Identification and Characterization of Host Genes Involved in Regulating Replication of Brome Mosaic Virus

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ABSTRACT

Brome mosaic virus (BMV) belongs to a viral class called positive-strand RNA [(+)RNA] viruses. This is the largest class of viruses and includes numerous important pathogens. BMV infects monocotyledonous plants and its replication can be recapitulated in the baker's yeast (*Saccharomyces cerevisiae*) under laboratory conditions to use yeast as an experimental model system. BMV generally does not infect dicotyledonous plants including the model plant *Arabidopsis thaliana*. One important shared feature of (+)RNA viruses is that they all make use of host proteins to aid in their own viral replication. In particular, (+)RNA viruses use host intracellular membranes for their replication and lipid composition of these membranes is crucial for viral replication. I show here that BMV replication protein 1a causes redistribution of host Lam5 (Lipid transfer protein Anchored at a Membrane contact site 5) and that Lam5 is necessary for BMV replication in yeast. Furthermore, in the absence of Lam5, BMV 1a affects the distribution of lipid droplets throughout yeast cells. Host factors also play critical roles in defense against viruses. Although wild-type Arabidopsis is not a host for BMV, the Arabidopsis *cpr5* (Constitutive expression of Pathogenesis-Related genes 5) mutant can support systemic infection of BMV. I performed screens in Arabidopsis and have identified four genes that contribute to defense against BMV. These include two RNA-binding proteins, a lectin superfamily protein, and an alternative oxidase. My results also contribute to the growing evidence that reactive oxygen species play a key role in BMV replication. In summary, my work provided new insights into BMV replication in hosts and plant defense against BMV infection. The information gained from these projects aids in our understanding of (+)RNA virus biology in general and may identify targets for developing broad-spectrum antiviral strategies.

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GENERAL AUDIENCE ABSTRACT

Viruses are important pathogens that can cause devastating diseases not only in humans, but also in animals and plants. It is important to study viruses and their interactions with their hosts to develop antiviral drugs and engineer plant resistance to viruses. Positive-strand RNA viruses are the largest class of viruses and are responsible for numerous human, animal, and plant diseases. Brome mosaic virus (BMV) belongs to this class of viruses and has a simple genome organization. Furthermore, the baker's yeast can support BMV replication. BMV serves as a model to study (+)RNA virus replication and virus-host interactions. I show here that BMV depends on the lipid transport protein Lam5 for proper replication in yeast. Furthermore, although much is known about BMV replication in yeast, more information is needed on BMV replication in plants. To aid in this goal, I performed screens in the model plant *Arabidopsis thaliana* to identify Arabidopsis mutants that could allow for systemic BMV infection. This project has uncovered four new genes that contribute to defense against BMV. This research aids in our understanding of how BMV replication works and also how plants defend themselves against viruses. This work is important for understanding of the biology of (+)RNA viruses and the plant immune system in general.

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List of Abbreviations

(+)RNA:	positive-strand RNA
ACBP:	acyl coA binding protein
AOX1a:	alternative oxidase 1a
APX1:	ascorbate peroxidase 1
BMV:	brome mosaic virus
Cho2:	choline-requiring 2
CMV:	cucumber mosaic virus
COPI:	coatomer protein I
DENV:	dengue virus
ER:	endoplasmic reticulum
ERMES:	ER/mitochondria encounter structure
ERO1:	ER oxidation 1
FHV:	Flock House virus
GFP:	green fluorescent protein
LAM:	lipid transfer protein anchored at a membrane contact site
NVJ:	nuclear vacuolar junction
PC:	phosphatidylcholine
PE:	phosphatidylethanolamine
Pgk:	phosphoglycerate kinase
PVDF:	polyvinylidene fluoride

RTNs:	reticulons
TMV:	tobacco mosaic virus
VRC:	viral replication complex
SARS-CoV:	severe acquired respiratory syndrome-coronavirus
vCLAMP:	vacuole and mitochondria patch
WT:	wild type

Chapter 1: Introduction

1.1 Classification of viruses

Viruses are obligate intracellular microbes. According to the Baltimore Classification, proposed by virologist David Baltimore in the 1970s, there are seven classes of viruses (fig. 1.1). These classes are based on the genetic material (e.g. DNA or RNA) and the sense of that genetic material (Baltimore, 1971). His classification system originally only concerned animal viruses, but actually applies well to all types of viruses, including plant viruses. This system is based on the fact that even though viruses have a diverse array of genomes, they all aim to make mRNAs that can be recognized by cellular ribosomes to make viral proteins (Koonin et al., 2021).

1.2 Positive-strand RNA viruses

My work focuses on Class IV: positive-strand [(+)] RNA viruses, whose RNA genome can be directly used for translation. This class includes many significant viruses based on their impact on science and on human health and the economy. Some examples include SARS-CoV-2, tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), barley yellow dwarf virus (BYDV), and potato virus Y (PVY). This is the largest of all the seven classes of viruses. In fact, (+)RNA viruses make up more than one-third of all known viral genera (Ahlquist et al., 2003). (+)RNA viruses constitute the majority of known plant viruses (Newburn and White, 2015).

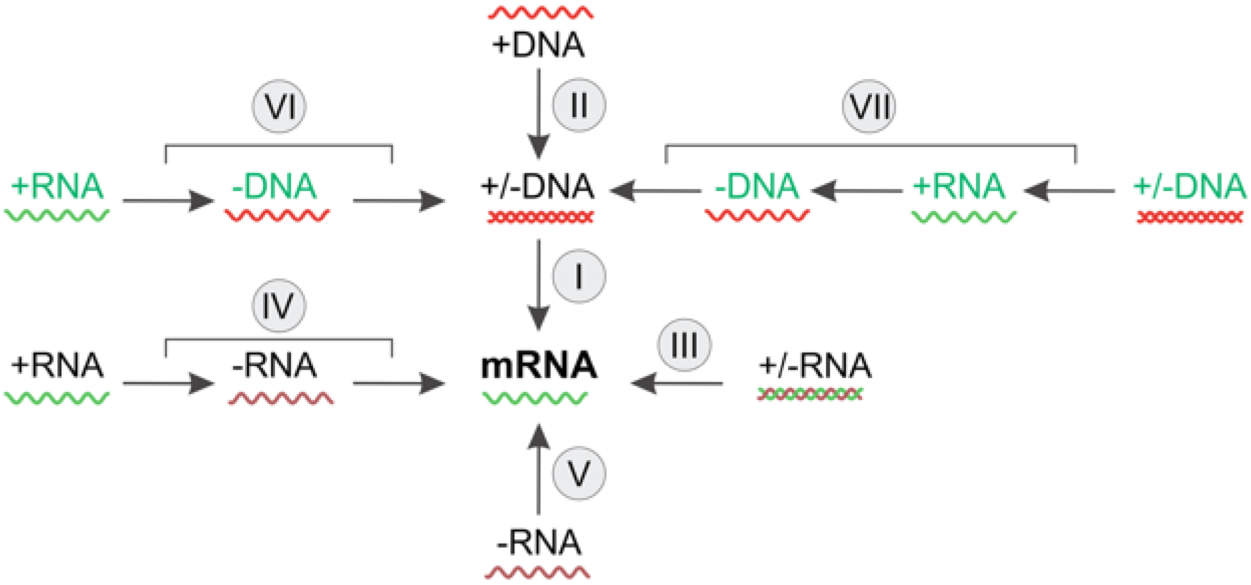

Figure 1.1 Seven classes of viruses. The Baltimore classification system classifies viruses based on how they use their genetic material to make mRNAs. These mRNAs are recognized and bound by cellular ribosomes to translate viral proteins. Roman numerals depict the viral class. Adapted from Koonin et al., 2021.

