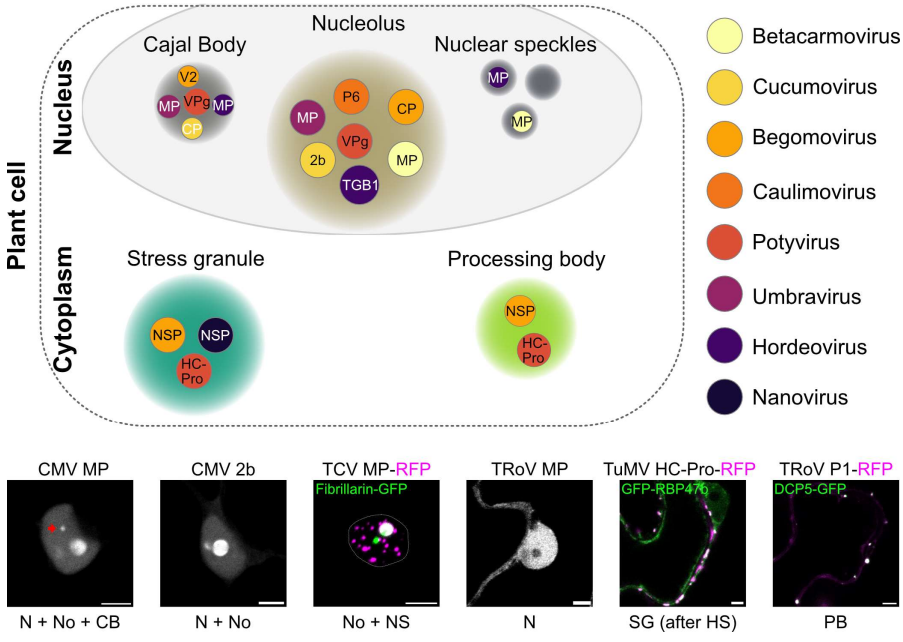

Figure 9: Phase separated compartments in the nucleus and cytoplasm targeted by viral proteins. Viral proteins from diverse viral groups (coloured circles) localize to one or several plant phase-separated compartments. MP = movement protein, CP = coat protein; VPg = viral protein genome-linked; NSP = nuclear shuttle protein. Images below the graphic are single plane confocal images of plant viral proteins (indicated above images) localizing to different compartment 3 days after agro-infiltration in *Nicotiana benthamiana*. CMV = *Cucumber mosaic virus*, TCV = *Turnip crinkle virus*, TRoV = *Turnip rosette virus*. Phase-separated compartments in planta are indicated below the images. N = nucleoplasm; No = nucleolus; CB = Cajal body (indicated by red arrow); NS = Nuclear speckle, SG = Stress granule (imaged 30 min after heat stress [HS]), PB = Processing body. NS, SG and PBs are counterstained with plant marker proteins to help visualization. Images = Gesa Hoffmann, unpublished. Figure is based on information from Ding & Lozano-Duran, 2020; Krapp et al., 2017; Hafrén *et al.,* 2015; Ye *et al.,* 2015; Semashko *et al.,* 2012; Tailansky *et al.,* 2010.

2. Aims of the study

The intimate relationship between a virus and its host suggests that both have profound impacts on each other. While the virus manipulates its host into enabling its replication and exploits host machineries for its own benefit, the host fights the virus directly through the disruption of viral replication and destruction of viral components or indirectly through evading compatibility with viral proteins.

In this study we aimed to elucidate the interplay of *Cauliflower mosaic virus* with its natural host *Arabidopsis thaliana* from two sides:

I. How does CaMV manoeuvre the RQC pathways of its host?

It is apparent that efficient translation and the protection of its genomic information is imperative for a virus to persist in a host. While cytoplasmic RNA granules, namely PBs and SGs, are at the heart of the cytoplasmic RNA quality control and translational regulation, not much is known about the role of these biomolecular condensates during plant virus infection. CaMV, because of its prominent viral factories in the cytoplasm, developed into an especially interesting model to study in this context.

II. How are host genetics influencing CaMV disease?

Through an unbiased approach using 100 natural accessions of Arabidopsis, we opted to discover the range of CaMV disease severity and virus accumulation to identify underlying genes and pathways implicated in the CaMV replication cycle.

3. Results and Discussion

This section briefly describes and discusses the main findings of the thesis work. It will connect the projects and manuscripts and sets the results in the broader context of the scientific field. Figures corresponding to the discussed findings are indicated throughout the text and can be found in the compiled papers at the end of this thesis.

3.1 RNA granules on a scale from pro- to anti-viral – learning from animal viruses

The roles of RNA granules during virus infections are as diverse as their compositions and dependent on the nature of the infecting virus. It is evident that both, virus and host, aim to use these granules to gain advantage during the infection. Much work has been done in animal virus systems, from which two main themes have emerged: The manipulation of RNA granule numbers during infection and the re-purposing of RNA granule components for viral replication (Figure 10).

In animals, viruses are challenged with a translational shutdown of the cell upon infection initiation (Rozman *et al.,* 2022), often leading to an initial increase of SGs, before viral counter mechanisms reduce SG numbers, either through cleavage of SG components (White *et al.,* 2007), forced translation (Montero *et al.,* 2008) or antagonizing key signalling steps in SG formation (Khaperskyy *et al.,* 2012). Mammarenaviruses can evade even the initial SG induction through rapid and effective suppression of SG formation (Linero *et al.,* 2011). Interestingly, Hepatitis virus chronic infections lead to an oscillation of SG numbers within the cell, possibly to balance virus translation with cell survival (Ruggieri *et al.,* 2012). No initial PB increase was reported for animal virus infections, but several viruses decrease, or even

completely disrupt PB numbers (Kleer *et al.,* 2022; Dougherty *et al.,* 2011). The induction of canonical RNA granules is an antiviral response of the animal host, whereas viruses have evolved several ways to subvert this response and use RNA granule components for their own benefit.

Viruses rely on the host machinery to complete their life cycle and transit from translation to replication. They can exploit the RNA regulatory functions of RNA granule proteins and divert them into their replication complexes to possibly serve pro-viral roles, but at least to be sequestered from their antiviral functions. Viruses with different replication strategies divert the SG and PB core components from canonical granules to viral replication complexes (Poblete-Durán *et al.,* 2016). Common targets include the SG proteins G3BP1 and TIA1 (homologous to the Arabidopsis UBP1 family), the shared proteins RAP55 (homologous to Arabidopsis DCP5) and XRN1 (homologous to XRN4) and the PB components GW182 (no homologue in plants), Lsm1-7 and HDELS/Ge-1 (homologous to Arabidopsis VCS) (Gaete-Argel *et al.,* 2019; Figure 10).

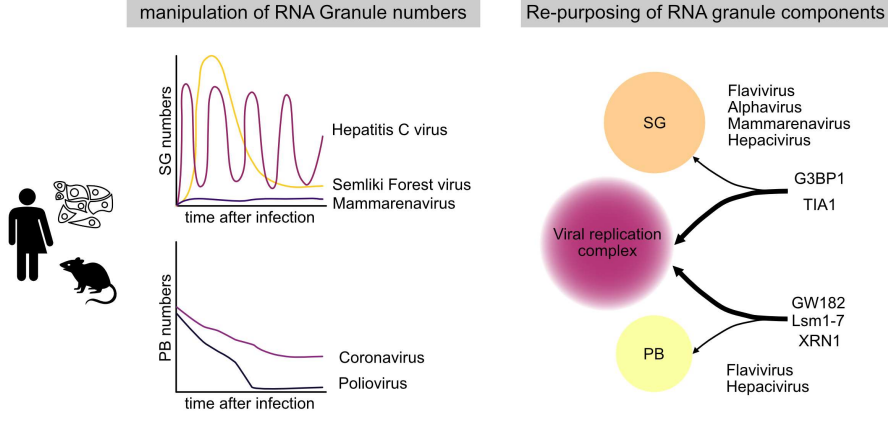

Figure 10: RNA granule manipulation by animal viruses. Two major ways of RNA granule manipulation by animal viruses have emerged through a large body of studies. Viruses mainly supress SG and PB numbers to subvert their antiviral functions and enhance translation. On the other hand, viruses channel SG and PB components into their own replication factories to facilitate replication and possibly serve other functions. Figure based on Poblete-Duran *et al.,* 2016 & Lloyd, 2013.

RNA granule dynamics and functions during plant virus infections are much less studied compared to animal and yeast systems. We started this project with a screen using the canonical PB marker DCP1-GFP (Motomura *et al.,*

2012) to visualize the effect of systemic plant virus infections on DCP1-marked PBs. In the lab we have a collection of diverse plant viruses inducing symptomatic infections in Arabidopsis (Table 2).

Table 2: Plant viruses used in the Hafrén lab.

Nomenclature	Acronym	Genome	Genus
Cardamine chlorotic fleck virus	CCFV	ssRNA(+)	Betacarmovirus
Turnip crinkle virus	TCV	ssRNA(+)	Betacarmovirus
Cauliflower mosaic virus	CaMV	DNA-RT	Caulimovirus
Cucumber mosaic virus	CMV	ssRNA(+)	Cucumovirus
Turnip mosaic virus	TuMV	ssRNA(+)	Potyvirus
Turnip rosette virus	TRoV	ssRNA(+)	Sobemovirus
Oilseed rape mosaic virus	ORMV	ssRNA(+)	Tobamovirus

In contrast to animal virus infections, none of the tested plant viruses decreased the number of DCP1-foci, but four of the seven viruses drastically increased their abundance at 21 days after infection (Figure 11A). Interestingly, the variation in DCP1-foci numbers was not dependent on transcriptional activation of *DCP1*, indicating that these foci assemble from a pre-existing soluble protein pool (Figure 11B).

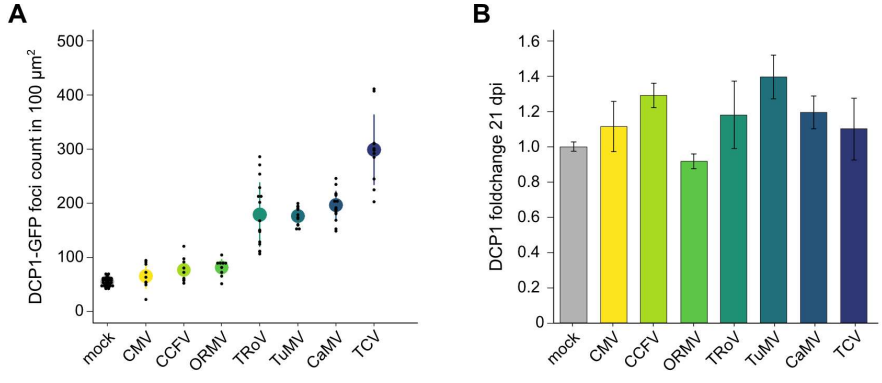

Figure 11: Several plant virus infections induce de novo assembly of DCP1-foci [A] DCP1-foci were counted in randomly chosen leaf areas at 21 dpi with indicated viruses. [B] Relative expression (foldchange) of DCP1 in virus infected rosette tissue compared to mock infected Col-0. Counts and expression analysis were performed as described in Hoffmann *et al.,* 2022.

We decided to focus our analyses on CaMV as it caused a strong induction of DCP1 foci (Paper I, Figure 1) and no dsDNA plant virus had been studied in this context before. Also, the interplay of CaMV VFs with cytoplasmic RNA granules was of special interest, since both are membrane-less organelles composed of proteins and nucleic acids, but the former attracts ribosomes, while the latter are proposed to be established in ribosome-free zones in the cells (Hamada *et al.,* 2018).

3.2 Virus infections - the fight for translation

Translation is the Achilles heel of any virus infection, as viruses completely rely on and compete with their host for the translation machinery (Walsh *et al.,* 2013). It is thus no wonder that most recessive resistance genes in plants encode for translation initiation factors (Machado *et al.,* 2017). Protein translation is one of the costliest processes and errors can have detrimental effects in cells (Drummond & Wilke, 2009). Yet, the translation process is surprisingly error prone and consequently tightly monitored by the cell's machinery. Viruses need to subvert and manipulate the production, as well as the monitoring of the translational machinery, to ensure their own protein translation.

In plants, Begomoviruses, a class of ssDNA viruses that cause great agricultural losses, are the only viruses known to cause a translational shutdown. They are recognized by the leucine-rich repeat receptor like kinase (LLR-RLK) protein NSP INTERACTING KINASE (NIK1), which triggers the translation repression (Zorzatto *et al.,* 2015; Brustolini *et al.,* 2015). In contrast, neither infection with the Tobravirus *Tobacco rattle virus* (TRV) nor the betacarmovirus *Turnip crinkle virus* (TCV) alter global translation patterns *in planta* (Meteignier *et al.,* 2016; Ma *et al.,* 2015). CaMV is an oddity, as its infection induces global translation rates in turnips (Park *et al.,* 2001) and Arabidopsis (Paper I, Figure 7), an effect that can be attributed to the function of the viral P6 protein as the translational transactivator (Paper II, Figure 6). So far, CaMV is the only plant virus known to trigger an increase in polysomes and it does so independent of the amount of viral RNA that is translated (Paper I Figure 7), indicating that host protein production will also be enhanced during CaMV infection. This would shift the balance of polysome- and RNA granule associated RNA in CaMV infected cells.

The fight for translation not only entails how much gets translated, but also what. The right for translation is decided by a triage between polysomes and the cytoplasmic SGs and PBs. Again, the responses in plants vary greatly from those observed in the animal field. PB numbers, for example, can increase and remain elevated for a long-time during plant virus infection (Paper I, Figure 1). PB induction upon TRV and TCV infection is not dependent on the increased amount of RNA in the cell, but the recognition of the virus by an R-gene, indicating a specifically triggered response rather than a monitoring of RNA abundance in the cell (Meteignier *et al.,* 2016). PB components have been found to serve mainly pro-viral functions for several viruses in different plant systems, including the facilitation of virus replication for *Barley yellow striate mosaic virus* (BYSMV, Zhang *et al.,* 2020b), aiding infection of PVA and TuMV (Zuo *et al.,* 2022; Hafrén *et al.,* 2015) and suppressing RNA silencing during CalCuV infection (Ye *et al.,* 2015).

We found that the PB proteins LSM1 and DCP5 are required for efficient CaMV translation and that the amount of viral protein is reduced in the mutants, even though the viral RNA remains at wild type level (Paper I, Figure 3). This was surprising as PB components are generally seen as antagonists to translation, by storing and degrading RNA, functions for which we found no evidence on CaMV RNAs (Paper I, Figure 4). However, our results do suggest that through the presence of PB proteins, the viral RNA is protected from the RNA silencing machinery (Paper I, Figure 5&7). CaMV appears resistant to RNA silencing, because genetic studies with *rdr6* and *dcl2/3/4* mutants did not show an increase in virus accumulation and because most antiviral siRNAs are generated against the noncoding *8S* RNA (Paper I, Figure 5 & 6; Blevins *et al.,* 2011). We found that CaMV uses PB components to hide from the RDR6-governed RNA silencing machinery (Paper I, Figure 6 & 7), possibly through preferred binding of the viral RNA (Paper II, Figure 5). In the single *dcp5* or *lsm1* mutants, ribosome occupancy on the CaMV *35S* RNA was reduced in line with reduced protein accumulation, however the double mutant with *rdr6*, but not the nuclear *rdr2*, rescued the ribosome occupancy and protein levels, indicating that PB components act as suppressors of RNA silencing mediated translational repression during CaMV infection (Paper I). A profiling of *dcp5* polysome-bound seedlings found that while most mRNAs had increased polysome occupancy in the *dcp5* mutant during seedling development, a cluster of

about 100 RNAs showed decreased polysome occupancy (Jang *et al.,* 2019). This indicates that a class of endogenous mRNAs exists whose translation is increased by the PB component DCP5 and it is possible that CaMV highjacks this mechanism to win the fight for translation in the presence of RNA silencing.

3.3 The CaMV viral factory – a viral RNA granule?

A striking finding was the massive, but selective, translocation of PB and SG components into the VFs of CaMV (Paper I, Figure 1; Paper II, Figure 2). DCP1, a canonical PB marker used for PB visualization in many studies (Yu *et al.,* 2019; Motomura *et al.,* 2015; Gutierrez-Beltran *et al.,* 2015; Moreno *et al.,* 2013) is the only PB marker tested that was excluded from VFs (Paper I, Figure 1 & 2), which could either indicate that PB proteins are not sequestered as pre-assembled PBs, or that DCP1 is specifically barred from entry. Similarly, the SG protein eIF4a-II did not enter VFs in ambient temperatures or after arsenite treatment, but readily re-localized after heat stress (Paper II, supplemental Figure 2). Interestingly, the exon-junction complex protein eIF4a-III is highly conserved in sequence to the cytoplasmic eIF4a-II, but distinct in function. eIF4a-III is localized diffusely in the nucleus, under stress however, it assembles into the nucleolus and nuclear speckles, where its mobility is reduced (Koroleva *et al.,* 2009a, Koroleva *et al.,* 2009b). This behaviour is analogous to our observations of eIF4a-II in the VFs and could point towards stress induced affinity for phase-separated compartments that is conserved in their sequence.

The accumulation of RNA binding proteins in viral replication complexes is shared between animal and plant viruses, even though the replication complexes are only functionally homologous, but not architecturally (Rodriguez-Peña *et al.,* 2021; den Boon *et al.,* 2010). In the early days of CaMV research, the large VFs were thought to be mainly inert and to not exchange components with the surrounding cytoplasm (Kitajima *et al.,* 1969; Conti *et al.,* 1972), an assumption that is unfounded in hindsight and was first disproved when Bak *et al.,* 2013 showed that VFs act as a reservoir for viral particles (~50 nm in diameter) that can be released upon aphid feeding and taken back up by the VFs after the transmission trigger subsides. This particle mobility within VFs shows that the P6 matrix is not a closed environment and can allow for the traffic of large compounds within the VF

and their surroundings, possibly including ribosomes and other multi-molecule compounds.
Unlike the rigid P6 matrix, PB and SG proteins are mobile within VFs and between VFs and their surroundings (Paper II, Figure 4). The diffusion rate of a protein is influenced by its size, its interaction with other molecules and the grade of molecular crowding of its immediate environment (Miyoshi & Sugimoto, 2008), but we found similar mobility in VFs compared to PBs or arsenite induced SGs. Severe stress, however, can drastically influence the cellular environment and thus the mobility of proteins. Heat stress is such a severe stress, leading to complete polysome disassembly, as well as influx of eIF4a and possibly more proteins into VFs (Paper II, supplemental Figure 2), increasing the molecular crowding within. Similar to canonical SGs, heat stress drastically decreases the mobility of SG proteins within VFs, while the arsenite-induced oxidative stress does not (Paper II, Figure 4; Frydrýšková *et al.,* 2020). Since PB and SG proteins behave similar inside VFs, as they do within their canonical granules, we speculate that VFs provide a phase separated environment, facilitating rapid movement and interaction of host and viral proteins. Like the well-studied P granules in *Caenorhabditis elegans*, VFs could consist of a solid shell (the P6 matrix) and a liquid core. While most BioMCs described to date have an approximate spherical shape, like SGs and PBs, amorphous and even tubular structures can occur, especially in solid shell / liquid core scenarios (Fare *et al.,* 2021). Also, P6 condensates in transgenic plants, as well as during early infections are spherical, but become distorted during disease progression and fusion of smaller condensates to the large amorphous VFs, which is consistent with the immobility of P6 within these foci. The importance of phase-separation and condensation during virus infection is becoming more and more evident in animal virus systems (Sagan & Weber, 2022) and the CaMV VFs provide a powerful model to study these processes during plant virus infections.

3.4 PBs and SGs – the Janus face of dynamic condensates

The brilliance of RNA granules is their reactivity, adaptability and versatility. The headache of studying RNA granules is their reactivity, adaptability, and versatility. SGs and PBs are generally described as two different entities, defined by a different subset of core proteins, different

RNA clients and different roles in RNA metabolism (Stoecklin & Kedersha, 2012). Yet, apart from a few core-proteins, several studies have identified widely differing protein compositions within one granule type (Liu *et al.*, 2022; Gutierrez-Beltran *et al*., 2021; Kershaw et al., 2021; RNA granule database: http://rnagranuledb.lunenfeld.ca/), which could be a consequence of differences in the purification methods or reflect the adaptive and diverse nature of them. With the multitude of proteins described as PB and/or SG localizing it is well possible that subtypes of these RNA granules can exist within one cell, that are not easily distinguishable by our current methods. These subtypes could perform different roles in RNA metabolism, contain different RNA clients and even contain signatures of both SGs and PBs. The docking and fusion of SGs and PBs depends on the stress exerted on a cell and expands the possibilities for functional divergence even more, to the point that it is not always clear whether under certain conditions, an RNA granule is an SG, a PB, a chimera, or something different (Burke *et al.,* 2020; Souquere *et al*., 2009; Kedersha *et al*., 2005). DCP1 is a widely used marker for PBs in plant and mammalian cells. The prominent DCP1-bodies are frequently targeted to VFs during CaMV infection and decorate their exterior extensively. We did, however, not observe this behaviour with any other PB marker, indicating that these DCP1-bodies do not contain the other decapping components, at least not in a detectable amount. The number, but not the localization of DCP1-bodies was also dependent on the viral strain (Paper I, Figure 1), a phenomenon that we cannot explain at the moment. In unstressed conditions, PBs are proposed to exist in phase-separated foci at a basal level in plant and mammalian cells. This observation holds true for the decapping proteins DCP1 and DCP5, but not for the decapping activator LSM1 and scaffold protein VCS (Paper I, Figure 1) which remain soluble in unstressed conditions. Do DCP1/DCP5-foci still qualify as PBs without the other components and vice-versa, does the viral VF with DCP5/VCS/LSM1, constitute a PB, even though DCP1 is excluded, while several SG proteins are present? We will need to understand the nature and interaction of RNA granules better to be able to answer these questions, but our study with CaMV exemplifies the importance of using several marker proteins, when working with RNA granules and cellular stress and to refer to the observed foci by the markers used.

3.5 *8S* RNA – 600 nucleotides to rule them all

Currently we lack a genetic system for SG knockouts and do not know whether mutant lines of single SG components are impaired in overall SG biology. While PB proteins serve pro-viral roles during CaMV infection, we could not detect a direct effect of SG proteins on virus accumulation (Paper II, Figure 5). But how can two protein classes with similar features and localized to the same compartment serve drastically different roles during infection? It is possible that CaMV uses a similar mechanism of avoidance as is known for the RNA silencing machinery, to escape antiviral SG protein functions. The mobility of PB and SG proteins within VFs suggests that these proteins are not stably bound to the rigid P6 matrix, making the viral RNA a likely culprit for causing the translocation to VFs. RNA binding proteins, including the SG and PB proteins DEAD box RNA helicases and mammalian G3BP1 have been described as nucleo-cytoplasmic shuttles (Pérez-Ortín & Chávez, 2022; Wang *et al.,* 2009; Cristea *et al.,* 2006), making them ideal for a virus to guide viral RNAs to replication factories. It is also possible that these proteins piggy-back on the viral RNA while it is translocated by viral proteins into the VFs or are transported through direct binding of viral proteins.

Proteins that bind RNA through their IDRs generally exhibit little sequence specificity (Corley *et al.,* 2020; Järvelin *et al.,* 2016). This makes them especially suitable for bulk RNA regulatory units like RNA granules and a prime target for regulation by viruses. Several RBPs bind to viral RNA during ssRNA virus infection in mammalian cells, although their composition is variable and dependent on the infecting virus (Iselin *et al.,* 2022). The CaMV noncoding *8S* RNA attracts the RNA silencing machinery to sequester it from the coding *35S* viral RNA (Blevins *et al.,* 2011; Paper I Figure 5). We additionally found that *8S* is preferentially associated with SG proteins compared to PB proteins (Paper II, Figure 5). Endogenous long noncoding RNAs (lncRNA) are broadly defined by a length >200 nt that are not transcribed into functional proteins (Statello *et al.,* 2021), criteria that the viral *8S* RNA (~600 nt) fulfils. LncRNA can act as sponges for endogenous microRNAs and RBPs through their binding and thereby supress their functions on other targets (Militello *et al.,* 2017; HafezQorani *et al.,* 2019). It is possible that the highly expressed *8S* sponges SG proteins in a manner similar to the RNA silencing machinery, to sequester them from their canonical functions. This likely takes place within the VFs, but further

experiments are needed to confirm. Our findings thus expand the functional repertoire of the CaMV *8S* RNA and strengthen its analogy of a eukaryotic lncRNA. If *8S* binding subverts SG protein functions, this would also explain the absence of virus accumulation differences in SG mutants, while PB proteins DCP5 and LSM1 gain access to the CaMV genomic *35S* RNA from which all viral proteins are translated and thus effect virus replication (Paper II, Figure 5).

3.6 CaMV on a scale from pro- to anti-granule – when in doubt: P6

It is difficult, if not impossible, to disseminate whether an induction of RNA granule-like foci during virus infection is initiated by the virus to induce pro-viral roles or orchestrated by the host to enhance antiviral pathways and both motives could be true at the same time.

Virus infection exerts extreme stress on a cell and while we did detect PB-like and SG-like foci in infected tissue, these foci largely contained P6 protein (Paper I, Figure 2 & Paper II, Figure 2). Within VFs, SG proteins are protected from and do not respond quantitatively to stresses like arsenite and heat application, or Cycloheximide treatment (Paper II, Figure 3). However, outside the infection context in transgenic P6 lines, the number and size of SGs marked by RBP47b and G3BP7 were drastically reduced, and the remaining foci contained P6 (Paper II, Figure 6). Expression of P6 increases the global translation levels in Arabidopsis, which are retained after arsenite, but not after heat stress. Conversely, P6 can fully supress the induction of SGs after arsenite, but only partially after heat-stress (Paper II, Figure 6). Importantly, expression of P6 does not alter protein levels of RBP47b or G3BP7 (Paper II, supplemental Figure 4), only their ability to assemble microscopic granules, a process that is dependent on non-translating RNA supply (Decker *et al.,* 2022). The ability of P6 to induce translation depends on the stabilization on ribosomes on the RNA which is mediated by the interaction with host translation factors, including eIF3g (Park *et al.,* 2001; Pooggin & Ryabova, 2018). A point mutation in P6 T305P abolishes this interaction and transgenic plants expressing the mutated P6 did not display a defect in RBP47b-foci generation after arsenite treatment (Paper II, Figure 6). Together, we could show that CaMV targets SGs through the P6 protein and inhibits their assembly in a partially translation dependent manner. This

mechanism however does not explain the assembly of SGs during CaMV infection, when translation levels are elevated and thus indicate the presence of a second mechanism.

3.7 Self-attenuation – a viral balancing act

For the obligate intracellular virus, a dead host is a bad host. Many lethal plant virus interactions occur because pathogen and host had not evolved together and adapted to each other's presence. This is one reason for the frequent outbreaks of virus disease in agricultural settings when naïve crop plants are introduced to new environments (Jones, 2009). With their limited coding capacity, viruses must employ their full force early in infection to overcome plant defences and establish the infection. However, many viruses self-attenuate during prolonged infections to ensure host survival during systemic infection. Especially the potent VSRs become inactivated over time. The CMV 2b protein is attenuated through interaction with the viral coat protein in systemic infection (Zhang *et al.,* 2017), as well as susceptible to degradation by the plant's autophagy pathway over time (Shukla *et al.,* 2021). The potyviral HC-Pro, is part of a polyprotein and only activated after cleavage. A gradual transition from fused HC-Pro and cleaved HC-Pro over time modulates its VSR strength (Pasin *et al.,* 2014). P6 is the VSR of CaMV and serves a plethora of functions that need to be regulated over time and space during CaMV infection. Over the infection time course, it undergoes a transition from mainly soluble to mainly condensed in large inclusions (Paper II, Figure 1). We propose that this transition helps in the attenuation of at least two major functions of P6, the translational transactivation and the suppression of canonical SGs (Paper II, Figure 6 & 7). Condensation levels can greatly influence the activity of a protein, as exemplified by Arabidopsis auxin response factors (Jing *et al.,* 2022; Powers *et al.,* 2019). We can mimic P6 condensation differences by fusion with fluorescent proteins, the condensation prone tagRFP and the soluble mRFP to the c-terminus of P6 (Paper II, Figure 7). These fluorescent tags are both monomeric, share the same weight (~25 kDA) and 56% sequence identity, but are derived from different organisms (Campbell *et al.,* 2002; Merzylak *et al.,* 2007) and in the case of P6 can drastically alter the behaviour of their fusion protein. P6-tagRFP localized mainly in large inclusions, while P6-mRFP remained mainly soluble with small foci formation. The soluble P6-mRFP was a

stronger suppressor of SG numbers after heat stress and showed a remarkedly stronger ability to transactivate *in planta*. P6-tagRFP was still able to supress SG numbers, albeit not as strongly as P6-mRFP, and the capacity for transactivation was drastically reduced, indicating that P6 functions are modulated and in the case of SG suppression and transactivation reduced by increasing levels of condensation during CaMV infection (Paper II, Figure 8). Importantly, we could disconnect P6 capacity for transactivation and SG suppression with a condensation-deficient n-terminal deletion mutant, that lost the ability for transactivation, but was still capable to repress SG induction after heat shock (Paper II, Figure 7). Whether P6 condensation affects its many other functions, including symptom elicitation (Figure 3), remains to be tested. Arabidopsis is a highly susceptible host of CaMV and does not exhibit recovery phenotypes, that is, newly emerging leaves remain symptomatic even in prolonged infections (Figure 2). It is hence not a suitable model to study symptom recovery, and it is possible that the pool of soluble P6 stays potent enough for symptom elicitation over the infection period. Kohlrabi (*Brassica oleracea-gongylodes*) on the other hand, is a tolerant host and exhibits recovery after infection with several CaMV strains (Al-Kaff & Covey, 1995). It could provide a good system to study the role of P6 condensation and its interplay with the RNA silencing machinery during symptom recovery (Ghoshal & Sanfaçon, 2015). The proposed self-attenuation mechanism provides CaMV with the means to balance host fitness and virus replication during prolonged infections, a battle that will entail many host factors which remain to be identified.

3.8 CaMV infection and natural variation - into the unknown

For me there are three major reasons to study plant viruses (and arguably many more):

I) Viruses cause damaging plant diseases that threaten current agricultural practices and must be contained to ensure food security.

II) They have evolved protein structures and mechanisms like no other organism that hold the potential to solve the very problem they cause.

III) Viruses are masters of manipulation and by understanding virus disease we can unravel hidden facts about their host organisms.

The CaMV *35S* promoter is invaluable for molecular biology and "*since its description in 1985 [...] has been the standard promoter used in all plant science and plant biotechnology, and has certainly propelled the research field forward like hardly any other discovery*" (direct quote from Somssich, 2019). While the economic impact of CaMV infections pales in comparison to other plant viruses, it occurs widespread in agriculturally used fields, causes symptoms, and can lead to drastic yield losses when co-infecting with other viruses (Bak & Emerson, 2020; Farzadfar et al, 2014; Spence *et al.,* 2007). But how does CaMV interact with and manipulate its hosts, and which factors influence CaMV disease? Through untargeted genotype-phenotype associations we can unravel the genetic bases of complex traits, like CaMV disease. In Paper III we use the disease differences of Arabidopsis accessions in response to CaMV infection to find underlying genes through GWA mapping. Plants exhibit phenotypic plasticity in response to changing environmental factors (Valladares *et al.,* 2007) and virus disease is no exception. A remarkable spectrum of disease outcomes dependent on host genotype was reported in crop plants, like Cassava (*Manihot esculenta*) in response to *Cassava brown streak virus* (CBSV, Sheat *et al.,* 2019), wheat (*Triticum aestivum*) in response to *Wheat dwarf virus* (WDV, Pfrieme *et al.*, 2022) and peanut (*Arachis hypogaea*) in response to *Tomato spotted wilt virus* (TSWV, Li *et al*., 2018). In Arabidopsis, several studies have focused on potyvirus disease, but they have mostly examined either plant or virus performance, not their correlation (see Table 1 for references). One strength

of our study is the simultaneous recording of plant and virus fitness, enabling us to identify resistant (high plant fitness, low virus accumulation), tolerant (high plant fitness, high virus accumulation), as well as susceptible (low plant fitness, high virus accumulation) and hypersensitive (low plant fitness, low virus accumulation) Arabidopsis genotypes (Paper III, Figure 7). We used relative fresh weight and macroscopic symptom severity to assess the plant fitness during CaMV infection (Paper III, Figure 1). CaMV is not transmitted vertically, making plant fecundity an irrelevant trait for the virus, while symptoms and growth (approximated through relative fresh weight) are well correlated with plant mortality and thus a good indicator for plant fitness (Doumayrou *et al.,* 2013). Importantly, symptom severity was poorly correlated with the amount of virus accumulating within the plant, indicating that resistance, as well as tolerance mechanisms shape the disease outcome (Paper III, Figure 2). This co-operation of tolerance and resistance within the same plant species is emerging as a common phenomenon in plant virus infections and exemplifies how dependent viruses are on the interactions with host components within their respective background and that a range of factors, including the viral and host proteins determine which mechanism will be the main driver in individual infections (González *et al.*, 2020).

Virus disease in plants can be affected by large effect size loci, like the recessive resistance against potyviruses or dominant resistance gene Tm-2 against Tobamoviruses. In many contexts however it is influenced by several smaller effect size loci, like we observed for CaMV (Paper III, Figure 3). GWA mapping associated several SNPs with differences in virus accumulation and subsequent testing of knock-out mutants of a subset of underlying genes confirmed a decrease in CaMV accumulation in about 50% of tested lines (Paper III, Figure 3). The strongest suppressor of CaMV accumulation was the *nced9* mutant, a gene implicated in ABA synthesis in the seed. *NCED9* transcripts are not detected in rosette tissue under undisturbed growth conditions, but can be induced through drought (Iuchi *et al.,* 2001) and CaMV infection (Paper III, Figure 5). Plant virus infections cause large disturbances in the plant transcriptome that can lead to ectopic accumulation of organ or development specific transcripts in the plant (Chesnais *et al.,* 2022; Liu *et al.,* 2019; Ghorbani *et al.,* 2018; Li *et al.,* 2017). Liu and colleagues for example, identified the seed germination modulator REDUCED DORMANCY 5 (RDO5) as an enhancer of resistance against CMV (Liu *et al.,* 2022; Xiang *et al.,* 2014). This begs the question whether

both, plant and virus could reactivate transcripts to either enhance resistance, as in the case of *RDO5* or activate susceptibility, as for *NCED9*. To our surprise, the resistance of *nced9* against CaMV was not based on reduced ABA levels, as exogenous application did not rescue virus accumulation in *nced9*. This contrasts virus behavior in Col-0 and the severe ABA mutant *aba2-1* where exogenous application of ABA rescued the virus and, in both backgrounds, increased the amount of viral DNA in a dose-dependent manner (Paper III, Figure 6). The *nced9* mutant is not insensitive to ABA (Paper III, Figure 6) and does not have altered ABA levels in mature leaves per se (Lefebvre *et al.,* 2006), but the pro-viral effect of ABA does not reach the virus. Additional experiments are required to dissect the effect of NCED9 on CaMV multiplication in Arabidopsis, but despite the uncoupling of NCED9 and ABA in this background, ABA did have a positive effect on CaMV accumulation in Col-0 and *aba2-1* (Paper III, Figure 6). ABA was shown to increase during the infection time course with different plant viruses (Cui *et al.,* 2021; Alazem *et al.,* 2014; Whenham *et al.,* 1986) although other viruses cause a downregulation of ABA-responsive genes (He *et al.,* 2021) and the pre-treatment of plants with ABA often increases plant resistance against invading viruses (Pasin *et al.,* 2020; Iriti & Faoro, 2008; Chen *et al.,* 2013).This is the first report of this important plant hormone positively influencing CaMV accumulation. Interestingly, it seems to be that the disruption of ABA pathways either through overstimulation by exogenous ABA application or downregulation through blocking of ABA biosynthesis, rather than the hormone level itself aids virus multiplication. This is exemplified by the large number of ABA responsive genes that are deregulated during CaMV infection (Paper III, Figure 6). A similar phenotype was observed in Arabidopsis with CMV, where virus infected tissues did not accumulate different levels of ABA, but the expression of ABA responsive genes was altered by virus infection, or the expression of the viral VSR protein (Westwood *et al.*, 2013). It is interesting to speculate whether P6 is responsible for the disruption of ABA signaling during CaMV infection. CaMV virulence in Arabidopsis accessions changes in response to water deficit (Bergès *et al.,* 2021; Bergès *et al.,* 2020; Bergès *et al.,* 2018). Berges and colleagues showed that 16 out of 24 tested Arabidopsis accessions accumulated more CaMV DNA under drought stress compared to well-watered conditions. The drought-stressed plants are likely to contain more ABA which could aid CaMV to accumulate to higher levels.

3.9 Main conclusions in a nutshell

I. CaMV VFs are (exclusive) melting pots for PB and SG proteins that can move within the structure.

II. PB proteins DCP5 and LSM1 serve pro-viral roles during CaMV infection by shielding the viral RNA from RDR6 mediated translational repression.

III. CaMV uses the nonspecific RNA-binding capacity of RNA granule proteins to sequester SG proteins, while PB proteins access the viral *35S* RNA.

IV. CaMV *8S* RNA possibly acts as a lncRNA sponge for SG proteins.

V. CaMV interferes with SG assembly through two independent P6 mechanisms that involve translation and condensation.

VI. PBs and SGs should always be referred to by the marker-line used in the study, ideally several markers should be tested.

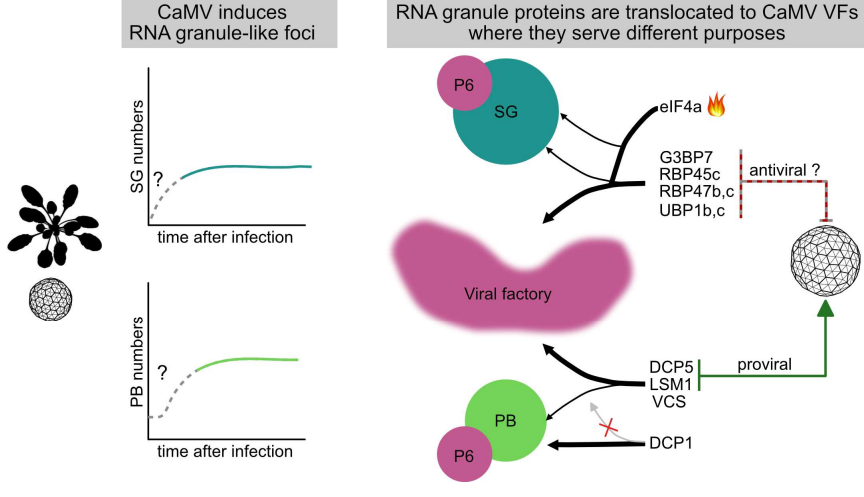

Figure 12: Graphical summary of the main findings in Paper I and Paper II (points I-VI). CaMV infection induces SG-like and PB-like foci and accumulates several RNA granule proteins within its VFs. PBs serve pro-viral roles, while antiviral SGs are disarmed.