1.3 Viral replication complexes

One shared feature of (+)RNA viruses is that they replicate at specific organelle membranes where they form viral replication complexes (VRCs). These VRCs are formed by the modifications of host cellular membranes that serve to compartmentalize viral replication, protect viral nucleic acids from host defense mechanisms, and to organize the viral/host factors to promote viral replication (den Boon et al., 2024). These membranes serve as scaffolds for anchoring VRCs to the cell. Viruses can often cause the redistribution of host proteins from areas throughout the cell to the VRCs so they can be utilized for viral replication (Rodriguez-Peña et al., 2021). Electron microscopy is often used to study VRCs. Recently, cryo-EM and tomography have revealed that the neck of VRCs contains “crown” complexes that stabilize the necks and form channels (den Boon et al., 2024).

Different viruses use different but specific organelle membranes as the site to form their VRCs. For example, poliovirus makes VRCs at the ER and Golgi membranes (Bienz et al., 1987; Belov et al., 2012), while Flock House Virus (FHV) replicates at the mitochondria (Miller et al., 2001). As examples of plant viruses, CMV replicates at the tonoplast (Hatta and Francki, 1981), while turnip yellow mosaic virus replicates at the chloroplast (Bhattacharyya and Chakraborty, 2018). Host membranes can be easily expanded and modified, thus explaining why viruses replicate there. Since viruses form VRCs at host membranes, it is clear that host membranes and thus lipids play significant roles in virus replication.

There are two major classes of VRCs. One class is called single-membrane-vesicles (SMVs) or spherules. These result from invaginations of a single membrane. Viruses that have this class of VRCs include brome mosaic virus (BMV), West Nile virus, and FHV. The other class of VRCs is called double-membrane-vesicles (DMVs). Viruses that generate DMVs include poliovirus, SARS-CoV-1, and hepatitis C virus (Shulla and Randall, 2016).

Formation of VRCs depends on both viral proteins and hijacked host proteins. Accumulating evidence indicates that expressing certain viral proteins in cells can cause membrane rearrangements. For example, expression of poliovirus protein 2BC in yeast causes membrane proliferation and disrupts exocytosis (Barco and Carrasco, 1995). Expression of replication protein 1a of brome mosaic virus in yeast is known to induce BMV spherule formation (Schwartz et al., 2002). However, these viral proteins are not inducing membrane rearrangements all on their own; they recruit host proteins to aid in the VRC formation.

1.4 Host factors involved in viral replication/infection

Since (+)RNA viruses have small genomes, a crucial aspect of virus infection is the use of host factors. Host factors play important roles in facilitating virus entry into cells, translating the viral genome, forming VRCs, modifying lipid composition of VRCs, preventing viral RNA from degradation, and several other processes (Ahlquist et al., 2003; Nagy and Pogany, 2012; Rodriguez-Peña et al., 2021; den Boon et al., 2024). Here I will discuss general groups of host factors involved in the replication of several viruses. Later, I will discuss host factors specifically involved in BMV replication.

Several viruses have been shown to use Coatomer protein I (COPI) for their replication (Jay C Brown, 2012). COPI is a protein that coats certain types of vesicles, termed COPI vesicles. These vesicles typically are used for retrograde transport within the cell: from the Golgi back to the ER (Taylor et al., 2023). Some studies have implicated COPI in anterograde transport, meaning transport from the ER to the golgi, and in endosomal sorting (Orci et al., 2000; Gabriely et al., 2007; Park et al., 2015). COPI has also been shown to affect plasma membrane lipid composition; disruption of COPI causes cholesterol to move away from the plasma membrane (Misselwitz et al., 2011).

Several viruses have also been shown to use GTPases, which are enzymes that convert GTP to GDP. GTPases are often involved in intracellular transport (Spearman, 2018). This may suggest that viruses use GTPases in relation to COPI vesicle components for intracellular transport.

Another class of host factors linked to virus replication is host chaperone proteins. Chaperones are proteins that assist with protein folding (Kim et al., 2013; Tittelmeier et al., 2020). It was found that inhibiting the chaperone protein Hsp-90 using geldanamycin greatly reduces FHV replication in Drosophila cells (Kampmueller and Miller, 2005). It was later found that inhibition of Hsp-90 decreases the synthesis of the RNA polymerase of FHV (Castorena et al., 2007). Hsp90 prevents proteasome-mediated degradation of the rift valley fever phlebovirus RNA polymerase (Alem et al., 2024). It has also been shown that assembly of the tomato bushy stunt virus replicase in vitro requires heat shock protein 70 from yeast (Pogany et al., 2008).

Genome-wide screens tell us that different viruses use mostly different sets of host proteins for their replication (Nagy and Pogany, 2012). Much remains to be studied on why different viruses prefer to hijack different proteins and what allows a virus to do so. Learning more about this and virus host factors in general would be of great benefit to developing antivirals that could target only the virus and leave the host undisturbed.

1.5 VRCs and Autophagy

There are important connections between viral replication and autophagy of several viruses. Autophagy is a process in which certain cytosolic contents are enveloped into vesicles and then degraded. This allows the cell to recycle cellular components. There is always a basal level of autophagy occurring within the cell, but it is especially induced under nutrient-stress, a time when recycling nutrients within the cell would be especially beneficial. Targets for autophagy can include proteins, lipids, or even whole organelles (Youle and Narendra, 2011; Chipurupalli et al., 2021). The double-membraned vesicles formed during autophagy, termed autophagosomes, could serve as scaffolds for viral replication, protect viral genomes from RNAses and host immune signaling, and could help with release of the virus outside the host cell (Jassey and Jackson, 2024). Viruses that use autophagy to aid in their replication will typically help initiate the formation of the double-membrane vesicles, but block the lysososmal degradation aspects. For example, influenza A virus prevents the double-membrane vesicles of autophagy from fusing to the lyusosome (Gannagé et al., 2009). Another example comes from Hepatits C virus, which induces the formation of autophagosomes to benefit its replication, but does not always let those autophagosomes mature (Wang et al., 2015).

However, there is evidence that autophagy plays antiviral roles as well and that some viruses attempt to block autophagy. One example is that a viral protein of barley stripe mosaic virus (BSMV) directly interacts with host Autophagy Protein 7 to disrupt a signaling pathway that would have led to autophagy (Yang et al., 2018).

1.6 The engineered BMV-yeast system

A good model to study (+)RNA virus replication has been brome mosaic virus. BMV is the type member of the bromoviridae family and a representative member of the alphavirus-like superfamily (He et al., 2020). This virus has a small genome, robust replication, and its replication can be recapitulated in yeast, thus making it a useful model virus (Diaz and Wang, 2014). BMV has 3 genomic RNAs: RNA1, 2, and 3, which are used to translate the viral proteins 1a, 2a, and 3a respectively. There is also subgenomic RNA 4, which codes for the capsid protein. RNA 4 also plays a role in regulating expression of other BMV RNAs via translational inhibition (Shih and Kaesberg, 1973). 1a and 2a are necessary and sufficient for BMV replication (Diaz and Wang, 2014). 3a is responsible for cell-to-cell movement. BMV 1a localizes to the perinuclear ER and is able to induce spherule formation without expression of other viral factors in yeast (Schwartz et al., 2002).