VII. CaMV induces a wide spectrum of symptoms in natural Arabidopsis accessions that are only weakly correlated with virus accumulation, indicating that tolerance and resistance govern CaMV disease in Arabidopsis.

VIII. Disruption of *NCED9* provides recessive resistance against CaMV, but not other viruses.

IX. The *nced9* resistance cannot be reversed by exogenous ABA application, indicating an independent mechanism.

X. Disruption of ABA homeostasis is beneficial for CaMV and is reflected in the massive deregulation of ABA responsive genes.

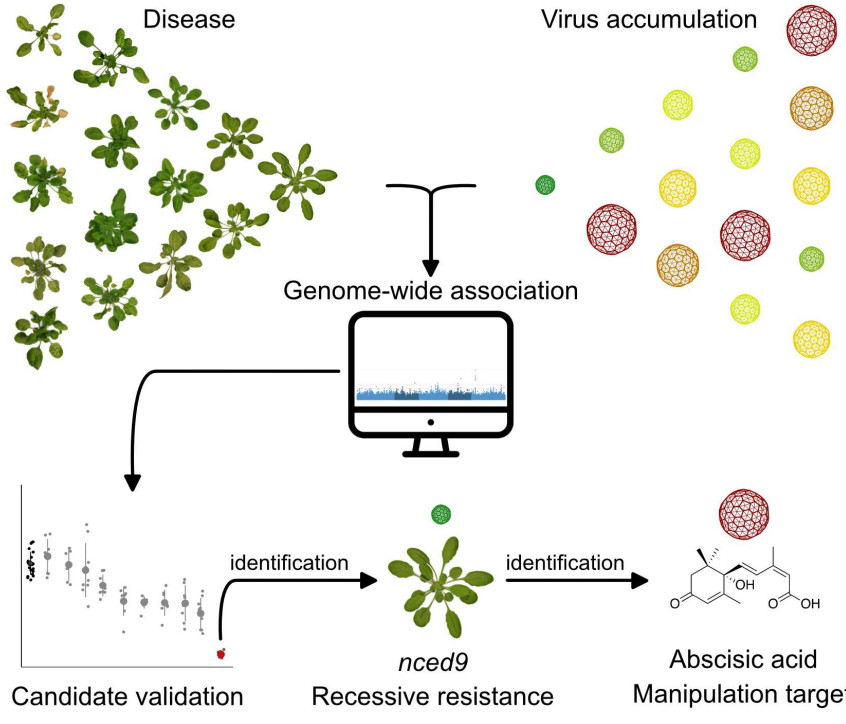

Figure 13: Graphical summary of the main findings in Paper III (points VII-X). CaMV disease spectrum in Arabidopsis does not reflect viral accumulation within single accessions. GWA-mapping identified several susceptibility factors, the strongest, *NCED9*, led to the discovery of ABA manipulation by CaMV.

4. Future perspectives

When I started my Bachelor in 2012, we had never heard about *Tomato brown rugose fruit virus* (ToBRFV). By the time I began my Master degree in 2016, ToBRV had spread from its origin in Israel to Jordan. In 2018, when I moved to Sweden for my PhD, the virus was reported in Germany, Italy, the US, Mexico and Palestine. Now that I am finishing my studies, the *European and Mediterranean Plant Protection Organization* (EPPO) lists 31 countries that have reported ToBRV outbreaks. This is one example of how (plant) viruses can emerge suddenly, spread rapidly, cause devastating epidemics and are notoriously difficult to control (Jones, 2021).
But how to fight the hydra when for every solved problem two new ones emerge? It will quite literally be a Herculean task requiring several disciplines to come together to understand virus epidemiology, transmission and biology in a race against evolving viruses and a changing climate.

Building on Paper I, viral RNA homeostasis and translation are two promising targets for virus resistance, as every single virus independent of their replication strategy, host range or transmission mode must use the host's machinery to translate viral RNA into viral proteins in large quantities. CaMV employs unique translation strategies that can teach about translation regulation and manipulation.

We have started an experiment to profile translation by ribosome footprints in conjunction with transcription by total RNA deep sequencing in mock and CaMV infected Col-0 and *dcp5* plants. The samples were taken together with the siRNA data used in Paper I and as a compendium will enable us not only to identify splice variants and translational transactivation marks on the viral genome, but also the effect on global translation *in planta* (Ingola, 2016). Possibly, we will be able to disseminate a group of RNAs

with an unchanged abundance between Col-0 and *dcp5*, but a reduced translation in the mutant, similar to CaMV *35S* (Paper I, Figure 7). This could point us to an endogenous pathway that CaMV is exploiting to ensure efficient translation through PB components in the presence of RNA silencing.

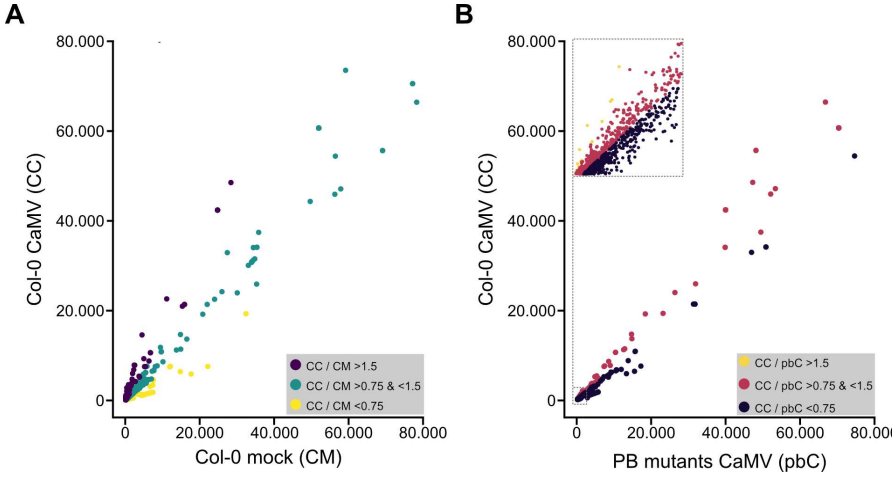

Figure 14: CaMV infection alters siRNA pressures on endogenous genes dependent on the host's genetic background. [A] DeSeq2 normalized counts of siRNA (18-26 nts) on Arabidopsis genetic elements in mock and CaMV infected Col-0 plants. [B] DeSeq2 normalized counts of siRNA (18-26 nts) on Arabidopsis genetic elements in CaMV infected Col-0 and CaMV infected *dcp5/lsm1* infected plants. Genetic elements were considered for this analysis if they behaved similar in *dcp5* and *lsm1* (ratio between 0.75-1.5) and averaged as "PB mutants". In both plots, each dot represents a distinct genetic locus with at least 50 normalized counts in CaMV Col-0 samples and averaged between two biological replicates. Colours indicate the ratio of normalized counts between CaMV infected and mock plants as indicated in the legends. For bioinformatic pipeline refer to Paper I Material & Methods section.

The siRNA data generated for Paper I did not show altered siRNA targeting of the viral RNA (Paper I, Figure 5), but CaMV does induce changes in siRNA populations on endogenous genes (Figure 14A) that are to some extend dependent on the genetic background of the plant (Figure 14B). Combining the siRNA data with translational data will help to identify siRNA pressures exerted on endogenous genes and their potential for translational repression in undisturbed growth and during virus infection. In addition, these profiles can be used as an untargeted approach to identify host genes under transcriptional or translational regulation during CaMV

infection and point towards novel pathways influencing virus disease. Altogether, genome-wide siRNA, total RNA and translating RNA levels will provide a powerful opportunity to find the hidden sides of CaMV infection in Arabidopsis.

The nature and composition of CaMV VFs still remains enigmatic despite six decades of research. VFs differ in size and numbers depending on the CaMV isolate, as well as on the host (Shalla *et al.,* 1980; Paper I Figure 1), indicating that virus- as well as host factors shape their morphology. Based on Paper I and II, we have identified several host RBPs present within VFs during infection, but these proteins likely represent only the tip of the iceberg. Proteomic methods to identify host proteins present in CaMV VFs would greatly further our understanding of the viral replication strategy and which host proteins are involved. However, this approach comes with many caveats that need to be addressed with the three main concerns being:

I) Purification of VFs without contamination of nuclei or transmission bodies
II) Elimination of viral proteins that will mask low abundance host proteins
III) Fixation of transient interactors during the isolation process

It is possible to enrich cell fractions for VFs by low-speed centrifugation of crude extracts, but these cell fractions will contain plant nuclei too. Arabidopsis INTACT lines (Deal & Henikoff, 2011) or an additional fluorescence activated cell sorting (FACS) step could purify the enriched fractions from nuclei, to avoid contaminations. Several methods have been developed to eliminate highly abundant proteins in animals (Bellei *et al.,* 2011) and plants (Li *et al.,* 2008) that could be adapted to remove viral proteins from purified VF fractions, as these proteins will constitute the vast majority and mask the presence of host proteins. We found for most proteins that they are highly mobile within VFs and likely associate with it transiently. Fixation methods in the beginning of the isolation are likely needed to not lose transiently interacting host proteins that could constitute a major part of the VF interactome, as they do in other phase-separated compartments (Gutierrez-Beltran *et al.,* 2021). This fixation needs to be reversible though to ensure viral protein depletion at a later step. When the isolation of pure, host factor enriched VFs is successful, it will further our understanding of

these unique inclusions tremendously and bring us one step closer to deciphering the biology of its main constituent – the P6 protein.

CaMV is widespread in domesticated and wild Brassicaceae species (Farzadfar et al, 2014; Pagán *et al.,* 2010; Raybould *et al.,* 1999), often without any apparent symptom development. A survey of 14 farms located around Nairobi, Kenya revealed that of 18 vegetable crops virtually all were infected with CaMV or a combination of CaMV and TuMV (Spence, 1999). A field study with wild *Brassica oleracea* in Dorset, UK found that CaMV was the most prevalent virus in four out of five sampled populations and that CaMV infection positively correlated with the presence of TuMV, *Beet western yellows virus* (BWYL), and *Turnip yellow mosaic virus* (TYMV) (Raybould *et al.,* 1999). Similar results were obtained in wild Arabidopsis populations on the Iberian Peninsula were CaMV was the second most frequent virus after CMV and CaMV infection was positively correlated with TuMV, CMV and TCV infections (Pagán *et al.,* 2010). Our results in Paper III show that CaMV infections cause many different disease outcomes that are dependent on the host's genetic background. Judging from the earlier fieldwork, the importance of CaMV in co-infections with other viruses should be addressed in the context of resistant genotypes and natural variation in *Arabidopsis thaliana*, as well as other Brassicaceae species. CaMV appears to facilitate higher accumulation levels of co-infecting viruses and it is possible that the translational transactivator functions of CaMV P6 are exploited by these other viruses to facilitate their own translation. An independent study on *Plantago lanceolata* virus communities on the island of Åland also detected a positive correlation of the Caulimovirus *Plantago lanceolata latent virus* (PLLV) with several co-occurring viruses (Sallinen *et al.,* 2020), indicating that phenomenon could be widespread for the Caulimoviruses. This would make Caulimoviruses, like CaMV potent helper viruses and a threat for emerging plant diseases, some of the most devastating of which are caused by viral co-infections (Jones, 2021).

Viruses are ancient. With their origins possibly dating back to the precellular time (Koonin & Dolja, 2014), signatures of past virus infections can be found in all cellular organisms (Diop *et al.,* 2018; Mushegian & Elena, 2015; Patel *et al.,* 2011). Viruses had ample time to evolve and diversify mechanisms to interact with their hosts, especially with conserved structures like the

cytoplasmic RNA granules, which are themselves proposed to be "*remnants of an ancient RNA world*" (Anderson & Kedersha, 2009). The diverse ways viruses use to subvert RNA silencing (Figure 5), another broadly conserved mechanism, shows the versatility of a comparative virology approach to uncover mechanisms of virus infections (Shukla *et al.,* 2019). In this thesis we have established several marker lines and experimental pipelines to facilitate the screening of PB and SG responses to viral infections, as well as study their connections with other RNA regulatory pathways. This, together with the collection of viruses available in the lab, enables us to efficiently and systemically unravel SG and PB dynamics in Arabidopsis during diverse virus infections, we can identify commonalities and differences in viral mitigation strategies and further our understanding of how viruses achieve RNA homeostasis during prolonged infections. By finding common targets of viruses within RNA granules, we could identify ancient partners in the virus-host interactome, because nothing in biology makes sense except in the light of interaction.

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Popular science summary

When humans inhabit new land, we scout the area for food and water, for dangers and hazards. After finding a suitable patch of land to settle down, we build shelters against the weather, build infrastructures, and change the environment to sustain our living. In this aspect, viruses are no different from us, except that viruses are tiny. So tiny that a single cell can sustain them, and a plant is like a continent waiting to be conquered. Within a cell, viruses build shelters, the viral replication factories, to shield themselves from the cellular defences and facilitate their rapid multiplication. They build infrastructure to move from cell to cell and quickly throughout the host, like boats on a river and they change their host's biology to allocate resources for the virus and provide for its needs. To do so viruses come with a very limited, but extremely versatile toolset and millennia of conquering new hosts have made them the master manipulators of biology.

Architecturally, viruses are simple beings. They consist of information in the form of nucleic acids, which is basically the blueprint of the virus and an executive force, the proteins. Because this blueprint is limited in size, viruses rely on their hosts to provide more workforce.

But plants do not share their resources freely and have set up several monitoring and defence outposts to stop the invading virus, to slow it down or to starve it out. Every virus carries signatures, like a banner, that plants recognize and try to destroy. One of these banners is the viral RNA, a molecule that contains the information for the workforce. No matter how the blueprint of a virus looks, all must rely on the use of RNA at one point in their replication cycle. In this thesis we looked at how the *Cauliflower mosaic virus* (CaMV) hides its RNA from the plants defence systems by beating the plant at its own game. CaMV uses the plants own workforce and

invites them into its shelter to provide for it within the save walls of the viral replication factory, while staying hidden from others.
Different plants react differently to an invading virus based on their own blueprint. By using plants from different regions of the world, we could show a dramatic range of symptoms after CaMV infection, as well as differences in how well the virus conquered the new territory. By comparing 100 plant populations we could find that a small part of the plants own blueprint was responsible for some of the variation we saw and that when we deleted this part, CaMV could not accumulate anymore. We do not yet know what this little piece of information does for the virus, but it shows that plants all over the world have already come up with solutions to our questions, we just need to find them.

Populärwissenschaftliche Zusammenfassung

Wenn Menschen neue Gebiete besiedeln, erkunden wir die Landschaft, suchen nach Nahrung und Wasser und halten Ausschau nach potenziellen Gefahren. Nachdem wir ein geeignetes Stück Land gefunden haben, um uns niederzulassen, bauen wir Unterkünfte zum Schutz vor der Witterung, etablieren Infrastrukturen und verändern die Umwelt, um unseren Lebensunterhalt zu sichern. In dieser Hinsicht unterscheiden sich Viren nicht von uns, abgesehen davon, dass Viren winzig klein sind. So winzig, dass eine einzelne Zelle sie ernähren kann, und eine Pflanze wie ein Kontinent ist, der darauf wartet, erobert zu werden. Innerhalb einer Zelle bauen Viren Schutzräume auf, die viralen Replikationsfabriken, um sich vor der zellulären Abwehr zu schützen und ihre schnelle Vermehrung zu erleichtern. Sie bauen eine Infrastruktur auf, um sich von Zelle zu Zelle und schnell durch den Wirt zu bewegen, wie Boote auf einem Fluss, und sie ändern die Biologie ihres Wirts, um Ressourcen für sich selbst bereitzustellen und ihre Bedürfnisse zu befriedigen. Zu diesem Zweck verfügen Viren über ein sehr begrenztes, aber äußerst vielseitiges Instrumentarium und Jahrtausende der Eroberung neuer Wirte haben sie zu Meistermanipulatoren der Biologie gemacht.

Viren sind architektonisch einfache Wesen. Sie bestehen aus Informationen in Form von Nukleinsäuren, die im Grunde der Bauplan des Virus sind, und einer ausführenden Kraft, den Proteinen. Da dieser Bauplan sehr begrenzt ist in seiner Größe, verlassen sich Viren darauf, dass ihre Wirte mehr Arbeitskräfte zu ihrer Verfügung bereitstellen.

Aber Pflanzen teilen ihre Ressourcen nicht freiwillig und haben mehrere Überwachungs- und Verteidigungsposten eingerichtet, um das eindringende Virus zu stoppen, zu verlangsamen oder auszuhungern. Jeder Virus trägt Signaturen wie ein Banner, das Pflanzen erkennen und versuchen zu

zerstören. Eines dieser Banner ist die virale RNA, ein Molekül, das die Informationen für die Proteine enthält. Egal, wie der Bauplan eines Virus aussieht, alle müssen sich an einem Punkt ihres Replikationszyklus auf die Verwendung von RNA verlassen. In dieser Doktorarbeit untersuchten wir, wie das Blumenkohl Mosaik Virus (CaMV) seine RNA vor den Abwehrsystemen der Pflanze verbirgt, indem es die Pflanze mit ihren eigenen Waffen schlägt. CaMV nutzt die werkseigenen Arbeitskräfte und lädt sie in seinen Unterschlupf ein, um innerhalb der sicheren Mauern der viralen Replikationsfabrik von ihnen zu profitieren, während sie vor anderen verborgen bleiben.

Verschiedene Pflanzen reagieren je nach ihrem eigenen Bauplan unterschiedlich auf ein eindringendes Virus. Durch die Verwendung von Pflanzen aus verschiedenen Regionen der Welt konnten wir ein weites Spektrum an Symptomen nach einer CaMV-Infektion zeigen, sowie Unterschiede darin, wie gut das Virus das neue Territorium erobert hat. Durch den Vergleich von 100 unterschiedlichen Pflanzengruppen konnten wir feststellen, dass ein kleiner Teil des Bauplans für einen Teil der Variation verantwortlich war, die wir sahen, und dass sich CaMV nicht mehr vermehren konnte, als wir diesen Teil aus dem Bauplan löschten. Wir können noch nicht sagen, was diese kleine Information für das Virus bedeutet, aber sie zeigt, dass Pflanzen auf der ganzen Welt bereits Antworten auf unsere Fragen haben, wir müssen sie nur finden.

Populärvetenskaplig sammanfattning

När människor bosätter sig i nya områden utforskar vi landskapet efter mat, vatten, och faror. Efter att ha hittat en lämplig plats att slå sig ner på, bygger vi skydd mot vädret, bygger infrastruktur och förändrar miljön för att möta våra behov. I denna aspekt liknar virus oss, förutom att virus är små. Så små att en enda cell kan upprätthålla dem och en växt är som en kontinent som väntar på att bli erövrad. Inom en cell bygger virus skyddsrum, virusreplikationsfabrikerna, för att skydda sig från cellulära försvar och underlätta deras snabba förökning. De bygger infrastruktur för att flytta från cell till cell och snabbt genom hela värden, som båtar på en flod, och de ändrar sin värds biologi för att allokera resurser till viruset och tillgodose dess behov. För att göra det har virus en mycket begränsad, men extremt mångsidig verktygslåda och årtusenden av erövring av nya miljöer (nya värdar) har gjort dem till biologins mästermanipulatörer. Arkitektoniskt är virus enkla varelser. De består av information i form av nukleinsyror, som i grunden är virusets ritning, och en verställande kraft; proteinerna. Eftersom denna ritning är begränsad i storlek, litar virus på att deras värdar ska tillhandahålla mer arbetskraft (proteiner) som tillhandahålls av växten. Men växter delar inte sina resurser fritt och har satt upp flera övervaknings- och försvarsposter för att stoppa det invaderande viruset, för att bromsa det eller för att svälta ut det. Varje virus bär signaturer, som en banderoll, som växter känner igen och försöker förstöra. En av dessa banderoller är viral RNA, en molekyl som innehåller information för arbetsstyrkan. Oavsett hur ritningen av ett virus ser ut, måste alla förlita sig på användningen av RNA vid ett tillfälle i sin replikationscykel. I avhandlingen undersökte vi hur blomkålsmosaikviruset (CaMV) döljer sitt RNA från växternas försvarssystem genom att slå växten i sitt eget spel. CaMV använder växternas egna arbetskraft och bjuder in den till sitt skydd för att försöja viruset inom virusreplikeringsfabrikens säkra väggar, samtidigt som de håller sig gömda från andra. Olika växter reagerar olika på ett invaderande virus baserat på växtens egen ritning. Genom att använda växter från olika regioner i världen kunde vi visa ett dramatiskt antal symtom efter CaMV-infektion, såväl som skillnader i hur väl viruset erövrade det nya territoriet. Genom att jämföra 100 växter kunde vi konstatera att en liten del av ritningen var ansvarig för en del av variationen vi såg, och när vi tog bort denna del från ritningen kunde CaMV inte ackumuleras längre. Vi vet ännu inte vad

den här lilla informationen gör för viruset, men den visar att växter över hela världen redan har kommit med lösningar på våra svar, vi behöver bara hitta dem.

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I can't believe it's been 10 years since I was rejected from studying psychology in Berlin. 10 years since I started my bachelor's in biology, being sure I will go into Neuroscience. 9 years since I took my first botany class, falling deeply in love with plants and 8 years since I my first internship working with plant pathogens. And now here I am at the end of this long and winding road, becoming a Doctor of Biology and not of Psychology, as 18-year-old me thought.

O Captain! my Captain! our fearful trip is done,
The ship has weather'd every rack,
The price we sought is won.

- Walt Whitman

Anders, I want to thank you for celebrating our wins with me and building me up after our losses. For asking me "what's the worst thing that could happen?" when I was already expecting everything short of a zombie apocalypse. For sharing your excitement for viruses and boats with me. For encouraging my independence. For your trust. For staying calm, when I couldn't. And through all the difficulties we had and the stress and the conflicts that a PhD and a life in academia brings with it, thank you for steering this ship, thank you for your supervision and for continuing the tradition of being a super-supervisor ;).

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I am rooted, but I flow.
The waves - ***Virginia Woolf***

I am incredibly privileged to have people in my corner who believe in me and encourage me every step of the way.
To my family, a million thank you's!
To Mimi & Opa, who taught me that love can be unconditional.
To my Muzz, who taught me to go my way and enjoy life unapologetically.
To Günter for his incredible kindness.
To Paps & Katrin for sharing their love for nature and biology with me.
To Dea for always seeing the best in things.
Danke, dass ihr an meiner Seite seid.

These pages are the third thesis that Bente had to read from me. And as much as I enjoy making you read fun facts about plants, fungi and viruses and you making me read (not so fun) facts about chromatin and epigenetics – let's not do that again any time soon. Let's go and enjoy the world. Because no matter if in Vienna, Tokio, Nairobi or Golm – *I choose you. Thank you.*

Friends are the family you choose, and I am so grateful to all the people I have met along my studies. To my Schweinebären in Cologne, to the A-lab survivors in Vienna, to the German connection in Uppsala – I couldn't have done it without you.
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To Anis, I have no words. Thank you for letting me be part of your world and for expanding my horizon. ♪*I love a song, I love a dance, I love a laugh*♪

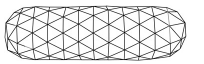
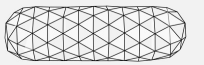
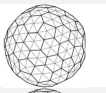
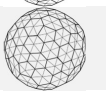
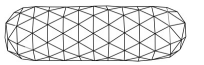
To all the great people in the department, especially my PhD-cohort: Thank you for the nice hallway chats, the Fikas, the late-night labs, the beer hours and the parties. I love our cultural mash up and I will take so many memories with me. Keep on keeping on!

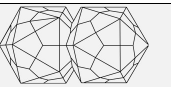
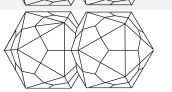
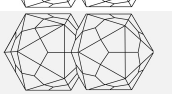
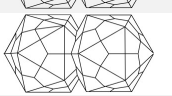
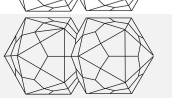
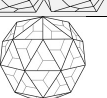
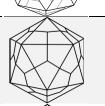
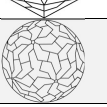
the future
world of our dreams
can´t be built on the
corruptions of the past
Tear it down – ***Rupi Kaur***

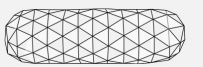
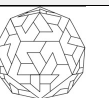
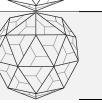
I do not want to gloss over the struggle.
There were times I was sure I was not going to complete this PhD. Times that I came home crying because I felt inadequate, overworked, directionless and alone. No matter how good your social network is, how great your supervisor, the academic system is built on exploitation, nepotism, and insecurity. We are playing by an antiquated rule book with toxic power dynamics. We pretend to be oblivious to the dynamics in society & politics and stay frozen in our comfortable ivory tower when we should be taking to the streets and leading the fight for progress. I do not believe, no, I do not accept that it has to be this way! Science is a beautiful endeavour, it is a privilege to explore and learn, but the system we are forcing it into is suffocating. It is on us to change it; it is on us to build the future world of our dreams!

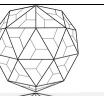
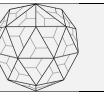
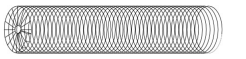
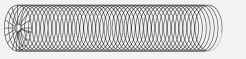
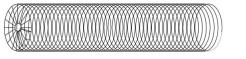
Appendix

Table 3: List of plant viruses mentioned in this thesis, sorted by family. Particles were designed based on the Expasy Viral zone page. (https://viralzone.expasy.org/.)

ACRONYM	VIRUS	GENUS	FAMILY	GENOME	PARTICLE
PVX	*Potato virus X*	Potexvirus	Aplhaflexivirus	ssRNA(+)	
BMV	*Brome mosaic virus*	Bromovirus	Bromoviridae	ssRNA(+)	
CMV	*Cucumber mosaic virus*	Cucumovirus	Bromoviridae	ssRNA(+)	
BSV	*Banana streak virus*	Badnavirus	Caulimoviridae	DNA-RT	
CSSV	*Cocoa swollen shoot virus*	Badnavirus	Caulimoviridae	DNA-RT	
CAMV	*Cauliflower mosaic virus*	Caulimovirus	Caulimoviridae	DNA-RT	
PLLV	*Plantago lanceolata latent virus*	Caulimovirus	Caulimoviridae	DNA-RT	
RTBV	*Rice tungro baciliform virus*	Tungrovirus	Caulimoviridae	DNA-RT	
SPCSV	*Sweet potato chlorotic stunt virus*	Crinivirus	Chlosteroviridae	ssRNA(+)	

CTV	*Citrus tristeza virus*	Closterovirus	Closteroviridae	ssRNA(+)	
AMV	*Abutilon mosaic virus*	Begomovirus	Geminiviridae	ssDNA	
CALCUV	*Cabbage leaf curl virus*	Begomovirus	Geminiviridae	ssDNA	
ACMV	*African cassava mosaic virus*	Begomovirus	Geminiviridae	ssDNA	
TYLCV	*Tomato yellow leaf curl virus*	Begomovirus	Geminiviridae	ssDNA	
WDV	*Wheat dwarf virus*	Mastrevirus	Geminiviridae	ssDNA	
BWYL	*Beet western yellows virus*	Luteovirus	Luteoviridae	ssRNA(+)	
BBTV	*Banana bunchy top virus*	Babuvirus	Nanoviridae	ssDNA	
PNYDV	*Pea necrotic yellow dwarf virus*	Nanovirus	Nanoviridae	ssDNA	
WCCV	*White clover cryptic virus*	Alphapartitivirus	Partitiviridae	dsRNA	
CBSV	*Cassava brown streak virus*	Ipomovirus	Potyviridae	ssRNA(+)	

PPV	*Plum pox virus*	Potyvirus	Potyviridae	ssRNA(+)	
PVA	*Potato virus A*	Potyvirus	Potyviridae	ssRNA(+)	
PVY	*Potato virus Y*	Potyvirus	Potyviridae	ssRNA(+)	
TBV	*Tulip breaking virus*	Potyvirus	Potyviridae	ssRNA(+)	
TEV	*Tobacco etch virus*	Potyvirus	Potyviridae	ssRNA(+)	
TUMV	*Turnip mosaic virus*	Potyvirus	Potyviridae	ssRNA(+)	
BYSMV	*Barley yellow striate mosaic virus*	Cytorhabdovirus	Rhabdoviridae	ssRNA(-)	
ALV	*Arabidopsis latent virus 1*	Comovirus	Secoviridae	ssRNA(+)	
TOBRV	*Tomato black ring virus*	Nepovirus	Secoviridae	ssRNA(+)	
RTSV	*Rice tungro spherical virus*	Waikavirus	Secoviridae	ssRNA(+)	
TROV	*Turnip rosette virus*	Sobemovirus	Solemoviridae	ssRNA(+)	

CCFV	*Cardamine chlorotic fleck virus*	Betacarmovirus	Tombusviridae	ssRNA(+)	
TCV	*Turnip crinkle virus*	Betacarmovirus	Tombusviridae	ssRNA(+)	
PLPV	*Pelargonium line pattern virus*	Pelarspovirus	Tombusviridae	ssRNA(+)	
GRV	*Groundnut rosette virus*	Umbravirus	Tombusviridae	ssRNA(+)	
TSWV	*Tomato spotted wilt virus*	Orthotospovirus	Tospoviridae	ssRNA(-)	
TYMV	*Turnip yellow mosaic virus*	Tymovirus	Tymoviridae	ssRNA(+)	
ORMV	*Oilseed rape mosaic virus*	Tobamovirus	Virgaviridae	ssRNA(+)	
TOBRFV	*Tomato brown rugose fruit virus*	Tobamovirus	Virgaviridae	ssRNA(+)	
TRV	*Tobacco rattle virus*	Tobravirus	Virgaviridae	ssRNA(+)	

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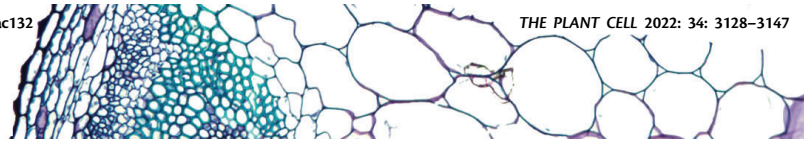

Arabidopsis RNA processing body components LSM1 and DCP5 aid in the evasion of translational repression during *Cauliflower mosaic virus* infection

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G.H. and A.H. designed the experiments and wrote the manuscript. G.H. conducted the experiments, analyzed the data, and designed the figures. Polysome analysis was conducted and analyzed together with A.M. and J.H. H.B. advised on library preparation and analyzed the sRNA data together with G.H. D.G. provided the *upf1*, *upf1 dpc5*, and DCP5-GFP Arabidopsis lines. All authors edited the manuscript and approved the final version.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plcell) is: Anders Hafrén (anders.hafren@slu.se).

Abstract

Viral infections impose extraordinary RNA stress, triggering cellular RNA surveillance pathways such as RNA decapping, nonsense-mediated decay, and RNA silencing. Viruses need to maneuver among these pathways to establish infection and succeed in producing high amounts of viral proteins. Processing bodies (PBs) are integral to RNA triage in eukaryotic cells, with several distinct RNA quality control pathways converging for selective RNA regulation. In this study, we investigated the role of *Arabidopsis thaliana* PBs during *Cauliflower mosaic virus* (CaMV) infection. We found that several PB components are co-opted into viral factories that support virus multiplication. This pro-viral role was not associated with RNA decay pathways but instead, we established that PB components are helpers in viral RNA translation. While CaMV is normally resilient to RNA silencing, dysfunctions in PB components expose the virus to this pathway, which is similar to previous observations for transgenes. Transgenes, however, undergo RNA quality control-dependent RNA degradation and transcriptional silencing, whereas CaMV RNA remains stable but becomes translationally repressed through decreased ribosome association, revealing a unique dependence among PBs, RNA silencing, and translational repression. Together, our study shows that PB components are co-opted by the virus to maintain efficient translation, a mechanism not associated with canonical PB functions.

Introduction

Eukaryotic gene expression is tightly regulated from RNA transcription to translation and decay. The importance of posttranscriptional control, especially during stress-induced cellular reprogramming, is becoming increasingly evident, as several studies have revealed extensive uncoupling between transcriptomes and translatomes (Branco-Price et al., 2005; Tebaldi et al., 2012; Liu et al., 2013; Zid and O'Shea, 2014; Xu et al., 2017).

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IN A NUTSHELL

Background: Viruses are unique in their ability to reuse and recycle host proteins and other components for their own benefit. *Cauliflower mosaic virus* (CaMV) forms special structures inside the host cells known as viral factories (VFs) to facilitate efficient replication and escape defense. VFs consist of viral proteins, as well as particles and nucleic acids, but also numerous host proteins and ribosomes that are co-opted into these structures. Building on knowledge from the animal field, RNA granules, including stress granules and processing bodies (PBs), are at the forefront of viral disease regulation. Several granule-localized proteins directly interact and influence virus replication.

Question: We investigated the role of PB components in CaMV infection. We wanted to elucidate the interplay from two sides: What is the effect of CaMV infection on the localization and abundance of PB components, but also how do these proteins influence CaMV replication and especially viral protein production?

Findings: Decapping proteins DCP5 and LSM1 localize to VFs during CaMV infection. CaMV DNA and protein accumulation, but not RNA levels, are reduced in Arabidopsis *dcp5* and *lsm1* mutants. We found that viral RNA is not a target of LSM1-mediated decapping and that RNA stability is not affected in either mutant. We examined *dcp5* and *lsm1* single mutants as well as double mutants with *RNA-dependent RNA polymerase 6* (*rdr6*), finding that less viral RNA was associated with ribosomes in the single but not double mutants. Thus, PB proteins help the virus evade translational repression by RDR6.

Next steps: We do not yet know how RDR6 mediates translational repression of viral RNA in the absence of DCP5 or LSM1. Elucidating the exact mechanism and which roles the VF and viral proteins play in this interaction will help further our understanding of plant virus infections.

Due to the high energy cost and possible detrimental effects of uncontrolled protein translation, eukaryotic cells have evolved a network of pathways to govern and regulate mRNA translation, including the "mRNA cycle" (Buchan and Parker, 2009). Here, cytoplasmic mRNAs are channeled between ribosomes and phase-separated cytoplasmic ribonucleoprotein (RNP) complexes, the RNA granules, in a triage between translation, nontranslating storage, and degradation. Several types of RNA granules have been identified and defined by their core protein constituents (Chantarachot and Bailey-Serres, 2018; Xing et al., 2020). The mRNA cycle involves two major types of RNA granules, processing bodies (PBs) and stress granules (SGs). RNAs are thought to shuffle between active translation at ribosomes and translationally repressed states at SGs (Buchan and Parker, 2009). In contrast, the localization of RNAs to PBs is mainly associated with RNA degradation owing to the absence of translation initiation factors and the highly conserved PB core components involved in RNA nonsense-mediated decay (NMD), miRNA-targeted gene silencing, deadenylation, and decapping (Anderson and Kedersha, 2009). Yet, while PB proteins can facilitate translational repression (Xu and Chua, 2009), recent studies have shown that PB-associated mRNAs can be stabilized and return to translation, expanding the multifunctionality of these RNA granules (Hubstenberger et al., 2017; Wang et al., 2018; Jang et al., 2019).

One hallmark of PBs is the accumulation of proteins required for mRNA decapping. This process involves the removal of the 7-methyl-guanosine 5′-diphosphate (cap) and is essential for subsequent 5′- to 3′-end mRNA degradation. In *Arabidopsis thaliana* (Arabidopsis), decapping is carried out by the nudix hydrolase DECAPPING2 (DCP2) and its cofactors DCP1 and VARICOSE (VCS; Xu et al., 2006). Several proteins function in decapping activation and PB assembly, including DCP5 and the SM-like (LSM) 1–7 complex (Xu and Chua, 2009; Perea-Resa et al., 2012). Uncapped RNAs are degraded by the cytoplasmic EXORIBONUCLEASE 4 (XRN4), which was also shown to accumulate in PBs (Souret et al., 2004; Yu et al., 2019). The decapping machinery is one part of the extensive RNA surveillance network present in PBs and is tightly connected to NMD (Chicois et al., 2018). NMD is governed by the surveillance protein UP FRAMESHIFT1 (UPF1), which in combination with other factors monitors RNAs for insufficient translation termination or the presence of exon junction complexes in the 3′-untranslated region (UTR) and subsequently induces their degradation. Interestingly, UPF1 not only associates with PBs but was also found to co-localize and shuffle between another class of cytoplasmic RNP granules, the small interfering (si)RNA bodies (Moreno et al., 2013). siRNA bodies are condensates of RNA-DEPENDENT POLYMERASE6 (RDR6), SUPRESSOR OF GENE SILENCING3 (SGS3), and ARGONAUTE7, as well as other posttranscriptional gene silencing (PTGS) factors (Jouannet et al., 2012). These bodies can localize adjacent to PBs and are proposed to store translationally repressed RNAs to triage them between PBs and RDR6-dependent PTGS, potentially through their interactions with UPF1 (Jouannet et al., 2012; Moreno et al., 2013).

Apart from their physical association, several connections and a tight inter-dependence of the RNA quality control (RQC) machinery and PTGS have been discovered in plants (Liu and Chen, 2016). An initial observation was the

susceptibility of transgenes to suppression by RNA silencing in Arabidopsis *dcp2* mutants (Thran et al., 2012). Subsequently, decapping mutants were found to accumulate novel classes of endogenous siRNAs that arose through the cytoplasmic RDR6 pathway (Martinez de Alba et al., 2015). In line with the central role of RDR6 in this process, its knockout rescued the seedling lethality in the severe decapping mutants *vcs6* and *dcp2* (Martinez de Alba et al., 2015). The fact that major cytoplasmic RQC pathways and PTGS converge in PBs (Chantarachot and Bailey-Serres, 2018) makes these RNA granules prime targets for virus resistance and manipulation by viruses.