The BMV-yeast system uses a cDNA derivative of BMV RNA3. This system makes use of the fact that the BMV coat protein gene is the second cistron of RNA3 and thus will only be translated from the subgenomic RNA, RNA4 (Janda and Ahlquist, 1993). RNA4 is only produced from negative-strand RNA3. Thus, it is possible to measure BMV replication by using BMV RNA3 derivatives that have reporter genes, such as luciferase or URA3, replacing the coat protein gene. The BMV-yeast system has served as a great tool to enhance our knowledge of viral replication complex formation, virus replication, and (+)RNA viruses in general.

1.7 BMV replication

BMV replication starts when 1a and 2a physically interact in the cytoplasm. These two proteins then travel to the perinuclear ER, where 1a induces invaginations of the outer perinuclear ER membrane (or outer nuclear membrane) into the ER lumen (Diaz and Wang, 2014). These invaginations become larger and eventually become spherules, which serve as the VRCs when 2a and RNA3 are recruited. When 1a is expressed with low levels of 2a, the spherules develop properly. However, if 2a is expressed under a stronger promoter, membrane layers/stacks form instead of spherules (Schwartz et al., 2004).

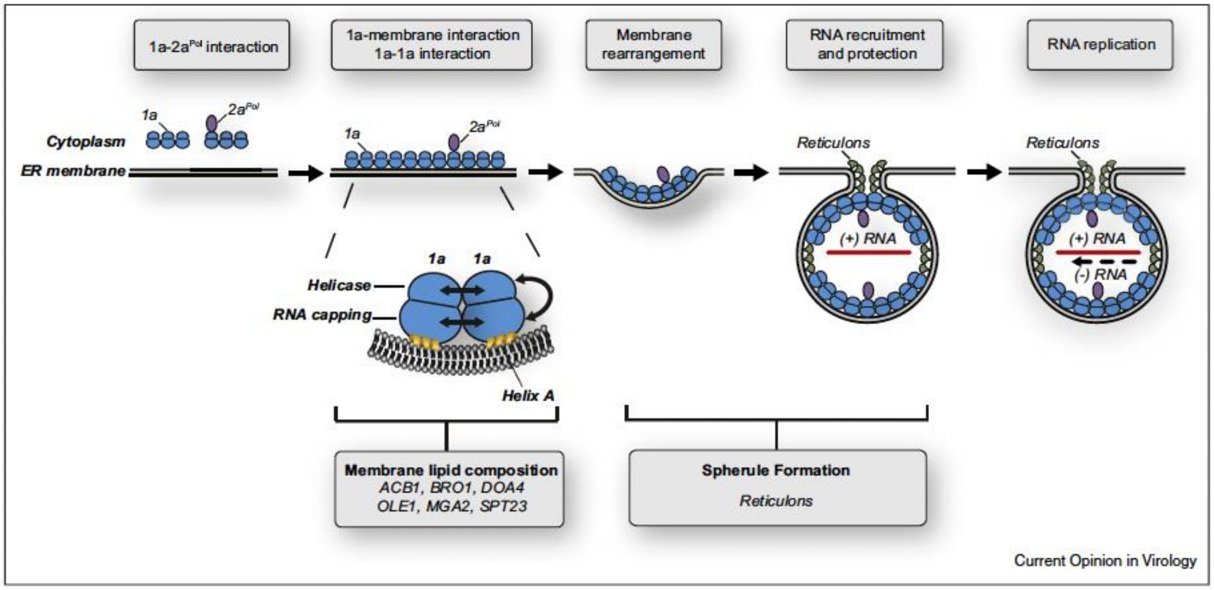

Figure 1.2 General replication process of BMV. BMV replication initiates when 1a and 2a interact and travel to the perinuclear ER, where they form invaginations in the membrane. These invaginations develop into the spherules, which serve as BMV's VRCs. Image adapted from Diaz and Wang 2014.

Once the VRCs are formed, BMV (+)RNA is replicated in those VRCs to produce (-)RNA by using (+)RNA as a template. Then, the (-)RNA serves as a template to produce many more offspring (+)RNAs and BMV subgenomic RNA4 (sgRNA4). Translation of viral proteins occurs and afterwards, the viral genome is encapsulated by coat protein to form the virion. These new virions can move cell to cell via plasmodesmata and vascular tissue. For viruses to move from one plant to another, vectors such as insects are typically required.

1.7.1 Host factors in BMV replication

Like other (+)RNA viruses, BMV makes use of multiple host factors to aid in its replication (Kushner et al., 2003). One class of these proteins is called the reticulons. These proteins function to cause and maintain ER membrane curvature and also function to form nuclear pores (Voeltz et al., 2006; Dawson et al., 2009). Another study revealed that reticulons help facilitate lipid transfer between the ER and mitochondria (Voss et al., 2012). Yeast cells have 3 reticulons: Rtn1, Rtn2, and Yop1. Expression of BMV 1a relocalizes all 3 reticulons from the peripheral ER to the interior of BMV VRCs (Diaz et al., 2010). In yeast cells without functional reticulon genes, VRCs do not form properly and thus BMV replication is inhibited. It has been hypothesized that reticulons are involved in the formation of the necks of the BMV VRCs (Diaz et al., 2010).

Another class of host proteins required for BMV replication is the ESCRT (endosomal sorting complexes required for transport) proteins. In cells, this class of proteins play roles in the formation of multivesicular bodies in cells. Furthermore, some ESCRT proteins play roles in the completion of cytokinesis, plasma membrane repair, and in the budding of enveloped viruses from the plasma membrane (Vietri et al., 2020). Individual deletions of several ESCRT genes result in decreased BMV replication. Of these deletion mutants, the *snf7* deletion mutant gave the largest decrease in BMV replication because VRC formation was affected. Snf7 is proposed to maintain the neck of VRCs (Diaz et al., 2015).

Expression of BMV 1a in yeast leads to an increase in total fatty acid levels and overall intracellular membranes (Lee and Ahlquist, 2003). In addition, BMV requires a high-level of unsaturated fatty acids (UFAs) as evidenced by a 20-fold reduction of BMV replication in a yeast *ole1* mutant. *OLE1* (Oleic acid requiring) is an essential gene encoding △9 fatty acid desaturase that converts saturated fatty acids (SFAs) to UFAs. The yeast *ole1w* mutant, which has a single mutation in *OLE1*, substantially inhibited BMV replication. However, this mutant grows as well as WT. Supplementing UFAs in the growth medium complemented BMV replication defects in *ole1w* yeast cells, indicating that OLE1's role in BMV replication has to do with UFA production (Lee et al., 2001). Further research showed that VRC membranes were preferentially deprived of UFAs in the *ole1w* mutant as demonstrated by electron microscopic analysis, suggesting that the lipid composition of VRCs is different from the rest of nuclear membranes (Lee and Ahlquist, 2003).

BMV also relies on phosphatidylcholine (PC) for its replication. BMV and other (+)RNA viruses promote PC synthesis near or at VRCs. BMV 1a interacts with Cho2, an enzyme in the CDP-DAG pathway of PC synthesis. Cho2 localizes to ER membranes but expression of 1a causes Cho2 to relocalize to the perinuclear ER and colocalize with BMV 1a. BMV replication is decreased in the yeast *cho2* mutant (Zhang et al., 2016). Phosphatidate (PA) is an important precursor of PC and other phospholipids. Pah1 converts PA to diacylglycerol, which is then converted to TAG for storage. Loss of function of *PAH1* increases levels of total phospholipids, increases the number of BMV VRCs, and increases BMV replication by more than 100% (Zhang et al., 2018), suggesting the availability of PC and phospholipids is a bottle neck of BMV replication.

An important question is how BMV viral proteins and associated host proteins are drawn to the VRCs. COPII vesicles are known to transport intracellular cargo to be secreted outside the cell. It was found that Erv14 (ER-vesicle protein of 14 kD), a receptor for COPII vesicles, interacts with BMV 1a. Furthermore, if *ERV14* is deleted, the localization of BMV 1a is disrupted (Li et al., 2016), suggesting that BMV uses Erv14 for its localization, thus possibly linking the COPII vesicle pathway to BMV replication.

1.8 Membrane contact sites

It was previously thought that eukaryotic cells were composed of different organelles simply floating through the cytosol. However, in the last few decades, a new perspective on this topic has emerged (Eisenberg-Bord et al., 2016). It turns out that the different organelles are physically tethered together throughout the cell. Different organelle membranes are held together in close proximity (typically 10-40 nm) by proteins that act as tethers (Eisenberg-Bord et al., 2016). These areas of close proximity between membranes are called membrane contact sites (MCS). Examples of MCS include the NVJ (nuclear vacuolar junction), vCLAMP (vacuole and mitochondria patch), and ERMES (ER/mitochondria encounter structure) (Elbaz-Alon et al., 2015). Organelles can form more than one MCS at a time and each of those can perform different functions. At MCS, although the membranes are very close and tethered together, the membranes do not fuse together. Near the MCS, the organelles contact the cytoskeleton. The tethers at MCS can endure even as the organelles travel long distances (Friedman et al., 2013). MCS can be within a single organelle, between multiple organelles, or even an organelle contacting a lipid droplet (Prinz et al., 2020). Lipids, such as cholesterol, can affect the composition of MCS (Rocha et al., 2009; Prinz et al., 2020). This potentially gives a clue as to if or how MCS could play a role in virus replication. Tomato bushy stunt virus (TBSV) interacts with MCS proteins and uses these proteins to transfer sterols to the peroxisome membrane, the site of TBSV replication (Levy and Tilsner, 2020).

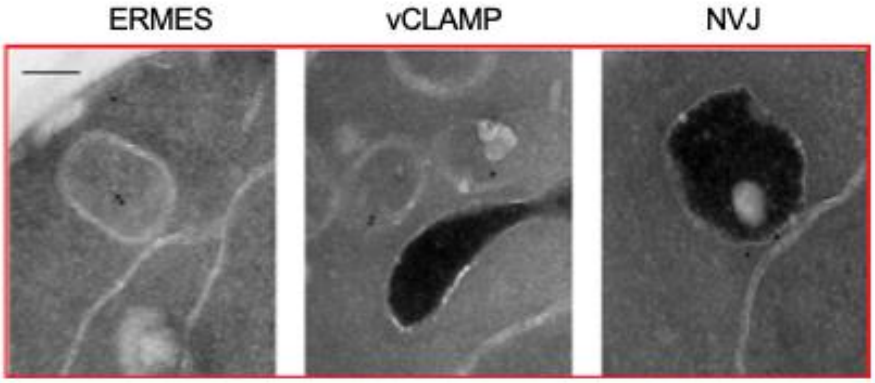

Figure 1.3 Examples of MCS. EM images depicting various MCS in WT yeast cells. Scale bar represents 200 nm. Image adapted from Elbaz-Alon et al., 2015.

MCS host a variety of different processes that are critical to the cell. Metabolite signaling is one of those processes. Calcium especially is an important metabolite transferred between the ER and other organelles, playing important roles in cell signaling. Calcium, an important secondary messenger, has been linked to signal transduction pathways involving ROS, oxidative phosphorylation, apoptosis, and several other important functions (Prinz et al., 2020). The cell stores a high amount of calcium in the ER lumen and usually very low calcium is in the cytosol (Phillips and Voeltz, 2016). When the cell needs to use calcium for the aforementioned signaling pathways, the calcium is released from the ER and sent to the cytosol or organelles to convey the signal.

1.8.1 Membrane contact sites and stress

Membrane contact sites have important roles in cellular stress responses (Prinz et al., 2020). I will discuss a few of the important details that are relevant to stresses because virus infection could be a source of stress to cells, so understanding how MCS change in response to stress could be useful to understanding how viruses impact the host cell.

One relevant type of stress is lipid stress, which occurs when there are too many lipids in the cell. Some lipids, though important to the cell, can become deleterious when lipid signaling becomes disrupted or lipids are in excess within the cell (Olzmann and Carvalho, 2019). To combat this, cells use lipid droplets (LDs) as structures to store excess lipids. LD biogenesis occurs in the ER. After budding from the ER, LDs mature and expand. ER phospholipid composition has been shown to affect LD budding from the ER (Ben M'barek et al., 2017). A large portion of LDs actually stay at the ER, but

LDs can form contacts with several other organelles. They can even form contacts with other lipid droplets (Olzmann and Carvalho, 2019). Under nutrient stress, there is an increase in LDs at the nuclear-vacuole junction (Hariri et al., 2018). Interestingly, several pathogens induce LD formation and exploit lipid droplets for nutrients (Bosch et al., 2021). During cell growth, LDs can be used as a source of lipids for membrane synthesis (Olzmann and Carvalho, 2019). Viruses can use this feature of LDs as well.

MCS have a role in plant response to mechanical stress. Mechanical stress can be caused by external factors such as wind, rain, or insects. Plants also have a degree of internal mechanical stress, which occurs during normal growth and especially during fruit development (Kouhen et al., 2023). *Arabidopsis thaliana* synaptotagmin 1 (*SYT1*) expression is upregulated in leaf cells that are experiencing mechanical stress (Pérez-Sancho et al., 2015). SYT1 is localized at ER-plasma membrane contact sites (Levy and Tilsner, 2020). Protoplasts prepared from the *syt1* mutant and leaves of the mutant are more susceptible to mechanical wounding than those of WT (Pérez-Sancho et al., 2015). It is hypothesized that SYT1 could act as a tether at the ER-plasma membrane contact site. SYT1 also regulates endocytosis and plays a role in cell-to-cell movement of Cabbage leaf curl virus (CLCV) and TMV. In fact, if *SYT1* is knocked down in Arabidopsis, CLCV infection is slowed (Lewis and Lazarowitz, 2010). It is possible that cell responses to mechanical stress are needed during formation of VRCs. SYT1 is needed for proper Turnip vein clearing virus (TVCV) VRC formation; in *syt1* the TVCV VRCs are smaller than in WT plants (Levy and Tilsner, 2020).

MCS have also been linked to important components of autophagy, which is also a major cellular stress response, as well. For example, mitophagy is a type of autophagy where mitochondria are degraded by the cell. This process is important to maintain steady-state levels of mitochondria and also to adjust mitochondria levels during important developmental processes (Wang et al., 2023). The ERMES colocalizes with mitoautophagosomes and if components of ERMES are knocked out, then mitophagy rates decrease (Böckler and Westermann, 2014).