Viruses challenge the RQC and PTGS machineries through their massive production of RNAs during replication, and the targeting of viral RNAs by RNA silencing is one of the major defense pathway plants employ against viruses. In turn, viruses have frequently evolved RNA silencing suppressors to overcome this silencing (Csorba et al., 2015). The roles of PBs during plant viral infections are currently not well understood, but initial findings suggest that some viruses may benefit from PBs or their components via reduced targeting by antiviral RNA silencing (Hafrén et al., 2015; Ye et al., 2015).

In this study, we investigated the roles of PBs and decapping components in viral infection using the pararetrovirus *Cauliflower mosaic virus* (CaMV; family *Caulimoviridae*) and the model plant Arabidopsis. CaMV is a double-stranded DNA virus that harbors seven open-reading frames in two mRNAs transcribed from two promotors (*19s* and *35s*). While *35s* RNA encodes all viral proteins, *19s* RNA only encodes the viral transactivator protein P6. P6 is a highly abundant, essential protein that assembles in large cytoplasmic aggregates termed viral factories (VFs) that are the site of viral translation, reverse transcription, and particle packaging (Schoelz and Leisner, 2017). We show that at least three hallmark proteins of PBs are targeted to the VFs of CaMV and that these proteins are important for virus accumulation. We demonstrate that PBs serve a pro-viral role during CaMV infection by alleviating translational repression through RNA silencing.

Results

PB components re-localize during CaMV infection

To visualize PB dynamics during CaMV infection, we used marker lines expressing GFP-tagged canonical PB proteins (DCP1pro:DCP1-GFP, UBQ10pro:DCP5-GFP, UBQ10pro:LSM 1a-GFP, and VCSpro:GFP-VCS) (Motomura et al., 2012; Roux et al., 2015; Chicois et al., 2018). Under mock conditions, the markers showed a cytoplasmic distribution with varying degrees of condensation into droplet-like foci (Figure 1A). LSM1a–GFP fusion protein accumulated evenly in the cytoplasm, with no visible PB assembly, while GFP-VCS and DCP5-GFP were both present in foci and soluble, and DCP1-GFP mainly assembled in foci. These localization patterns were similar to those described previously (Motomura et al., 2015; Roux et al., 2015; Perea-Resa et al., 2016; Chicois et al.,

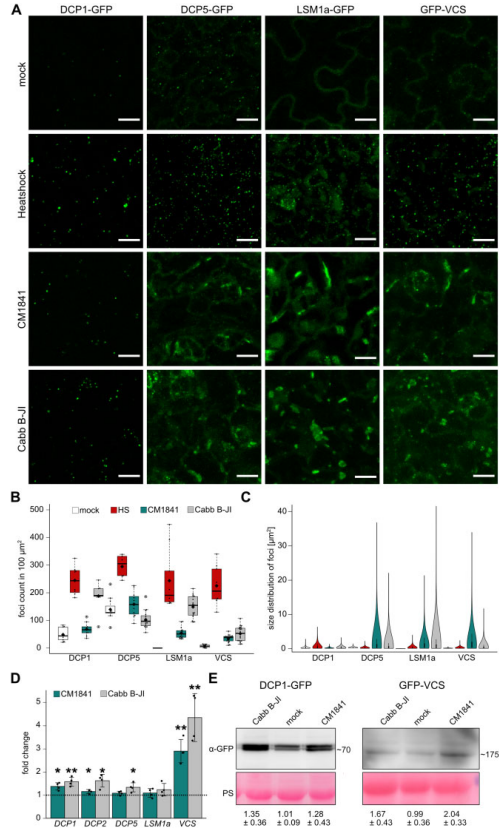

Figure 1 CaMV infection induces PB protein re-localization. A, Localization of four canonical PB markers under control conditions, after HS, and 21 dpi with CaMV strains CM1841 and Cabb B-JI. The representative images are composed of confocal Z-stacks (Scale bars = 10 µm). B, Count of fluorescent foci in 100 µm^2 corresponding to the treatments in (A). Counts were performed from randomly chosen areas using ImageJ and a custom pipeline. The box represents the interquartile range (IQR), the solid lines represent the median, diamonds the average. The whiskers extend to a maximum of 1.5× IQR beyond the box. C, Size distribution of detected foci corresponding to (B). B and D, Values calculated from nine z-stacks of three plants per replicate. All experiments were replicated at least 3 times independently. D, Relative expression (fold change) of PB components 21 dpi compared to mock (dashed line). Values represent means ± standard deviation (SD; $n = 4$) relative to Col-0 plants and normalized to *PP2a* as the internal reference. The experiment was repeated 3 times independently. Error bars represent SD. Statistical significance was determined by Student *t* test (*$P \leq 0.05$; **$P \leq 0.01$). E, Immunoblot analysis of DCP1-GFP and GFP-VCS in systemic leaves of infected marker lines. Total proteins were extracted at 21 dpi and probed with GFP-antibodies. Ponceau S (PS) staining served as a loading control. Numbers indicate average (±SD) of protein abundance from three independent blots from independent infections quantified with ImageJ. Numbers on the side of the blot indicate the molecular weights of fusion proteins (kDa).

2018). Before analyzing infection dynamics, we established how the markers behaved after heat shock (HS) application (Motomura et al., 2015). The number of detectable foci after HS increased drastically and was comparable for all markers, pointing toward directed co-assembly during stress (Figure 1, A and B). This is consistent with earlier findings that some PB proteins, including LSM1a, associate with PBs only upon stress (Perea-Resa et al., 2016; Guzikowski et al., 2019). Importantly, this analysis confirmed the functionality of the marker lines under our conditions.

Upon infection with two CaMV strains (CM1841 and Cabb B-JI), the PB marker proteins formed two morphologically distinct classes of visible structures in systemic leaves 21-day postinfection (dpi; Figure 1A), while free GFP localization remained unchanged (Supplemental Figure S1B). The number of DCP1-marked foci especially increased during Cabb B-JI infection, without any apparent change in morphology (Figure 1, A–C; Supplemental Figure S1A). The markers LSM1a, VCS, and DCP5 also accumulated in small DCP1-like foci upon CaMV infection, but most striking was their prominent assembly into large, irregularly shaped structures not seen with DCP1 (Figure 1, A–C; Supplemental Figure S1A). The large structures were less abundant than the droplets for the three markers and had a distorted circularity, which was not seen after HS or in the DCP1 marker (Figure 1B; Supplemental Figure S1A). We never detected comparable structures under either control conditions or after HS with any of the markers, while they were found abundantly with both CaMV strains, with slight variations in number and size. These structures grew in size and decreased in number during the infection time course, indicating their fusion in infected cells (Supplemental Figure S1B). To validate the findings and confirm that the same structures were indeed marked by different PB markers, we established two double marker lines with GFP-VCS/DCP1-RFP and GFP-VCS/LSM1a-RFP. DCP1 and LSM1a showed the same localization pattern regardless of which fluorescent marker was used. Interestingly, only a fraction of DCP1 and VCS co-localized under mock conditions, while co-assembly after HS again confirmed the stress-dependent co-accumulation of PB markers (Supplemental Figure S2). During CaMV infection, LSM1a-RFP and GFP-VCS both marked the same large, irregular structures, while DCP1-RFP localized to smaller foci adjacent to VCS structures (Supplemental Figure S2).

The localization of PB components to virus-induced structures led us to test whether the transcription of these components was altered during infection. The transcript levels were consistently elevated for *DCP1*, *DCP2*, and more strongly for *VCS* with both CaMV strains, while *DCP5* expression was only induced during Cabb B-JI infection, and *LSM1a* expression was not responsive to either strain (Figure 1D). Accordingly, immunoblot analysis confirmed that DCP1-GFP and GFP-VCS protein levels increased during infection (Figure 1E). In conclusion, CaMV infection causes condensation and a drastic re-localization of several PB proteins into large virus-induced structures.

CaMV sequesters PB components into VFs

The re-localization of LSM1, VCS, and DCP5 into novel structures during CaMV infection, suggested that these structures could be virus-induced inclusions. CaMV assembles two types of cytoplasmic inclusions: the spherical transmission bodies that are mainly formed by the viral protein P2, and the more irregularly shaped VFs that are mainly formed by the viral protein P6 (Martelli and Castellano, 1971; Espinoza et al., 1991). Heterologous co-expression of six CaMV proteins with PB proteins in *Nicotiana benthamiana* showed that viral P6 protein co-localized with DCP1, DCP5, and VCS (Supplemental Figure S3). This prompted us to investigate co-localization of PB markers with VFs during CaMV infection. We used transgenic P6-mRFP expressing PB marker lines to investigate the association of DCP1, DCP5, LSM1, and VCS with VFs. Under control conditions, P6-mRFP was mostly soluble in the cytoplasm, with occasional foci formation (Supplemental Figure S4). Some, but not all these foci co-localized with DCP1, DCP5, and VCS, indicating that these proteins already associated in the absence of infection (Supplemental Figure S4, white arrows). During infection, the P6-mRFP protein assembled to mark the characteristic large VFs, which also accumulated DCP5, LSM1a, and VCS (Figure 2A). DCP1 foci accumulated around, but not within the VFs.

Translation inhibition through the trapping of ribosomes on mRNA by Cycloheximide (CHX) leads to the disassembly of canonical PBs (Teixeira et al., 2005; Motomura et al., 2015). Under our conditions, CHX treatment of the DCP1-GFP and DCP5-GFP marker line after mock or CaMV infection confirmed the dissociation of canonical PBs after CHX treatment. However, the irregular VFs were still marked by DCP5 in CHX-treated samples, albeit at lower signal intensity (Figure 2B). DCP1 bodies disappeared after treatment regardless of viral infection (Figure 2B). These results indicate that DCP5 in VFs is dynamically less responsive to depletion of the RNA supply from ribosomes than canonical PBs, possibly owing to VF size or other distinct physicochemical properties, including interactions with the VF matrix.

Disruption of PB functions attenuates CaMV infection

The VFs formed by CaMV P6 protein are electron dense, RNA-, and protein-rich structures with essential roles in the viral lifecycle (Martelli and Castellano, 1971; Schoelz and Leisner, 2017). VFs are proposed to be sites of active viral RNA translation, reverse transcription, and packaging of viral genomic DNA in particles. Considering the re-localization of PB components to viral replication sites, we next investigated the role of PB components in CaMV disease by analyzing infection phenotypes in mutants affected in PB formation. The null mutant *lsm1a/b* (hereafter referred to as *lsm1*) and knockdown mutant *dcp5* were chosen for this study, because both mutations cause a reduction in PB

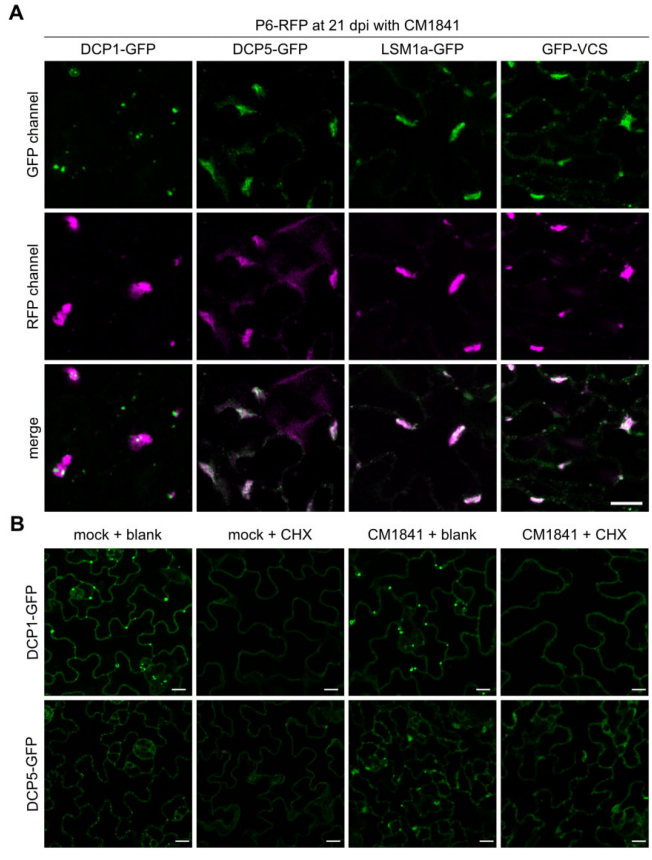

Figure 2 Virus-induced PB protein localization in viral factories. A, Co-localization of P6-RFP with GFP-tagged PB markers in transgenic Arabidopsis 21 dpi with CaMV strain CM1841. Representative single plane images are shown (Scale bars = 10 μm). The experiments were replicated in independent transformants. B, Distribution of DCP1-GFP and DCP5-GFP marker 21 days after mock or CaMV infection and 1 h after 200 μM CHX or blank infiltration. Images represent single plane micrographs (Scale bars = 10 μm). DCP1-GFP was imaged with a higher exposure to ensure visualization of the soluble fraction.

formation and PB size, as well as an over-accumulation of capped mRNAs (Xu and Chua, 2009; Perea-Resa et al., 2012, 2016). Importantly, these mutants are not postembryonic lethal, in contrast to null mutants of *DCP1*, *DCP2*, and *VCS* (Xu et al., 2006), and grow well enough for virus infection experiments. The *lsm1* and *dcp5* plants showed developmental phenotypes, including slightly delayed germination, mild dwarfism, and leaf serrations (Figure 3A). Additionally, the null-mutant of the cytoplasmic exonuclease *xrn4* was used; this mutant is not impaired in PB biogenesis and mRNA decapping, but it over-accumulates uncapped RNAs (Nagarajan et al., 2019). The *xrn4* plants were morphologically not distinguishable from Col-0 plants under short-day conditions but showed the typical serrations under long-day conditions.

Upon infection with CaMV, all mutants showed similar levels of stunting, vein bleaching, rosette distortion, and leaf wrinkling compared to Col-0 (Figure 3A), with prominent symptoms appearing at 12 (Cabb B-JI) or 14 (CM1841) dpi. The relative fresh weight of CaMV-infected compared to mock-inoculated plants was taken as a measure of disease severity. The fresh weight loss was less severe in all three mutants compared to Col-0 for the milder CM1841 strain and unaltered for Cabb B-JI (Figure 3B). In general, Cabb B-JI infection caused stronger but also more variable infection phenotypes, possibly masking potential effects of PB disruption on fresh weight loss.

To establish viral load in the mutants compared to Col-0, we measured viral DNA, RNA, and protein levels. Viral DNA accumulation was attenuated for both CaMV strains in *lsm1*

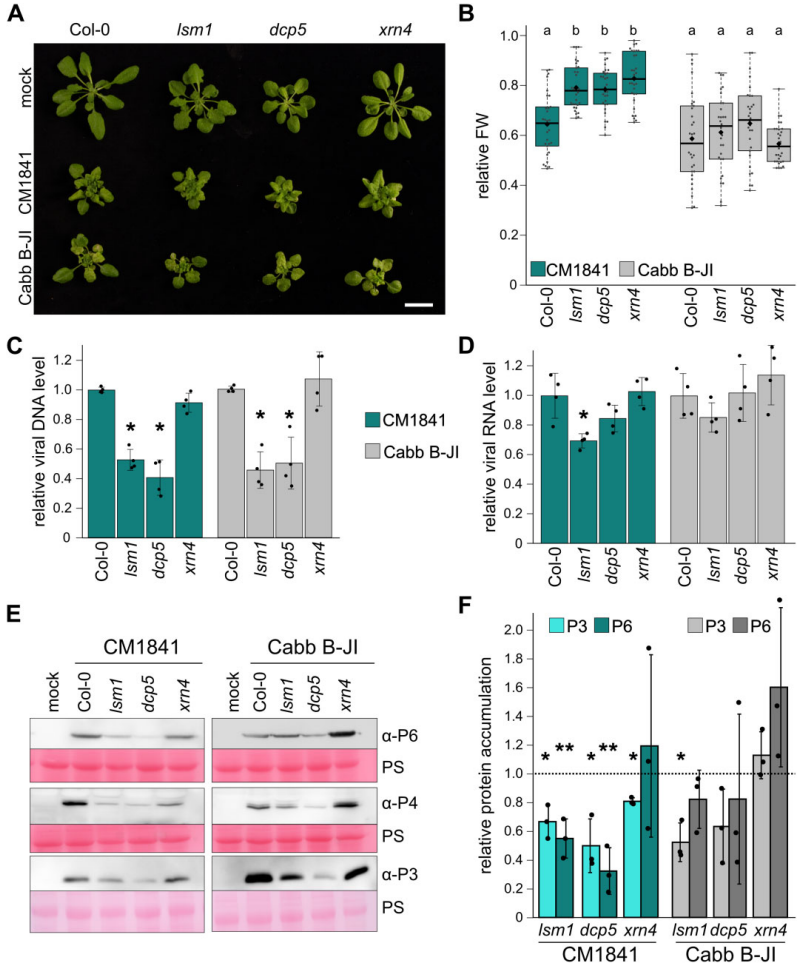

Figure 3 CaMV disease is attenuated in *lsm1* and *dcp5* mutants. A, Virus-induced symptoms in Col-0, *lsm1*, *dcp5*, and *xrn4* plants at 21 dpi. CM1841 and Cabb B-JI-infected plants are compared to mock-infected plants (Scale bar = 2 cm). B, Relative fresh weight of CaMV-infected compared to mock plants at 21 dpi (n = 30). The box represents the IQR, the solid lines represent the median, diamonds the average. The whiskers extend to a maximum of 1.5× IQR beyond the box. Statistical significance was determined by one-way ANOVA coupled with Tukey's HSD test (α = 0.05), letters indicate statistical groups. C, Viral DNA accumulation in systemic leaves of Col-0 and mutant plants at 21 dpi, determined by qRT-PCR. Values represent means ± SD (n = 4) relative to Col-0 plants and normalized to *18S* ribosomal DNA as the internal reference. D, 35s RNA levels of CaMV were determined by qRT-PCR in systemic leaves at 21 dpi. Values represent means ± SD (n = 4) relative to Col-0 plants and normalized to *PP2a*. E, Immunoblot analysis of CaMV P3, P4, and P6 proteins in the systemic leaves of Col-0, *lsm1*, *dcp5*, and *xrn4* plants Total proteins were extracted at 21 dpi and probed with specific antibodies. Mock-infected plants were used as a control for signal background. Ponceau S (PS) staining served as a loading control. F, Accumulation of CaMV P3 and P6 proteins in all genotypes in systemic leaves at 21 dpi quantified by direct ELISA. Values represent means ± SD (n = 3) in arbitrary units relative to Col-0 plants (dashed line). Statistical significance was determined by Student's t test for (C, D, and F) (*$P \leqslant 0.05$; **$P \leqslant 0.01$). All experiments (A–F) were repeated at least 3 times from independent infections.

and *dcp5*, but not in the *xrn4* mutant (Figure 3C). In addition, we analyzed CM1841 DNA levels in heterozygous plants of the embryo lethal *dcp1-1*, *dcp2-1*, and *vcs6* mutants as well as the homozygous knockdown mutant *dcp1-3* (Martinez de Alba et al., 2015), but we did not detect a defect in viral titer in any of these lines (Supplemental Figure S5). This suggests that *dcp1-3* and the heterozygous lines are weaker mutants compared to *dcp5* and *lsm1*, as

supported by the absence of morphological defects. Alternatively, there may be a specific involvement of LSM1 and DCP5, independent of decapping, but the localization of VCS along with these components to VFs would argue against this. Viral DNA is produced through reverse transcription of the viral *35s* RNA. Interestingly, the levels of *35s* RNA were only mildly reduced for CM1841 and remained unaffected for Cabb B-JI in *lsm1* and *dcp5* (Figure 3D), suggesting that reduced DNA levels could be caused by defects in viral RNA usage in translation or reverse transcription rather than RNA production.