Another possible connection MCS could have to autophagy comes through TORC signaling. TORC1 and TORC2 (target of rapamycin complex 1 and 2) are two protein complexes that are important regulators of autophagy, as well as cell growth and proliferation. Compartments at ER-plasma membrane contact sites have been shown to affect TORC2 signaling via the LAM (Lipid transfer proteins anchored at membrane contact sites) family of genes. Loss of function of Lam1 and Lam3, proteins that reside at these MCS, affected the activity of TORC2 (Murley et al., 2017). Lam6 causes formation of sterol-enriched domains at ER-vacuole MCS and, through this activity, was found to regulate activity of TORC1 (Murley et al., 2017). Since these Lam genes affect TORC signaling, I hypothesize that these genes affect autophagy as well. It will be interesting to learn more in the future how MCS, stress, and autophagy all interact to regulate virus replication.

1.9 Plant viruses

While BMV is well-known for its ability to serve as a model virus in the yeast system, its natural hosts are plants. It mainly infects monocotyledon (monocot) plants, such as rice, wheat, and barley, but not dicotyledon (dicot) plants (He et al., 2020). Arabidopsis, the primary model organism of plant biology, is not susceptible to BMV. However, another model dicot plant *Nicotiana benthamiana* is susceptible to BMV and many other RNA viruses, and serves as a universal host for the majority of plant viruses (Yang et al., 2004).

Figure 1.4 BMV infection of barley. Section of barley leaf infected with BMV at 10 days post-infection. Characteristic of mosaic symptoms that plant viruses cause.

Viruses cause plant stunting and leaf discoloration, thus leading to decreases in crop yields (Hull, 2013). Viruses can also cause other abnormalities in plant growth, including formation of tumors or changes in cell size. Perhaps the most famous symptom of plant viruses is the mosaic pattern on leaves. Other macroscopic symptoms include necrosis, leaf chlorosis, or mottling (Jones, 2021). On a molecular level, there is evidence that plant virus infection coincides with altered plant hormone levels, decrease of photosynthetic components such as rubisco and chlorophyll A, and decrease in nutrient uptake (Hull, 2013).

Plant viruses are important to study for their significance to agriculture. Plant viruses cause decreases in crop yields estimated to amount to more than $30 billion across the world every year (Nicaise, 2014). Viruses can directly cause yield losses themselves. They can also aid other viruses or other pathogens in their infection (Syller, 2012). These are termed synergistic interactions between pathogens. Several synergistic interactions between viruses have been reported, often involving potyviruses. For example, Potato virus X and Potato virus Y, when infiltrated into *Nicotiana tabacum* together, cause dramatically symptoms than when each is alone (Rochow and Ross, 1955). It has been shown that HC-Pro (helper component-proteinase) of PVY inhibits host antiviral defense RNA interference and therefore, promotes replication of PVX (Bowman Vance et al., 1995).

Aside from their importance in agriculture, plant viruses also have been significant for the science of virology and molecular biology in general (Scholthof et al., 2011; Lomonossoff, 2018). For example, TMV was the virus at the foundation of modern virology research. BMV has also been important for molecular biology research. It was used for early research on eukaryotic translation, because its translation in a wheat-embryo system produces a large quantity of protein (Shih and Kaesberg, 1973). BMV RNA transcripts made from infectious cDNA clones were used to infect plants, being the first plant RNA virus to do so (Ahlquist et al., 1984). The BMV-yeast system has given several insights into molecular virology, including the identification of VRCs, virion assembly, and host genes involved in viral replication among others (Krol et al., 1999;

Schwartz et al., 2002; Kushner et al., 2003; Schwartz et al., 2004). Clearly, studying plant viruses is useful for agricultural benefits as well as for use as scientific tools.

1.10 Resistance to plant viruses

Resistance to plant viruses can be classified into two groups. Nonhost resistance and host resistance. Plants, like humans, are resistant to most of the microbes they encounter. This is termed nonhost resistance. In other words, it is when all genotypes of a plant species are resistant to a virus. Nonhost resistance is the most common case of resistance to plant viruses; typically, a plant is not a host to most viruses. In an extreme example of nonhost resistance, humans are non-hosts to plant viruses. This is why it is generally safe for humans to be in contact with plant viruses. Nonhost resistance is considered to be very potent, broad and durable. Nonhost resistance is also related to host range, which includes a set of plants that can be infected by a specific pathogen. Those that are not on the list of host range usually possess nonhost resistance (Panstruga and Moscou, 2020).

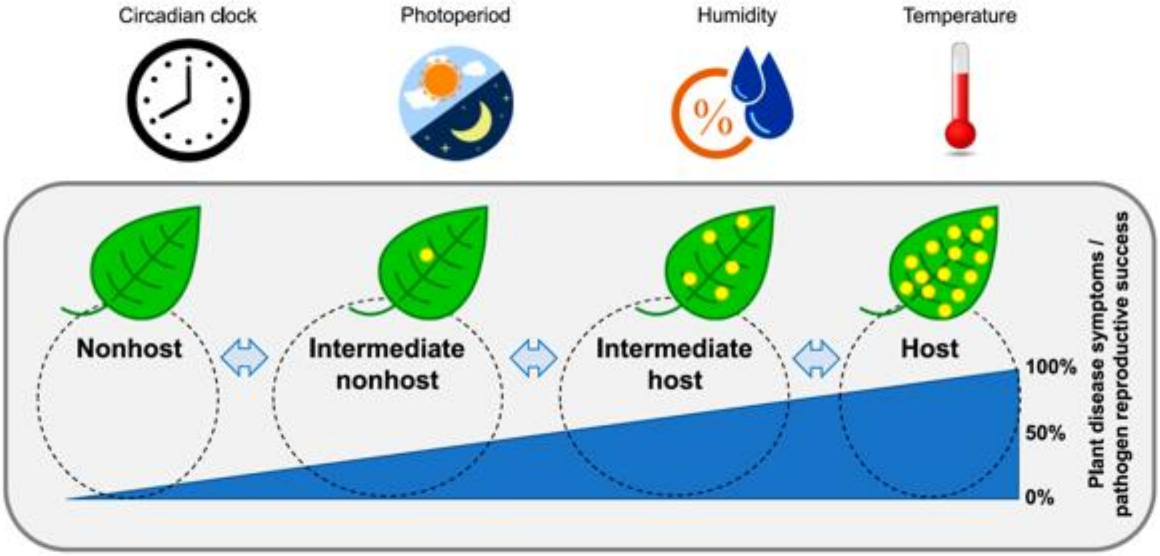

Figure 1.5 Factors that could affect nonhost resistance. Nonhost resistance can be controlled by various external factors, including time of day, photoperiod, humidity, and temperature. Image adapted from Panstruga and Moscou 2020.

Preformed physical barriers are a major component of nonhost resistance; they are a major obstacle for most microbes attempting to enter plant cells. The waxes on the cuticle of plants can make it difficult for bacteria and fungi to enter the plant (Ishiga et al., 2013). The tough and rigid cell wall is another obstacle pathogens go through to get inside plant cells (Bellincampi et al., 2014). However, these physical barriers are not critical to plants virus infection because plant virions (active viral particles) enter cells via wounding caused by mechanical damage or insect vectors. No receptors have been found involved in plant virus entry.

Components of both pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) play important roles in nonhost resistance. An example comes from bacterial flagellin, a component of flagella. Flagellin is the most studied PAMP (Pathogen associated molecular pattern), a highly conserved molecule from pathogens that is recognized by plants and leads to immune response (Li et al., 2005). Even purified flagellin can lead to defense response in tomato and in Arabidopsis (Zipfel et al., 2004). Effectors can also lead to nonhost resistance. One example is hopAS1, an effector from *Pseudomonas syringae.* This bacterial protein contributes to virulence in tomato, but contributes to nonhost resistance in Arabidopsis (Sohn et al., 2012).