Immunoblot analysis showed that less of the viral inclusion protein P6, the coat protein P4, and the virion-associated protein P3 accumulated in both *lsm1* and *dcp5* compared to Col-0 (Figure 3E). Viral protein accumulation in *xrn4* differed between the two strains, with CM1841 showing a mild reduction in P6 and P4 levels, while Cabb B-JI showed higher levels of P6 and P4. A direct enzyme-linked immunosorbent assay (ELISA) confirmed reduced P6 and P3 accumulation in *lsm1* and *dcp5* for CM1841 and also Cabb B-JI, albeit the effect was weaker (Figure 3F). In combination, the impairment of CaMV disease in these mutants indicates that PB components play a pro-viral role during CaMV infection. Virus accumulation was impaired in mutants defective in PB biogenesis and decapping (*lsm1* and *dcp5*), but not in exonucleolytic RNA decay (*xrn4*). Owing to the similarities between the two strains, we continued our subsequent analysis with the milder CM1841 strain.

LSM1 has no major role in viral RNA stability or decapping

The established role of LSM1 and DCP5 in RNA decapping and degradation led us to test whether these PB-associated factors were acting on viral RNA during infection, as the seemingly unaltered viral RNA levels in *lsm1* and *dcp5* mutants (Figure 3D) could still be explained by a combination of reduced transcription and a defect in RNA decay. To determine the capping levels of viral RNAs in Col-0 and *lsm1* plants, we performed an RNA-pulldown experiment with cap-specific antibodies (Golisz et al., 2013). We found known targets of LSM1-mediated decapping to be more abundant in their capped form in the *lsm1* mutant, as expected from previous studies (Perea-Resa et al., 2012; Golisz et al., 2013), while the capping levels of CaMV *35s* and *19s* RNA did not differ between Col-0 and *lsm1* (Figure 4A). Furthermore, a comparison of known LSM1 targets between the control and CaMV-infected samples showed that viral infection does not influence decapping of those endogenous targets, although we cannot exclude the possibility that other targets might be affected (Figure 4B).

Unaltered capping of viral RNA was further supported by a cap-sensitive exonuclease digestion of total RNA from infected plants, showing identical susceptibility of viral *35s* RNA isolated from the *lsm1* mutant compared to Col-0 (Figure 4C). Considering the possibility of decapping-independent RNA decay, we also tested whether the decay rate of viral *35s* RNA was altered in *lsm1* mutants by quantifying RNA from infected rosettes in a time course after inducing transcriptional arrest using Cordycepin (Sorenson et al., 2018). CaMV RNA was remarkably stable and showed no sign of degradation after 120 min of transcriptional inhibition in Col-0, *lsm1* (Figure 4D), *dcp5*, and *xrn4* (Supplemental Figure S6A). A longer treatment time of 8 h still showed no evident degradation of viral RNA (Supplemental Figure S6B), indicating that the viral RNA is strongly protected. The degradation profile of *AT4G32020*, a known target of LSM1-dependent decapping (Golisz et al., 2013), confirmed the transcriptional inhibition and LSM1-dependent effects (Figure 4D). Our results support that CaMV RNAs are not major targets of LSM1-dependent decapping or decay and thus, these dysfunctions in *lsm1* and *dcp5* are not likely to cause the reduced virus accumulation observed in these mutants.

Defects in LSM1/DCP5 expose CAMV to RNA silencing but not NMD

Since PBs are at the heart of RNA triage and a hub for major RNA surveillance mechanisms, we examined whether the reduced CaMV accumulation is dependent on NMD surveillance or mediated through the RNA silencing machinery. To this end, we characterized viral infections in combinatorial mutants. CaMV titers were not affected in the previously described NMD-regulator mutant *upf1-5* (Chicois et al., 2018; Figure 5A), although the plants showed a higher relative fresh weight compared to Col-0, which is similar to *dcp5* (Supplemental Figure S7, A and B). The double mutant *dcp5 upf1* showed the same titer defect as the *dcp5* single mutant, showing that this reduction is independent of UPF1-triggered NMD (Figure 5A). A previous study found that overexpression of CaMV P6 protein relieved the suppression of several NMD targets containing different NMD marks, including premature termination codons (PTCs) and long upstream open reading frames (uORFs) (Lukhovitskaya and Ryabova, 2019). During CaMV infection, however, we only detected de-repression of PTC-carrying targets *SMG7* and *RPS6*, but not uORF-containing genes, suggesting that CaMV specifically represses PTC-triggered NMD (Figure 5B), possibly to protect against the numerous PTCs present in polycistronic viral RNA. A comparison of transcript levels in infected tissues between Col-0, *dcp5*, and *upf1* revealed that the transcription profiles of NMD targets in *dcp5* are more similar to those of Col-0 than *upf1*, uncoupling NMD regulation during CaMV infection from DCP5 functions (Supplemental Figure S8A).

Because RQC mutants are generally prone to initiate RNA silencing against highly expressed RNAs such as transgenes and viral RNAs as well as endogenous genes (Liu and Chen, 2016), we tested whether the observed viral repression in

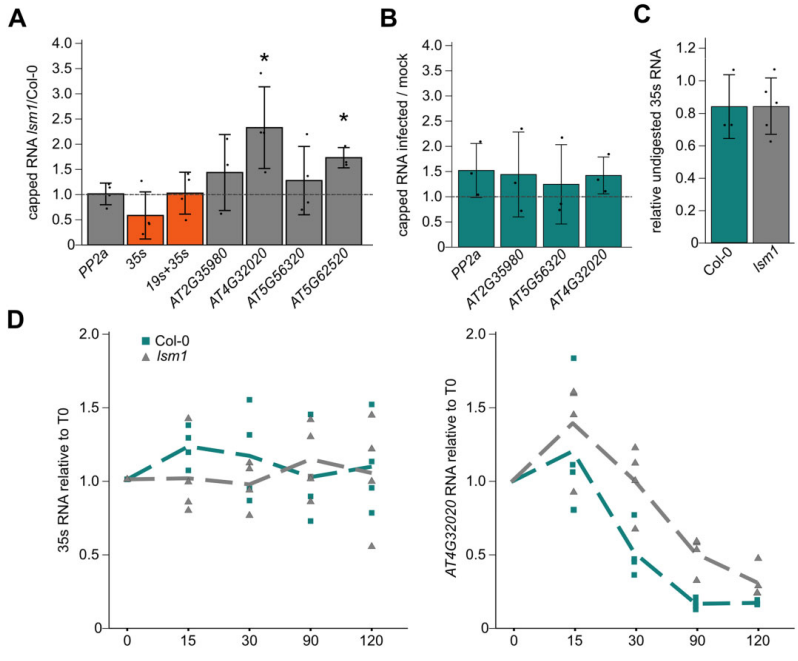

Figure 4 LSM1 does not regulate viral RNA stability. A, RNA levels detected after cap-dependent pulldown in infected *lsm1* compared to Col-0 plants for the housekeeping gene *PP2a*, viral RNA, and four previously described LSM1 targets. Bars represent mean from independent pulldowns from independent infections ($n = 4$). B, RNA levels detected after cap-dependent pulldown on endogenous RNAs from CaMV infected tissue compared to mock infected. Bars represent mean from independent pulldowns from independent infections ($n = 3$). C, Amount of viral *35s* RNA in Col-0 and *lsm1* mutant detected after 1h of XRN1 treatment. Bars represent the mean from independent digestions from independent infections ($n = 3$ for Col-0; $n = 5$ for *lsm1*). Statistical significance was determined by Student's *t* test for (A–C) (*$P \leqslant 0.05$). D, Transcript decay profiles for viral *35s* and *AT4G32020* RNA after transcriptional arrest using cordycepin. Dotted line represents the average of four independent experiments, single experiments are shown by circles (Col-0) and triangles (*lsm1*). Sampling timepoints are indicated on the *x*-axis (0- to 120-min past treatment).

mutants affected in PB formation was mediated by the RNA silencing machinery by establishing higher-order mutants of *lsm1*, *dcp5*, and *xrn4* with *rdr2*, *rdr6* and *dcl2 dcl3 dcl4* (*dcl234*; Allen et al., 2004; Xie et al., 2004; Deleris et al., 2006). These mutants, as well as their parental lines, were infected with CM1841, and virus disease was analyzed at 21 dpi. The *rdr2*, *rdr6* and *dcl234* alleles exhibited comparable fresh weight loss to Col-0 during CM1841 infection (Supplemental Figure S7D), and it is noteworthy that the RNA silencing mutants did not reverse the developmental phenotypes of *lsm1* and *dcp5* (Supplemental Figure S7C). Nonetheless, *rdr6* and *dcl234*, but not *rdr2*, rescued viral DNA accumulation in the *lsm1* and *dcp5* backgrounds while remaining at Col-0 levels in the *rdr2*, *rdr6*, and *dcl234* as well as *xrn4 rdr6* and *xrn4 rdr2* mutants (Figure 5C). The finding that comparable levels of CaMV RNA accumulated in all single and combinatorial mutants excludes the possibility that overcompensation via increased RNA content is the source of viral DNA rescue (Figure 5D). Importantly, it strengthens the notion that lower accumulation of CaMV DNA in *lsm1* and *dcp5* is a posttranscriptional effect.

sRNA accumulation against CaMV, tasiRNA suppression, and TRV-induced gene silencing remain intact in *dcp5* and *lsm1*

RNA silencing is frequently activated in RQC mutants and involves the biogenesis of small RNAs (sRNAs) against endogenous targets (Martinez de Alba et al., 2015). To determine whether viral sRNAs profiles and amounts were altered in *lsm1* and *dcp5* in an RDR6-dependent manner, we analyzed sRNAs in infected Col-0, *rdr6*, *lsm1*, *lsm1 rdr6*, *dcp5*, and *dcp5 rdr6* using sRNA-sequencing. We produced libraries from rosette samples at 21 dpi in duplicates and mapped 18–26 nucleotide (nt) reads to the TAIR10 Arabidopsis reference genome and against the CaMV genome (GenBank V00140.1). In agreement with previous observations, most viral sRNAs mapped against the highly abundant noncoding *8s* RNA (Figure 6, A and C). The

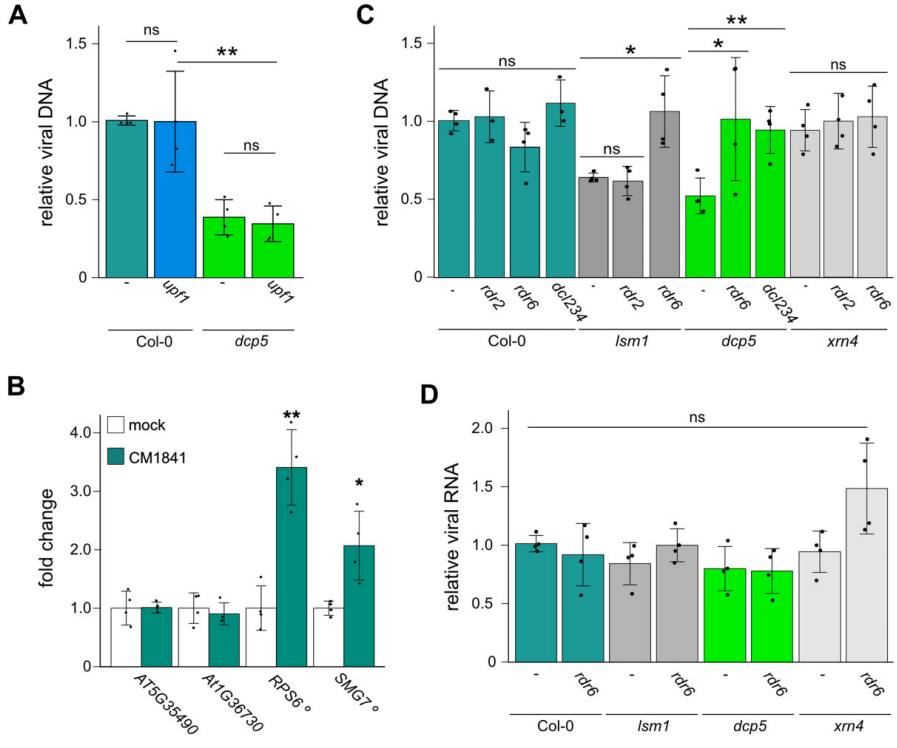

Figure 5 CaMV disease is rescued in combinatorial mutants with RNA silencing, but not NMD. A, Viral DNA accumulation in systemic leaves at 21 dpi in the indicated genotypes, determined by qRT-PCR. Values represent means ± SD ($n = 4$) relative to Col-0 plants and normalized to *18S* ribosomal DNA as the internal reference. B, Relative expression of NMD targets in mock and CM1841-infected rosettes 21 dpi determined by qRT-PCR. Values represent means ± SD ($n = 4$) relative to Col-0 plants and normalized to *PP2a* as the internal reference. Open circles indicate the two PTC-containing transcripts *RPS6* and *SMG7*. C, Viral DNA accumulation in systemic leaves at 21 dpi in the indicated genotypes, determined by qRT-PCR. Values represent means ± SD ($n = 4$) relative to Col-0 plants and normalized to *18S* ribosomal DNA as the internal reference. D, Viral *35s* RNA accumulation in systemically infected rosettes of the indicated genotypes at 21 dpi relative to Col-0, determined by qRT-PCR. Values represent means ± SD ($n = 4$) relative to Col-0 plants and normalized to *PP2a* as the internal reference. Statistical significance was calculated by two-sided Student's *t* tests (*$P \leqslant 0.05$; **$P \leqslant 0.01$; ns, no significant difference.) for (A–D). All infection experiments were replicated at least 3 times independently.

percentage of sRNAs mapping to the CaMV sequence compared to sRNAs mapping against the TAIR10 genome was consistently at ~20% (Figure 6D), with a similar size distribution (Figure 6E) as well as position and abundance across the viral genome in all genotypes and replicates (Figure 6, A–C; Supplemental Figure S9; Supplemental Data Set 1). This confirms that sRNAs mapping against the viral genome are generated independently of RDR6 and without synergistic effects in the double mutants. Hence, impairing LSM1 or DCP5 function does not have any major effects on the quantity, quality, or position of CaMV-related sRNAs.

Pathogenic plant viruses have commonly evolved viral suppressors of RNA silencing (VSRs) to counteract RNA silencing. For CaMV, the VSR protein P6 inhibits the generation of secondary RDR6-dependent trans-acting siRNAs (tasiRNA; Shivaprasad et al., 2008). To assess whether CaMV-dependent tasiRNA suppression is compromised in *lsm1* and *dcp5* mutants as a sign of a dysfunctional VSR, we counted the reads generated from the three *TAS1* and the *TAS2* loci, as well as selected tasiRNA target genes (minimal average count in Col-0 mock > 1,000 reads per million [RPM]) in noninfected Col-0 and infected Col-0, *lsm1*, and *dcp5*. CaMV infection led to a decrease in sRNA counts on *TAS*-loci and tasiRNA targets (Figure 6, F and G). The reduction in sRNA occupancy was consistent in *lsm1* and *dcp5*, suggesting that P6-mediated repression of RDR6-dependent tasiRNA generation is functional in these backgrounds. Furthermore, equal increases in the transcript levels of two

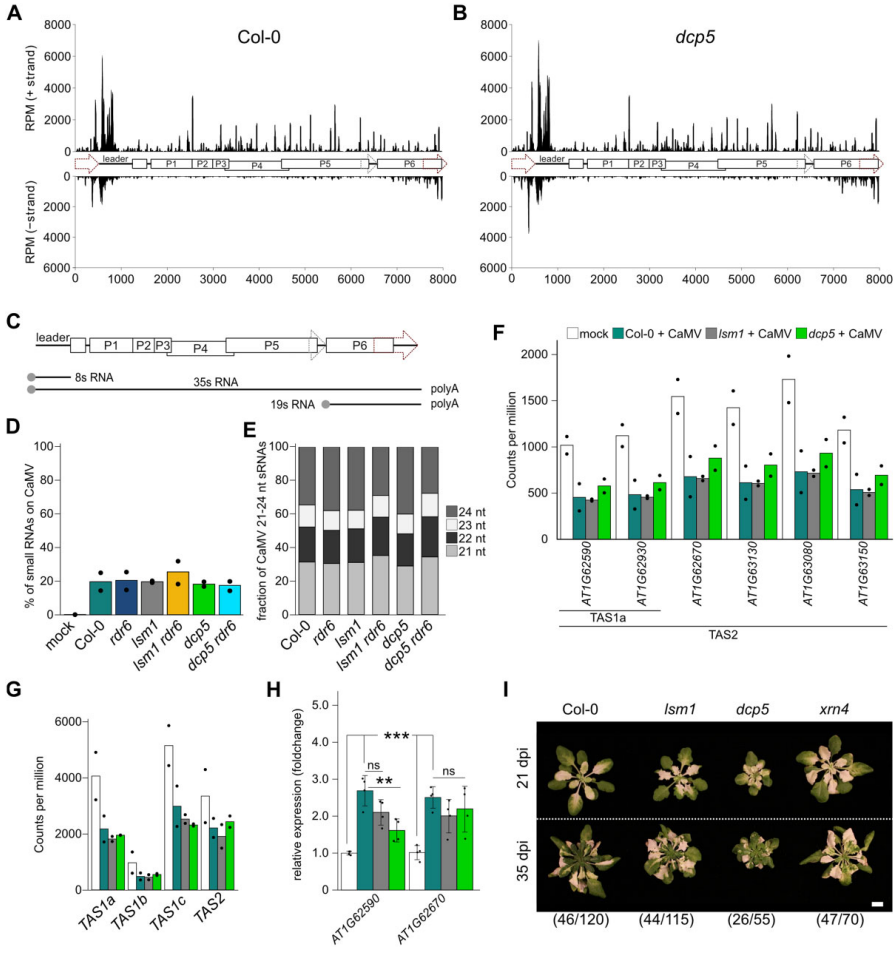

Figure 6 sRNA profiles on CaMV are not altered in PB and combinatorial mutants. A, Coverage plot of 24–25 nt sRNA profiles along the 8,031-bp viral genome in Col-0 21 dpi with CMI1841. The starting position was set to the beginning of the 35*s* promoter (genomic position 7090). Genomic features are annotated as depicted in (C). B, Coverage plot of 24- to 5-nt sRNAs along the viral genome in *dcp5* 21 dpi with CMI1841. C, Schematic depiction of the CaMV genome. ORFs are indicated by boxes, the 19s and 35*s* promotors by dashed arrows. Viral RNAs resulting from PolII transcription are depicted below the genome. D, Percent of viral sRNAs found in samples sequenced from rosette tissue 21 dpi with CMI1841 in the indicated genotypes. Bars represent the average of two biological replicates. Dots indicate single replicates. E, Fractions of 21- to 24-nt viral sRNAs in the indicated genotypes 21 dpi with CMI1841. Bars represent the average of two biological replicates. F, Normalized sRNA counts on tasiRNA target loci in the indicated genotypes. tasiRNA generating loci are depicted below the graph. Bars represent the average of two biological replicates. Dots indicate single replicates. G, Normalized sRNA counts on *TAS1a,b,c* and *TAS2* loci in the indicated genotypes. Bars represent the average of two biological replicates. Dots indicate single replicates. H, Expression of two tasiRNA targets at 21 dpi in Col-0, *lsm1*, and *dcp5* relative to mock-infected Col-0 ($n = 4$). Statistical significance was calculated by two-sided Student's *t* tests (**$P \leqslant 0.01$; ***$P \leqslant 0.001$). The experiment was repeated 3 times from independent infections. I, Representative images of VIGS phenotype in the indicated genotypes at 21 and 35 dpi with TRV-PDS (scale bar = 1 cm). Numbers indicate plants showing the phenotype/total number of plants scored.

tested tasiRNA target genes occurred during CaMV infection (Figure 6H), further supporting that CaMV-dependent *TAS* suppression and de-repression of tasiRNA target genes are functional in *lsm1* and *dcp5*. A recent study found little difference in the sRNA profiles of *lsm1* compared to Col-0 during undisturbed growth (Krzyszton and Kufel, 2022).

Whether certain endogenous sRNAs apart from tasiRNAs are misregulated in *lsm1* and *dcp5* during CaMV infection will be studied in detail in the future.

To test whether *lsm1* and *dcp5* have a general activation of virus-induced gene silencing (VIGS), as reported for other RQC mutants, we used the tobacco rattle virus-*Phytoene Desaturase* (TRV-PDS) system, which leads to leaf whitening through VIGS of *PDS* (Liu et al., 2002). We did not detect increased whitening, delayed recovery, or a higher number of symptomatic plants for *lsm1* and *dcp5*, whereas *xrn4* showed a clearly enhanced VIGS phenotype (Figure 6I). Previously, both *xrn4* and the hypomorphic *DCP2* mutant *increased transgene silencing 1* were shown to have enhanced TRV-PDS-induced VIGS (Ma et al., 2019), which was linked to higher silencing activity in these RQC-impaired backgrounds. Our results suggest that *lsm1* and *dcp5* plants do not have the same level of hyper-activated RNA silencing as the other mutants.

Taken together, *lsm1* and *dcp5* mutants (1) do not show altered viral sRNA quantities or profiles (2), do not show elevated VIGS, and (3) do not compromise the capacity of the viral silencing suppressor P6 to target the RDR6-dependent tasiRNA pathway, despite full rescue of virus DNA and protein accumulation by *rdr6* in double mutants. Importantly, based on the unaltered viral RNA levels and decay rates in *lsm1*, we propose that the RDR6-dependent suppression in the *lsm1* and *dcp5* backgrounds does not involve viral RNA degradation or transcriptional silencing.

LSM1 and DCP5 counter RDR6-dependent translational repression of viral RNA

The common modes of action of RNA silencing include transcriptional silencing, transcript degradation, and translational repression. After establishing the former two to be unlikely, we determined whether viral RNA translation was impaired in *lsm1* and *dcp5*. First, we performed polysomal fractionation of CaMV-infected Col-0, *lsm1* and *dcp5* samples from three independent infection experiments. Notably, CaMV-infected tissue showed markedly increased polysome abundance compared to the mock controls for both Col-0 and the mutants (Figure 7A), ruling out any global defect in translation. Fractions were collected from free and monosome-bound RNA, as well as from light, moderate, and heavy polysomes. In a first step, we confirmed the robustness of RNA content in the fractions by examining the housekeeping genes *SAND* and *PP2a* (Supplemental Figure S10A). SAND showed a stable distribution among all ribosome fractions and a decrease in abundance in the ribosome-free fraction. As control, we normalized *PP2a* expression to *SAND* expression in each fraction. PP2a abundance was comparable in the input (Supplemental Figure S10B), as well as along the gradient (Figure 7B), and had the same distribution as *SAND*. Additionally, we tested the polysome association of the four abovementioned NMD targets (Figure 5B; Supplemental Figure S8A) in two replicates of Col-0 and *dcp5*. The translation profiles for *AT5G35490* and *AT1G36730* did not differ between the genotypes, similar to their expression levels. Yet, the two PTC-carrying RNAs had a higher abundance in *dcp5* polysome fractions, strengthening the role of DCP5 as a translational repressor for endogenous targets (Supplemental Figure S10C). Importantly, these profiles validated our methodology.

We measured viral *35s* RNA in fractions from Col-0, *lsm1*, and *dcp5*. This RNA was mostly present in ribosome-bound fractions compared to free RNA, and in contrast to the tested endogenous RNAs, specifically enriched in the light polysome fraction (Figure 7C). Strikingly, the viral RNA content in ribosome-bound fractions was reduced in *lsm1* and *dcp5*, despite comparable RNA content in the input samples (Figures 7C and 5D). In accordance with the ELISA and immunoblotting results (Figure 3, E and F), the reduced ribosome association of viral RNA in the *lsm1* and *dcp5* mutants indicates that lower translation levels and not protein degradation are responsible for the decreased amounts of viral protein in these genotypes.

Finally, to confirm the notion that the rescue of viral DNA by *rdr6* is directly linked to translational efficiency, we performed polysome fractionations for the *rdr6*, *lsm1 rdr6*, and *dcp5 rdr6* mutants. The global polysome profiles were comparable among genotypes during infection (Figure 7D), and *rdr6* alone did not show an altered polysome distribution of viral RNA compared to Col-0 (Figure 7E). Intriguingly, the viral RNA in the *lsm1 rdr6* and *dcp5 dr6* double mutants showed fully restored polysome associations compared to their respective single mutants (Figure 7F), while *PP2a* remained unaffected in all tested genotypes (Figure 7G; Supplemental Figure S10B). Immunoblot analysis against viral P6 protein confirmed restoration of viral protein accumulation in the combinatorial mutants (Figure 7, H and I). Together, our results indicate that the defect in viral protein production in the *lsm1* and *dcp5* mutants is mediated through the cytoplasmic PTGS pathway governed by RDR6. In the *lsm1* or *dcp5* background, RDR6 promotes translational repression of viral RNA independently of sRNA abundance. This establishes the PB components LSM1 and DCP5 as antagonists to RNA silencing during CaMV infection and a shield to help the virus circumvent translational repression by the antiviral RNA silencing machinery.

Discussion

Animal viruses are commonly challenged with a global shutdown of translation as part of an antiviral defense response (Walsh et al., 2013). In plants, this has so far only been observed for geminiviruses (Zorzatto et al., 2015), and in general, plant virus infections do not induce evident effects on global translation levels (Ma et al., 2015; Meteignier et al., 2016; Li et al., 2019). CaMV is exceptional, as it causes a substantial increase in polysome levels indicative of hyperactivated translation in turnips (*Brassica rapa* ssp. *rapa*) (Park et al., 2001) and Arabidopsis (this study). Translation of CaMV's polycistronic *35s* RNA is a complex process, including mechanisms of leaky scanning and transactivation

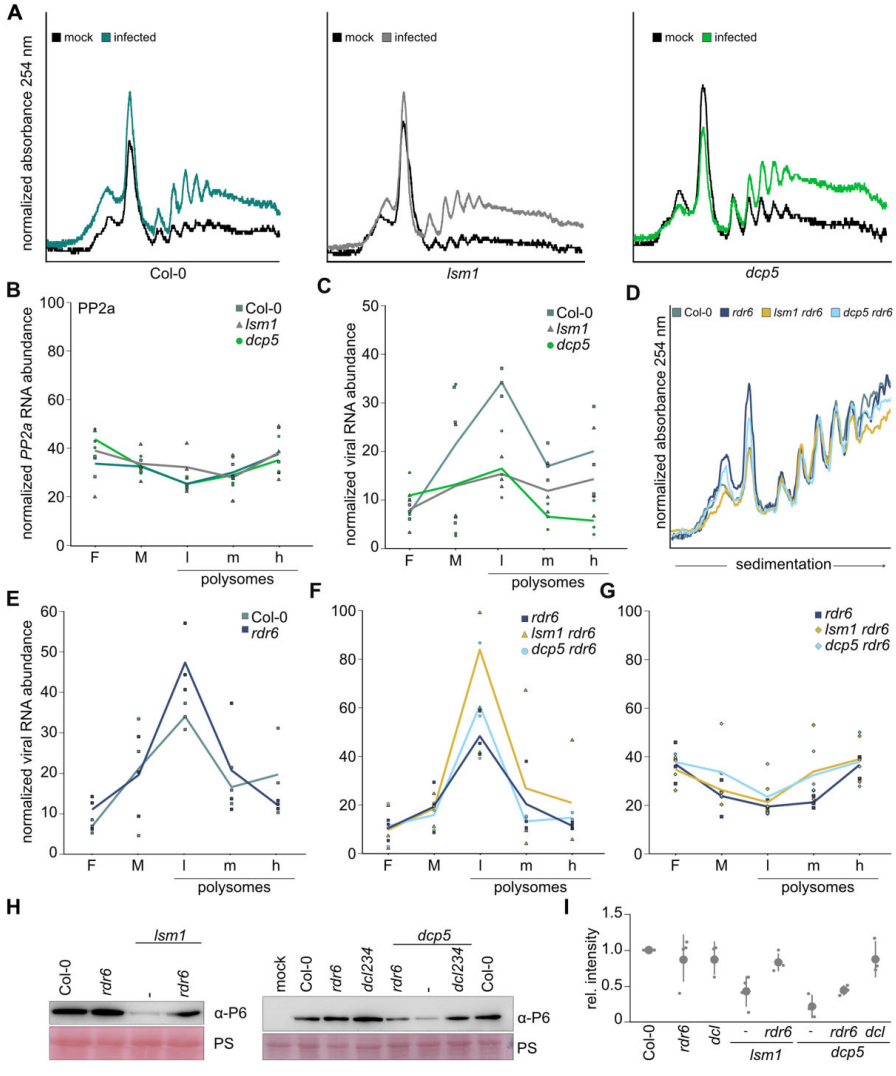

Figure 7 Ribosome association of viral RNA is reduced in *lsm1* and *dcp5*. A, Polysome Profiles of Col-0, *lsm1* and *dcp5* at 21-dpi CaMV (strain CM1841) infection. RNA samples were collected from unbound RNA, as well as along the gradient of ribosome-bound RNA. B, *PP2a* RNA abundance in collected samples in the indicated genotypes. The experiment was performed 3 times using material from independent infections. Fractionated RNA was normalized to *SAND* and depicted as fractions of total ribosome-bound RNA. Solid lines represent the average of biological replicates, characters represent single experiments of the indicated genotypes. Measured fractions represent free (F), monosome (M), light (l), medium (m), and heavy (h) polysome-associated RNA. C, *35s* RNA abundance in collected samples in the indicated genotypes. The experiment was performed as described in (B). D, Polysome Profiles of Col-0, *rdr6*, *lsm1 rdr6*, and *dcp5 rdr6* at 21 dpi CaMV infection. RNA samples were collected from unbound RNA, as well as along the gradient of ribosome-bound RNA. E and F, RNA abundance in collected samples measured for viral *35s* RNA in the indicated genotypes. The experiment was performed as described in (B). G, *PP2a* RNA abundance in collected samples of the indicated genotypes. The experiment was performed as described in (B). H, Immunoblot analysis of CaMV P6 protein in systemic leaves of the indicated genotypes. Total protein samples were extracted at 21 dpi and probed with anti-P6. Ponceau S (PS) staining served as a loading control. I, Quantification of signal intensity of the immunoblots in (H). Values indicate average (±SD) of protein abundance from three independent blots (for *dcp5* combinatorial mutants) or four independent blots (for *lsm1* combinatorial mutants) from independent infections quantified with ImageJ. Points represent single experiments.

(Pooggin and Ryabova, 2018). The viral transactivation factor P6 is essential for the translation of downstream ORFs in 35S RNA (Bonneville et al., 1989) through its interaction with a multitude of translation-associated proteins, including the translation initiation factor eIF3g, components of the large ribosomal subunit, the reinitiation supporting protein complex, and the TOR kinase (Park et al., 2004; Schepetilnikov et al., 2011).

In this study, we identified PB components as important factors that support CaMV infection via viral RNA translation, being in sharp contrast to their established function as selective repressors of endogenous mRNA translation (Brodersen et al., 2008; Xu and Chua, 2009; Jang et al., 2019). There are only a few reports identifying canonical PBs and their components as regulators of plant viral infections. Carbon Catabolite Repression 4 facilitates *Barley yellow striate mosaic virus* replication in barley (Zhang et al., 2020), *Cabbage leaf curl virus* induces RNA decay rates in PBs to reduce antiviral silencing (Ye et al., 2015), VCS supports *Potato virus A* (PVA) infection (Hafrén et al., 2015; De et al., 2020), and LSM1 strengthens *Turnip mosaic virus* infection (Zuo et al., 2022), which is in turn compromised by the overexpression of several PB components (Li and Wang, 2018). The similarities between CaMV and the fundamentally different positive-stranded RNA virus PVA are striking, as VCS promotes PVA translation in a manner closely associated with the RNA silencing pathway (Hafrén et al., 2015; De et al., 2020). Thus, it seems that plant viruses could more commonly exploit this pathway for translational targeting of their RNAs. However, as PB components were also found to limit plant viruses (Li and Wang, 2018), this interaction is more complex, and plant viruses probably evolved individually to cope with the many PB-associated functions, including more general plant innate immune responses (Chantarachot et al., 2020).

PB components are involved in several different RNA surveillance processes, including decapping, NMD, and RNA silencing, which all play major roles in translational regulation through direct degradation but also translational repression of endogenous mRNA targets (Brodersen et al., 2008; Isken et al., 2008; Lanet et al., 2009; Xu and Chua, 2009; Jang et al., 2019; Wu et al., 2020; Hung and Slotkin, 2021; Iwakawa et al., 2021). When using four established PB marker proteins, we found distinct localization patterns under non-stress conditions, and the co-assembly of VCS, LSM1a, DCP5 and DCP1 into granules after HS (Figure 8A). Our results thus support the notion that stress-induced PBs contain the higher-order decapping complex, in accordance with previous findings (Xu and Chua, 2012; Motomura et al., 2015; Perea-Resa et al., 2016), while the constitutive microscopic foci of DCP1, DCP5, and VCS are unlikely to have prominent decapping activity and may instead serve other functions, including the storage of translationally repressed RNAs (Hubstenberger et al., 2017; Courel et al., 2019). Three out of four known PB decapping components localized to VFs (Figure 8A), giving rise to the hypothesis that the mRNA decapping machinery localized here to promote viral RNA decay. However, we found that both viral RNA stability and its capping levels where unaltered in the *lsm1* knockout mutant, unlike the situation for the previously established endogenous decapping target *AT4G32020*. Indeed, mRNA degradation and translational repression are selective (Xu and Chua, 2009; Tani et al., 2012; Hubstenberger et al., 2017; Sorenson et al., 2018; Jang et al., 2019), and together with our finding that VFs lack the essential decapping activator DCP1, this function is unlikely to be associated with VFs.

The polycistronic viral 35S RNA contains several potential triggers for RQC mechanisms, including PTCs, a large stem–loop, and extremely high expression levels. PTCs trigger degradation through NMD (Peltz et al., 1993) and in plants, this pathway was shown to suppress infections of PTC-carrying RNA viruses (Garcia et al., 2014). The primary reasons for addressing the NMD regulator UPF1 in this study is its largely shared protein interactome with DCP5 (Chicois et al., 2018), the general coupling of NMD with PBs (Lejeune et al., 2003; Raxwal et al., 2020), and the proposed capacity of P6 to suppress NMD via a direct interaction with VCS (Lukhovitskaya and Ryabova, 2019). However, CaMV showed UPF1-independent accumulation in both the *upf1* and *dcp5 upf1* mutants, disconnecting the NMD pathway from the pro-viral function of DCP5. Furthermore, endogenous targets of NMD decay were stabilized during infection in a DCP5-independent manner, suggesting that CaMV suppresses the NMD pathway irrespective of this PB component. Intriguingly, while transcript accumulation of the selected endogenous NMD targets occurred irrespective of DCP5 during CaMV infection, two targets had increased polysome association in *dcp5*, suggesting that these targets are under PB translational repression, unlike CaMV.

Our results suggest that the RNA silencing component RDR6, likely in conjunction with DCLs, mediates translational repression of the viral RNA in *dcp5* and *lsm1*. A link between RDR6-dependent RNA silencing and PBs was initially established in plants from forward genetic screens of induced transgene silencing, identifying both *xrn4* (Gazzani et al., 2004) and *dcp2* (Thran et al., 2012). Subsequently, endogenous genes were also shown to become targets of RDR6-dependent sRNA biogenesis in more severe seedling-lethal decapping mutants (Martinez de Alba et al., 2015). Based on these and other findings, the current model postulates that when the capacity of mRNA decay is exceeded, for example, overloaded with substrate or functionally compromised, decay substrates leak into the RDR6/DCL2/DCL4 pathway for sRNA biogenesis and subsequent RNA silencing processes (Liu and Chen, 2016). Even though CaMV infection is analogously compromised by RDR6 in *lsm1* and *dcp5*, we obtained numerous lines of evidence that this phenomenon differs from the above-described canonical model: (1) There were no evident changes in viral sRNA quantity or profiles in the mutants; (2) viral RNA levels remained largely unaffected, unlike transgenes, which are degraded and transcriptionally silenced; (3) *xrn4* did not weaken CaMV

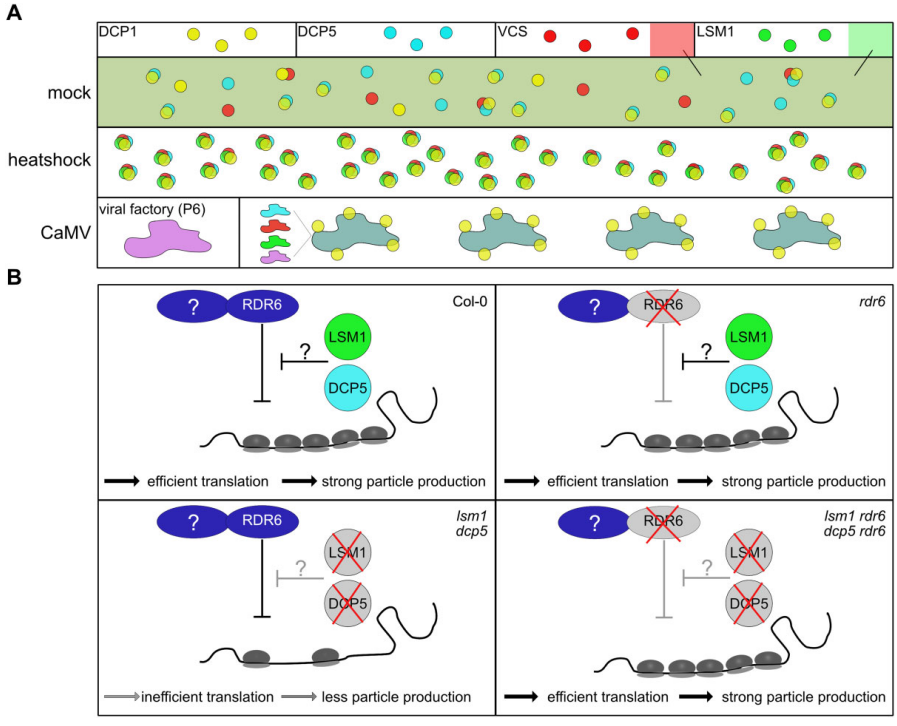

Figure 8 The role of LSM1 and DCP5 during CaMV infection. A, During undisturbed plant growth, PB components DCP1, DCP5, and VCS form foci that can be distinct for each protein or contain higher-order complexes of two or all three proteins, while LSM1 remains soluble in the cytoplasm. Upon heat stress, all four components assemble into higher-order complexes. CaMV produces viral factories in the cytoplasm that are sites of viral translation and replication. DCP5, VCS, and LSM1 are localized to these viral factories throughout the infection, while DCP1-marked foci assemble around, but not within viral factories. B, LSM1 and DCP5 aid viral translation by shielding the viral RNA from the repressive functions of RDR6 and possibly other proteins. Upon deletion of either DCP5 or LSM1, viral translation is impaired, leading to reduced particle production. While viral translation is not altered in the single *rdr6* mutant, it is rescued in *lsm1* and *dcp5* upon the additional deletion of RDR6, restoring the production of viral articles.

infection; and (4) viral RNA does not qualify as a substrate, as it showed no detectable levels of LSM1-dependent decapping and decay.