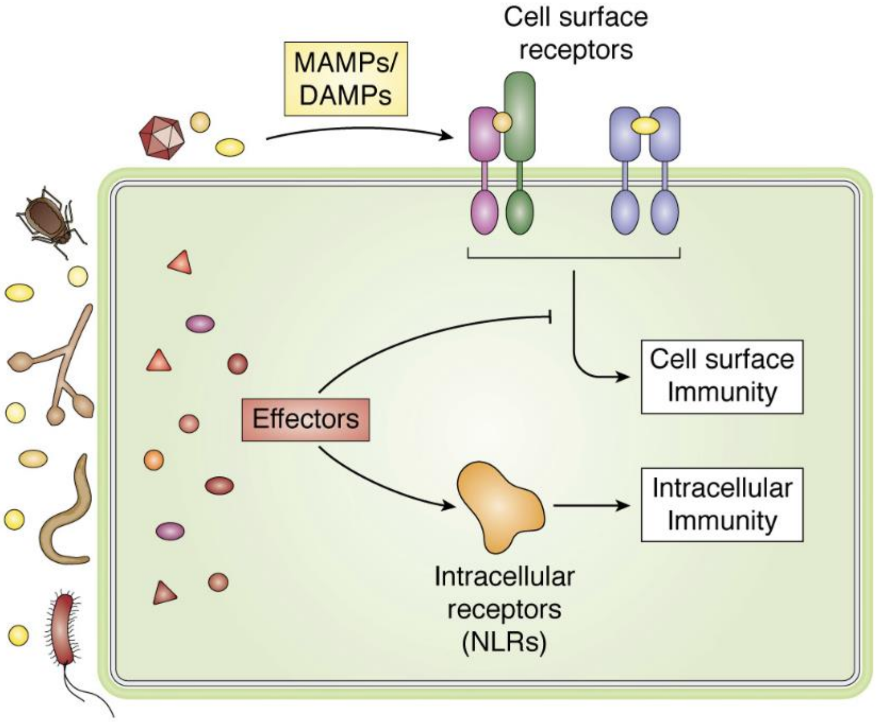

Figure 1.6 Overview of Plant immune system at a cellular level. Plants are constantly exposed to pathogens, some of which can enter the plant. Plant cells have cell surface receptors and intracellular receptors to recognize components of the pathogen or the

effectors the pathogens release. Recognition via these receptors triggers signaling pathways to initiate intracellular immunity. Image adapted from Bentham et al., 2020.

Mechanisms associated with nonhost resistance include ROS signaling and calcium signaling. These are both secondary messengers that help activate defense-related genes and production of defense-metabolites (Boller and Felix, 2009). Plant defense hormones salicylic acid (SA) and jasmonic acid (JA) are also parts of nonhost resistance. SA functions by activating pathogenesis-related proteins and helping the plant turn on systemic acquired resistance in upper uninfected leaves. JA is produced after plant wounding and induces expression of proteins that aid in defense against herbivores, especially insects (Halim et al., 2006).

1.10.1 Dominant resistance

Host resistance to plant viruses is when different genotypes within a plant species confers differing levels of resistance to the virus. In other words, there is a genetic polymorphism for resistance to the virus. Host resistance can further be split into two categories: dominant or recessive resistance. Dominant resistance involves the plant using Resistance (R) genes to sense the pathogen via the pathogen's avirulence (avr) factors. For viruses, these avr factors could be a variety of viral proteins, including replicases, movement proteins, or coat proteins (Sett et al., 2022).

The recognition of an Avr protein can lead to the plant hypersensitive response. This response is characterized by programmed cell death at the infection site; the plant tries to kill off the infected cells in order to prevent the spread of the pathogen. This is a relatively fast response; the HR response happens just a few hours after inoculation. Since viruses are obligate intracellular organisms, the HR response is very effective against viruses. This is in contrast to necrotrophic pathogens, which are able to survive on dead plant tissue. In fact, the hypersensitive response may actually help necrotrophic pathogens (Dalio et al., 2021).

Induction of the hypersensitive response in plants involves bursts of reactive oxygen intermediates (ROI) and also very rapid ion fluxes across the plasma membrane. These ion fluxes are mainly import of calcium into the cell and export of chlorine and potassium (Morel and Dangl, 1997; Dalio et al., 2021). Other features of HR-response-induced cell death include the shrinking of the cytoplasm, the stoppage of cytoplasmic streaming, shrinking of the nucleus, and formation of large vesicles within the cell (Balint-Kurti, 2019). The HR-response is very rapid and powerful, so it serves as a good way to restrict the growth of the pathogen so that the pathogen does not spread much farther past the site of entry. However, it is also very damaging to the plant, as evidenced by necrotic lesions appearing on the plant. Therefore, the activation of the hypersensitive response must be tightly regulated by the plant.

The hypersensitive response can often lead to a state called Systemic Acquired Resistance (SAR). In contrast to the HR response, SAR uses long distance signaling to confer immunity throughout the plant (de Ronde et al., 2014). This relies on a plant hormone called salicylic acid and a family of proteins called Pathogenesis Related (PR) proteins. In fact, well-controlled expression of Arabidopsis NPR1 (nonexpresser of PR genes 1), which is a master regulator of PR gene expression, in crops has been useful in combatting several diseases (Zavaliev and Dong, 2024). However, there is a fitness cost to SAR. Induction of SAR can have growth yield penalties. Mutants that have PR genes turned on constitutively, such as the *cpr5* mutant, have significant growth defects (Durrant and Dong, 2004). Overexpressing AtNPR1 has been shown to have harmful effects on growth and yield in certain crops. One way to manage that has been heterologous expression of AtNPR1 only in certain tissues where the pathogens reside and use uORF (upstream open reading frames) to express AtNPR1 in a low level (Xu et al., 2017; Zavaliev and Dong, 2024).

The majority of dominant resistance genes identified so far are NB-LRR (also called NLR) genes. These are intracellular immune receptors, which consist of a nucleotide binding site (NB-ARC) domain, a varying N-terminal domain, and a leucine-rich repeat (LRR) domain (Balint-Kurti, 2019). The N-terminal domain is often a Coiled-coil or Toll-interleukin receptor domain. The NB-ARC, which is made up of a nucleotide binding domain and an ARC domain, binds to and hydrolyzes ATP (de Ronde et al., 2014). The C-terminal of the NB-LRR protein contains the LRR domain, which is the part of these proteins that recognize the substrate. The LRR domain is highly variable. This diversity allows NB-LRR proteins to recognize a diverse array of pathogens (de Ronde et al., 2014). One potential explanation as to how these NB-LRR proteins became so diverse is alternative splicing (Peng et al., 2022). Although NB-LRR genes are activated by a specific pathogen, that activation induces responses (HR response and SAR) that provide defense against all types of plant pathogens.

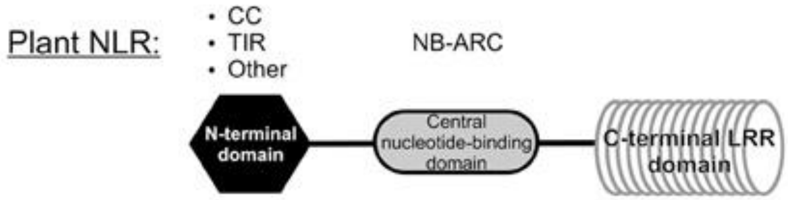

Figure 1.7 Structure of Plant NLR proteins. These consist of a variable N-terminal domain, a C-terminal LRR domain, and a nucleotide binding domain in between. Image adapted from (de Ronde et al., 2014)

Since too much activation of the hypersensitive response is detrimental to plant health and growth, plants tightly regulate the production of NB-LRRs at both the RNA and protein levels. Plants generally keep a low transcript abundance of NB-LRRs and once translated, the amount of NB-LRR protein is controlled by stabilization by chaperones and the ubiquitin-mediated degradation pathway (Balint-Kurti, 2019).

One example of a dominant resistance gene is the N gene from tobacco that confers resistance to TMV. If TMV is inoculated onto NN or Nn *N. tabacum,* then the spread of TMV is halted and necrotic lesions appear at the inoculated leaves, indicating the HR response occurred. However, for nn homozygous recessive plants, TMV spreads throughout the plant and mosaic symptoms occur (Whitham et al., 1994). Interestingly, the N-gene mediated-hypersensitive response to TMV by the N gene is temperature sensitive. At temperatures below 28 °C, the HR response occurs. However, at temperatures above 28 °C, even the NN or Nn plants do not have the hypersensitive response and TMV is allowed to spread throughout the plant. If those plants are then moved to a lower temperature, then the hypersensitive response can occur, indicating that the temperature sensitivity of the N gene is reversible. This phenomenon was used to identify tobacco loss-of-function mutants that could not generate the HR response to TMV, thus allowing for identification of the N gene product (Whitham et al., 1994). Isolation of the N gene and using agrobacterium to express that gene in nn plants allowed those nn plants to have a HR response (Dinesh-Kumar et al., 1995). Later on, it was found that this N gene from tobacco was able to confer resistance to TMV in *N. benthamiana* and tomato (Whitham et al., 1996; Liu et al., 2002).

Another example of a dominant resistance gene is RTM1 (restricted TEV movement 1) from Arabidopsis, which confers resistance to tobacco etch virus (TEV). This gene was identified in an Arabidopsis screen where they identified a mutant that was more susceptible to TEV. The RTM1-mediated response to TEV does not involve a hypersensitive response or SAR (Mahajan et al., 1998). Instead, RTM1 seems to function to restrict long-distance movement of TEV in the phloem. This was discovered by fusing a TEV protein to the reporter gene GUS (Mahajan et al., 1998). RTM1 has high similarity to jacalin or jacalin-like proteins, which play roles in the jasmonic-acid response (Chisholm et al., 2001). Like other jacalin-like proteins, RTM1 interacts with itself. It likely functions as aggregate complexes that reside in the phloem (Chisholm et al., 2001).

1.10.2 Recessive resistance

Recessive resistance is based on the fact that plant viruses thrive by making use of host factors in plant cells. In general, recessive resistance occurs when these host components, also termed susceptibility genes, are mutated, so that the virus cannot use that host component anymore.

Most of the recessive resistance genes identified so far for viral resistance are eukaryotic translation initiation factors, from the eIF4E or eIF4G family. In 2002, a forward genetic screen was conducted in Arabidopsis to identify Arabidopsis mutants with a decreased susceptibility to turnip mosaic potyvirus (TuMV). Typically, TuMV causes stunting in Arabidopsis. However, this screen was able to identify mutants that, when infected with TuMV, did not display stunted growth. In addition, the difference in TuMV replication among the mutants and WT was confirmed by using ELISA. The gene responsible for these mutants' phenotype was isolated as eIF(iso)4E (Lellis et al., 2002). Later on, recessive resistance genes that provide resistance to potyviruses in several crops, including melon, tomato, pepper, pea, and barley, were cloned and confirmed to be eIF4E or eIF(iso)4E (Wang and Krishnaswamy, 2012). In cells, eIF(iso)4E is an isoform of eIF4E that likely complements most functions of eIF4E, but may have other functions as well. Both eIF4E and eIF(iso)4E serve as cap binding proteins involved in translation initiation, but only one can be recruited for virus infection. One proposed mechanism for how the eIF4E family contributes to potyvirus replication is that one of these proteins could help the translation of viral RNA. Other hypotheses say that eIF4E can help the movement of the virus or disrupt host mRNA movement out of the nucleus (Rodriguez-Peña et al., 2021).

There are other examples of recessive resistance to viruses besides the eIF4E family. For example, loss of function of a DNA binding protein phosphatase (AtDBP1) allows for resistance to TuMV and plum pox virus (PPV). DBP1 is predicted to function in regulating transcription or have some role in signal transduction. Loss of DBP1 function caused significant decrease in protein levels of eIF(iso)4E, suggesting a link between DBP1 and the eIF4E family (Castelló et al., 2010).

Another example comes from the ER stress pathway. ER stress, which causes the unfolded protein response (UPR), is when there are too many unfolded proteins in the endoplasmic reticulum (Chipurupalli et al., 2021). Silencing BZIP60, a protein that plays a role in UPR, suppresses potato virus x replication in *N. benthamiana*. In fact, infecting *N benthamiana* with potato virus X caused upregulation of several genes related to stress, including calmodulin and a protein related to ubiquitin ligase-mediated degradation (Ye and Verchot, 2011). ER stress is also known to lead to autophagy (Chipurupalli et al., 2021). This could potentially be a link between ER stress and viral replication.

In summary, there is very interesting biology going on with plant resistance to viruses. There can be the hypersensitive response or SAR from dominant resistance. These are mostly driven by NB-LRR proteins, which are highly diverse. What creates this diversity still remains to be elucidated. HR response or SAR cause a very potent resistance, but can also significantly damage the plant, killing off infected areas of the plant or diminishing plant growth. On the other hand, there is recessive resistance. Most of the genes identified to contribute to recessive resistance so far come from a family of translation initiation factors. Recessive resistance is obtained by mutating host genes that are required for viral replication. Although this would suppress viral replication, it may cause harmful effects on the host.

Plants clearly have a very sophisticated immune system capable of responding to a plethora of pathogens. Yet BMV is remarkably capable of infecting plants and inducing immune responses while also being able to infect such a different organism as yeast. Additionally, BMV has such a small genome even compared to other viruses. Therefore, learning more about the host factors that BMV can manipulate in both yeast and in plants can be of great importance to the study of the plant immune system.

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Chapter 2: Deletion of host lipid transporter Lam5 inhibits proper BMV replication

2.1 Abstract

Lipids play critical roles for the replication of positive-strand RNA viruses. These viruses form viral replication complexes by rearranging host intracellular membranes, recruiting viral and host proteins as well as viral replication templates. The replication of brome mosaic virus can be recapitulated in the baker's yeast. I use the engineered BMV-yeast system to examine viral replication mechanisms and virus-host interactions of positive-strand RNA viruses. Here I report that a yeast lipid transfer protein, Lam5 (lipid transfer protein anchored at a membrane contact site), is crucial for BMV replication. Lam5 belongs to a six-member family that functions to transfer sterols at membrane contact sites, which are important for communication between organelles. I show that Lam5 is relocalized by BMV replication protein 1a: when BMV 1a is expressed, Lam5-GFP changes from a punctate localization to a ring localization. These rings colocalize with BMV 1a-mCherry. Furthermore, BMV replication significantly decreases in *△lam5* yeast. However, accumulation of BMV 1a and membrane-association of BMV 1a are similar in WT and the *△lam5* mutant. BMV RNA3 protection still occurs in *△lam5,* but to a lower extent than in WT cells. In *△lam5*, expression of BMV 1a causes neutral lipids to localize into ring structures in contrast to their normal punctate localization. Furthermore, *Arabidopsis thaliana* Lam2 is relocalized by BMV 1a in yeast cells, suggesting a Lam protein could be involved in BMV replication in plants. However, more work needs to be done to decipher the exact role Lam proteins could have in plants.