Plant viruses have frequently evolved means to suppress antiviral RNA silencing (Morel et al., 2002; Garcia-Ruiz et al., 2010; Garcia et al., 2014; Csorba et al., 2015). This includes CaMV, which is normally insensitive to DCL- and RDR6-dependent RNA silencing (Blevins et al., 2011), relying on at least two different strategies (Hohn, 2015). First, viral P6 suppresses the DRB4/DCL4 node of PTGS (Haas et al., 2008; Shivaprasad et al., 2008), a process that seems to also function in *lsm1* and *dcp5* judging from the comparably reduced levels of tasiRNAs along de-repression of their targets during infection. Second, these mutants show similar massive accumulation of viral sRNAs derived from *8s*, which are thought to constitute an important part of suppression by saturating and decoying the RNA silencing machinery with ineffective sRNAs (Blevins et al., 2011). Thus, both RNA silencing suppression strategies of CaMV appear to operate normally in the *lsm1* and *dcp5* mutants, prompting us to propose that PB dysfunction exposes the virus to a new, otherwise avoided RNA silencing-based translational repression mechanism (Figure 8B).

Having established a fundamentally novel framework around the balance between PB components and the RNA silencing machinery in CaMV RNA translation, the detailed mechanism becomes intriguing and requires further attention. RNA silencing involving RDR6, SGS3, and specifically DCL2-dependent 22-nt sRNAs were recently proposed to act together in translational repression during stress adaption and defense against transposons (Wu et al., 2020; Iwakawa et al., 2021; Kim et al., 2021). These studies identified abundant sRNA accumulation as part of the process, while CaMV sRNAs levels and profiles remained unaltered during translational repression in *lsm1* and *dcp5*. This is not necessarily a discrepancy, because all major size classes of

viral sRNAs are already highly abundant in wild-type plants and likely sufficient to drive the response with increased efficiency. Intriguingly, both RDR6 and SGS3 are well-established components of siRNA bodies (Jouannet et al., 2012; Kim et al., 2021), and the concept of substrate channeling and competition between PBs and siRNA bodies has been proposed (Jouannet et al., 2012), along with the general connection between RQC mutants and RDR6 (Liu and Chen, 2016). In summary, we propose that the association of PB components with CaMV VFs reduces viral RNA exposure, thereby evading translational repression by the RDR6 pathway (Figure 8B).

Material and methods

Plant material and growth conditions

All mutants used in this study were in the *Arabidopsis thaliana* accession Columbia-0 (Col-0) background, which was used as a control for all experiments (Supplemental Data Set 2). Mutants were checked for homozygosity using the primers described in Supplemental Data Set 3. Arabidopsis and *N. benthamiana* plants were grown in walk-in chambers under standard long-day conditions (120 mE,16-h light/8-h dark cycle) at 22°C day temperature (20°C night temperature) and 65% relative humidity for crossing, propagation, and transient expression assays. For infection experiments, plants were grown under short-day conditions (120 mE, 10-h light/14-h dark cycle) at 22°C day temperature (19°C night temperature) and 65% relative humidity. Light spectra in both conditions ranged from 400 to 720 nm.

Plasmid construction, generation of transgenic lines, and transient expression

The pENTRY clone containing the full-length Cabb B-JI P6 coding sequence (Hafren et al., 2017) was cloned into the pGWB654 or pGWB554 vector under the control of the 35*s* promoter (Nakagawa et al., 2007). Expressor lines were generated for this study by the floral dip method (Clough and Bent, 1998); all lines and constructs are listed in Supplemental Data Set 2. The coding sequences of *DCP1*, *DCP5*, *VCS*, and *LSM1a* were amplified from Col-0 plants (primers listed in Supplemental Data Set 4), cloned into pENTR/D-TOPO, and recombined in the pUBN/C-dest vector system for GFP fusions (Grefen et al., 2010). To establish PB double marker lines, *DCP1* and *LSM1* were cloned into the pUBC-mRFP vector and introduced into the GFP-VCS background by the floral dip method (Grefen et al., 2010). For transient expression, all coding sequences were cloned into pUBC for GFP fusions and pGWB654 for mRFP fusions. *Nicotiana benthamiana* leaves were infiltrated with resuspended Agrobacterium strain C58C1 cells (optical density (OD) 0.2, 10-mM $MgCl_2$, 10-mM MES pH 5.6, 150-μM acetosyringone) and the constructs analyzed after 48 h.

Virus inoculation and quantification

Arabidopsis plants were infected with CaMV or TRV 18 days after germination. The first true leaves were infiltrated with *Agrobacterium tumefaciens* strain C58C1 carrying CaMV strain CM1841 or TRV RNA1 and 2 (Liu et al., 2002) (OD 0.15) or mechanically rubbed with Cabb B-JI particles that were purified from turnip leaves and resuspended in carborundum-supplemented phosphate buffer (Martinière et al., 2009). Rosettes were harvested 21 dpi in four biological replicates, each containing two to three individual plants from which inoculated leaves were removed. All experiments were repeated from independent infections, each containing three to four biological replicates. For CaMV DNA quantification, 100 mg pulverized frozen leaf material was resuspended in 300 μL 100-mM Tris buffer (pH 7.5), supplemented with 2% sodium dodecyl sulfate (SDS) and treated with 0.2-mg/mL Proteinase K. Total DNA was precipitated with isopropanol 1:1 (v:v), and viral DNA levels were determined by quantitative Real-Time polymerase chain reaction (qRT-PCR) and normalized to *18S* ribosomal DNA (Hafren et al., 2017). RNA extraction from rosette tissue was performed with a Qiagen RNeasy kit and on-column DNase I digestion according to the manufacturer's protocol. About 500 ng of total RNA was used for first-strand cDNA synthesis with a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Waltham, MA, USA]). qRT-PCR analysis was performed with Maxima SYBR Green/Fluorescein qRT-PCR Master Mix (Thermo Fisher Scientific) using the CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with gene-specific primers (Supplemental Data Set 5). Viral transcripts were normalized to *PP2a* (*AT1G69960*) and expression levels determined as described by Livak and Schmittgen (2001).

Immunoblot analysis

Proteins were extracted from frozen rosette tissue in 100-mM Tris buffer (pH 7.5) supplemented with 2% SDS. Samples were incubated at 95°C for 5 min in 1× Laemmli sample buffer and cleared by centrifugation. The protein extracts were separated by SDS–PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, GE Healthcare, Amersham, UK), and blocked with 8% (w:v) skimmed milk in 1× PBS, supplemented with 0.05% Tween-20. Blots were incubated with 1:2,000 diluted primary antibodies α-P3 (Drucker et al., 2002), α-P4 (Champagne et al., 2004), α-P6 (Schoelz et al., 1991), or α-GFP (Santa Cruz Biotechnology, Dallas, TX, USA; sc-9996) before the subsequent addition of secondary horseradish peroxidase-conjugated antibodies (1:20,000; NA934 and NA931, Amersham, GE Healthcare). The immunoreaction was developed using an ECL Prime kit (Amersham, GE Healthcare) and was detected in the LAS-3000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). Quantification of band intensities was performed on blots using ImageJ 1.48v (Schneider et al., 2012). Band intensities were normalized to Ponceau S stain. An ELISA was performed for three independent experiments, with 100-mg infected plant material in 1 mL (w/v) 8M Urea buffer. Samples were incubated on high-binding ELISA plates for 6 h at 37°C before blocking in 5% skimmed milk.

Primary antibodies were added at 1:500 dilution overnight and secondary antibodies at 1:1,000 dilution for 3 h at 37°C. Absorbance was measured at 405 nm from 30 to 120 min after the addition of Substrate buffer (PNPP; Thermo Fisher).

Cap-dependent immunoprecipitation and XRN1 digestion

Immunoprecipitation of 7-methylguanosine (m^7G)-capped RNA was performed as described by Golisz et al. (2013). Anti-m^7G-Cap mAb (clone 150-15) was purchased from MBL International Corporation. For exonucleolytic digestion, total RNA was extracted from rosettes 21 dpi and incubated at 37°C with 1U XRN1 enzyme (Thermo Fischer) or in reaction buffer (mock) (Roux et al., 2015). cDNA synthesis and qRT-PCR were performed as described in the previous section. Transcript levels were normalized to *eIF4a* (*AT3G13920*; Perea-Resa et al., 2012; Roux et al., 2015).

RNA half-life measurement

Rosettes of CaMV-infected plants (21 dpi) were vacuum infiltrated with 1-mM Cordycepin (Sigma-Aldrich, St. Louis, MO, USA) in buffer (1-mM PIPES, pH 6.25, 1-mM sodium citrate, 1-mM KCl, 15-mM sucrose) and placed in a damp chamber. Two plants were harvested per sample corresponding to 0, 15, 30, 60, and 120 min after transcriptional inhibition. Total RNA was extracted using TRIzol reagent, followed by cDNA synthesis and qRT-PCR as described in the previous section. RNA levels were normalized to *eIF4a* (*AT3G13920*).

Preparation, sequencing, and analysis of sRNA libraries

sRNA libraries were prepared from 500-ng total RNA with a NEBNext Multiplex Small RNA Library Prep Set for Illumina (E7300; New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. The amplification step was set to 12 cycles, and amplified libraries were cleaned using SPRIselect beads (Beckman Coulter, Brea, CA, USA). Size selection was performed on a 6% polyacrylamide gel according to the manufacturer's protocol and eluted in 10-μL TE buffer. Size range and library concentrations were confirmed using the Bioanalyzer 2100 (Agilent Systems, St Clara, CA, USA). Libraries were sequenced on an Illumina Nextseq2000 system in paired-end 50-bp mode at the SciLifeLab facility, Solna, Sweden.

For sRNA-seq analysis, reads were obtained in fastq file format from the facility. Adapters were trimmed using flexbar with the -ap ON option (version 3.5.0; (Roehr et al., 2017)) and the corresponding adaptor sequences as indicated for the NEBnext E7300 sRNA preparation kit (read1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC, read 2 GATCGTCGGACTGTAGAACTCTGAACGTGTAGATCTCGG TGGTCGCCGTATCATT). Afterward, corresponding forward and reverse reads were combined using fastq-join (version 1.3.1; https://github.com/ExpressionAnalysis/ea-utils/blob/wiki/FastqJoin.md) and the option-v for illumina reads. Joint fastq files were size trimmed to obtain only sizes of 18–26 nt by utilizing cutadapt (version 1.9.1) (Martin, 2011) with the parameters -m 18 -M 26. Read size and quality were checked using FastQC in default mode (version 0.11.9; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Size trimmed fastq files were further processed as described in Bente et al. (2021) using publicly available scripts (https://github.com/AlexSaraz1/paramut_bot). To create sRNA profiles along the CaMV sequence, the hygromycin phosphotransferase transgenic sequence from Bente et al. 2021 was replaced by the genomic CaMV sequence (GenBank V00140.1) by setting the start position to the beginning of the 35*s* promoter. Reads were aligned to the TAIR10 genome including an extra contig containing the above-mentioned CaMV sequence. Alignment was done using Bowtie2 (version 2.3.5.1; Langmead and Salzberg, 2012) with the options -k 500 -no-unal. For read counts in the TAS genes and its targets, featureCounts (version v1.6.3; Liao et al., 2014) from the Subread package (http://subread.sourceforge.net/) was used with the options -t gene -s 1 -M on the aligned bam files (Liao et al., 2014). Graphical representation was achieved using R. For profile comparisons along the CaMV sequence, the axes were adjusted to 8,000 and 6,000 RPM on the positive and negative strands, respectively. An overview of the processed sRNA libraries is shown in Supplemental Data Set 1.

Polysome isolation

Polysome extraction was performed based on Mustroph et al. (2009) with some modifications. Briefly, 1-mL frozen leaf powder was thawed in 8 mL of polysome extraction buffer (200-mM Tris–HCl (pH 8.0), 200-mM KCl, 35-mM $MgCl_2$, 25-mM EGTA, 1-mM DTT, 1-mM phenylmethanesulfonylfluoride, 100-μg/mL CHX, 1% (vol/vol) detergent mix (20% (w/v) Brij-35, 20% (v/v) Triton X-100, 20% (v/v) Igepal CA630, and 20% Tween-20), 1% (v/v) polyoxyethylene 10 tridecyl ether), resuspended, and kept on ice for 10 min. The plant debris was removed by centrifuging at 16,000*g* for 15 min at 4°C in a JA-25.50 rotor and Avanti

J-20 XP centrifuge (Beckman Coulter). The clear supernatant was gently poured on top of an 8-mL sucrose cushion (100-mM Tris–HCl (pH 8.0), 40-mM KCl, 20-mM $MgCl_2$, 5-mM EGTA, 1-mM DTT, 100-μg/mL CHX in 60% sucrose) in a 26-mL polycarbonate tube (Beckman Coulter). After proper balancing, the samples were centrifuged at 35,000 RPM for 18 h at 4°C in a 70Ti rotor and L8-M ultracentrifuge (Beckman Coulter). The ribosome pellets were gently washed with RNase-free water and resuspended in 300 μL of resuspension buffer (100-mM Tris–HCl (pH 8.0), 40-mM KCl, 20-mM $MgCl_2$, 100-μg/mL CHX). The resuspended samples were kept on ice for 30 min, followed by centrifugation at 16,000*g* at 4°C to remove any debris. The RNA content was measured for each sample using a Qubit BR RNA assay kit (Thermo Fisher Scientific). The resuspended ribosome samples were loaded on 15%–60% sucrose gradients and centrifuged at 50,000 RPM in a SW55.1 rotor and L8-M ultracentrifuge (Beckman Coulter). The gradient samples were fractionated using an ISCO absorbance detector (model # UA-5, ISCO, Lincoln, NE) to obtain fractions of ∼250 μL.

The fractions were pooled before RNA extraction with TRIzol to obtain samples from free RNA as well as monosome-bound RNA and three pools of light, medium, and heavy polysome-bound RNA. RNA levels were normalized to *SAND* (*AT2G28390*) in each fraction and depicted as fractions of the sum of ribosome-bound RNA in Col-0 (for *dcp5*, *lsm1*, and *rdr6*) or *rdr6* (for *lsm1 rdr6* and *dcp5 rdr6*) to enable the comparison of total abundance on ribosomes, as well as relative abundance along the gradient, after testing for comparable RNA input.

Confocal microscopy and treatments

Micrographs from leaf abaxial epidermal cells were taken under a Zeiss LSM 780 microscope. GFP and RFP signals were detected at 488 nm/490–552 nm and 561 nm/569–652 nm, respectively. Co-visualization was achieved through sequential scanning mode. For HS conditions, leaves were kept in water at 38°C for 30 min (1 h for LSM1-GFP) before imaging. Translational inhibition treatment was achieved by infiltrating young leaves with 200 μM CHX (Sigma-Aldrich), followed by incubation for 1 h before imaging. Images were processed with ZEN black software (Zeiss) and ImageJ version 1.53b. For quantification, Z-stacks were Brightness increased and a median filter of 2 pixels applied. Stomata were manually deleted from micrographs, and a mask was generated through thresholding. Foci were counted using the "Analyze Particles" tool.

Data analysis and statistical methods

Statistical comparisons of two groups were performed by Student's t test in Excel. One-way analysis of variance (ANOVA) followed by a post-hoc Tukey HSD test ($\alpha = 0.05$) was performed with R version 4.0.02 and the R-package "agricolae" (version 1.3-3; https://cran.rproject.org/web/packages/agricolae/index.html). Test statistics are shown in Supplemental Data Set 6.

Accession numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: DCP1 (AT1G08370), DCP2 (AT5G13570), LSM1a (AT1G19120), LSM1b (AT3G14080), DCP5 (AT1G26110), XRN4 (AT1G54490), VCS (AT3G13300), RDR2 (AT4G11130), RDR6 (AT3G49500), DCL2 (AT3G03300), DCL3 (AT3G43920), DCL4 (AT5G20320), UPF1 (AT5G47010), TAS1a (AT2G27400), TAS1b (AT1G50055), TAS1c (AT2G39675), TAS2 (AT2G39681), RPS6 (AT5G4670), and SMG7 (AT5G19400).

sRNA sequencing data from this study were deposited in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE194186, and raw data were deposited in the Sequencing Read Archive (https://www.ncbi.nlm.nih.gov/sra) under accession number SRP356192.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Description of PB markers during CaMV infection.

Supplemental Figure S2. PB double marker lines show co-assembly after HS and during CaMV infection.

Supplemental Figure S3. Co-expression of PB components with CaMV proteins in *N. benthamiana*.

Supplemental Figure S4. Co-localization of P6 with PB components under mock conditions.

Supplemental Figure S5. Viral DNA accumulation in additional PB mutants.

Supplemental Figure S6. *35s* RNA decay after transcriptional arrest with cordycepin.

Supplemental Figure S7. CaMV disease in combinatorial mutants with NMD and RNA silencing.

Supplemental Figure S8. Expression of NMD targets in *dcp5* and *upf1* during CaMV infection.

Supplemental Figure S9. sRNA profiles in *lsm1* and combinatorial mutants.

Supplemental Figure S10. Translational profiling during CaMV infection.

Supplemental Data Set 1. Total number of sRNA reads in each sample and their mapping to the 8,031-bp CaMV genome.

Supplemental Data Set 2. Plant material used and generated in this study.

Supplemental Data Set 3. DNA oligonucleotides used in this study for genotyping.

Supplemental Data Set 4. DNA oligonucleotides used in this study for molecular cloning.

Supplemental Data Set 5. DNA oligonucleotides used in this study for expression analysis.

Supplemental Data Set 6. ANOVA tables.

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Viruses flood cells with foreign RNA during infection, which triggers several defence responses in the host. Plant cytoplasmic RNA granules are at the forefront of RNA regulation during development and stress responses. This thesis examines the role of RNA granules during *Cauliflower mosaic virus* infection in *Arabidopsis thaliana* and how both, plant and virus, struggle to win the upper hand in RNA expression and translation.

Gesa Hoffmann received her graduate education at the Department of Plant Biology, SLU, Uppsala. She obtained her M.Sc. degree in Molecular Biology from the University of Vienna and her B.Sc. degree in Biology from the University of Cologne.

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