2.2 Introduction

Lipids are one of the four classes of major macromolecules. They serve a variety of vital biological functions, including being components of biological membranes which serve as the boundary of the cell and also compartmentalize the cell into various organelles (Wong et al., 2019). Lipids are also a source of energy in cells and play important roles in signal transduction pathways within cells. Eukaryotic cells contain more than a thousand different types of lipids (Lev, 2010). Most lipids are produced at the ER, yet lipids are found throughout the cell. Different organelles and membranes contain different lipid compositions (Sleight, 1987; Egea, 2021). Thus, an important question in cell biology is how different lipids get to the cellular location where they function.

2.2.1 Lipid transport and viral replication

Understanding lipid transport is of great importance in virology. All (+)RNA viruses remodel host intracellular membranes to form viral replication complexes (VRCs) (den Boon et al., 2024). Viruses use these membranes as a physical scaffold for their replication and as a way to protect themselves from host antiviral proteins (Zhang et al., 2019).

As major components of cellular membranes, lipids play significant roles in viral replication. For example, hepatitis C virus (HCV) relies on cholesterols and lipid droplet metabolism for its entry, replication, and secretion (Ye, 2007; Vieyres et al., 2020). Another example is how phosphatidylethanolamine (PE) levels increase in plant cells infected with tomato bushy stunt virus (TBSV). During TBSV replication in yeast, there is increased PE levels at the peroxisomes, where TBSV replicates. Furthermore, increasing PE levels leads to an increase in TBSV replication (Xu and Nagy, 2015). Specific mutations in TBSV replication proteins p33 and p92 inhibited their binding to sterols, leading to a decrease in TBSV replication in yeast and plants (Xu and Nagy, 2017). Dengue virus (DENV) causes an increase in synthesis of phospholipid and fatty acids, corresponding with an increase of de novo lipids in the cell fraction where DENV resides (Heaton et al., 2010; Vial et al., 2021). Expression of just a single BMV protein, BMV 1a, causes increases in total fatty acid levels and intracellular membranes (Lee and Ahlquist, 2003). DENV promotes lipophagy, a type of autophagy that breaks down lipid droplets (Zhang et al., 2018a). This may be to increase the supply of phospholipids to make VRCs or to allow more fatty acid hydrolysis to generate more ATP (Zhang et al., 2017).

For eukaryotes, phospholipids are the most abundant type of lipids in cell membranes. Phosphatidylcholine (PC) is the most abundant phospholipid in cell membranes and has been shown to be important for several viruses (Zhang et al., 2018). In fact, BMV, HCV, and poliovirus have increased PC levels at their VRCs (Zhang et al., 2016). BMV replication is substantially decreased when PC synthesis is blocked (Zhang et al., 2016).

The ratio of saturated fatty acids (SFAs) to unsaturated fatty acids (UFAs) affects the fluidity and permeability of cell membranes, and surprisingly, also affects viral replication, as evidenced in BMV, which prefers a higher ratio of UFAs to SFAs. OLE1 is an enzyme that catalyzes the conversion of unsaturated fatty acids to saturated fatty acids. BMV replication is decreased by more than 20-fold in yeast *ole1w* mutant cells due to decreased UFA levels preferentially at VRC membranes.(Lee et al., 2001; Lee and Ahlquist 2003). Similarly, pharmacological inhibition of stearoyl-CoA desaturase decreases the synthesis of UFAs and decreases HCV replication (Lyn et al., 2014).

In fact, viruses compete with hosts for lipids and increased virus replication can come at the cost of host cell growth (Zhang et al., 2018b). Since different viruses use different specific membranes to replicate and that different species of lipids are found at different membranes, lipid composition could explain why different viruses preferentially make their VRCs in different areas within the cell. Understanding lipid transport could reveal insight into how viruses get to their VRCs, how they could form their replication sites within cells, and how they recruit host factors to their VRCs.

2.2.2 Lipid transport

Coordinated lipid transport through the cell could be classified into two broad categories: vesicular and non-vesicular transport (Wong et al., 2019). Vesicular transport is the transport of lipids between organelles by vesicle budding and fusing from and to the different organelles. This is an energy-dependent process and is also a major transport-method of protein transport. Vesicles typically have a protein coat that seem to dictate the destination of the vesicle. Types of vesicles include Clathrin, COPI (coat protein I) and COPII (coat protein II) (Cui et al., 2022).

Transport of the majority of cellular lipids is via non-vesicular transport (Wong et al., 2019), which primarily involves the use of lipid transport proteins and typically transfer a specific lipid species across a membrane (Lev, 2010; Reinisch and Prinz, 2021). These proteins use a hydrophobic pocket to protect the lipids from the aqueous cytosol (Prinz et al., 2020). Many lipid transport proteins have protein sequences that target them to two organelles, so they can travel between the donor membrane and the destination membrane (Wong et al., 2019). There are two general categories of lipid transporters. One category is “cup-like” transporters that typically bind to the donor membrane and pick up a specific single lipid molecule and subsequently travel and drop off the lipid molecule at the destination membrane. The other category is “bridge-like” transporters. These are large proteins that act as a hydrophobic bridge for many lipid molecules to pass through and likely facilitate more rapid and high-volume lipid transport than the cup-like transporters (Reinisch and Prinz, 2021).

Non-vesicular lipid transport especially occurs at membrane contact sites (Reinisch and Prinz, 2021). Examples of membrane contact sites include the ERMES (ER/mitochondria encounter structure), the NVJ (nuclear vacuolar junction), and the vCLAMP (vacuole and mitochondria patch) (Elbaz-Alon et al., 2015). Non-vesicular transport has the advantage of being quicker than vesicular transport and could be used in times of stress when vesicular transport is impaired (Reinisch and Prinz, 2021). This could be useful, for example, when the cell needs to rapidly adjust membrane contents in response to environmental stimuli or intracellular signaling (Wong et al., 2019).

Several lipid transfer proteins have been identified and investigated so far. These include the ORPs (oxysterol-binding protein related proteins), the TULIPs (tubular lipid binding proteins), and the StARkin (relatives of steroidogenic acute regulatory protein) family (Wong et al., 2019). Even if they are in the same family, lipid transfer proteins can bind different lipid molecules. Other functions of lipid transport proteins include lipid sensing, lipid presenting, and lipid modification (Wong et al., 2019).

Because of (+)RNA viruses' dependence on host membranes for formation of VRCs, and the fact that other host factors related to lipid composition have found to be involved in BMV replication, I screened a large number of other lipid-related proteins in yeast. My goal was to find new host factors that could be involved in BMV replication, so that we can better understand how BMV replication works and how BMV interacts with its hosts. One group I screened was the Lam (<u>L</u>ipid transfer proteins <u>A</u>nchored at a <u>M</u>embrane contact site) family of genes, which function to transport sterols specifically. These family members share key structural domains (Fig. 2.1). All six members of this family in yeast have StART (Steroidogenic Acute Regulatory Transfer)-like domains which bind sterols (Gatta et al., 2015) and a GRAM domain that controls the localization of these proteins (Murley et al., 2015). GRAM stands for glucosyltransferases, Rab-like GTPase activators, and myotubularins and are part of the pleckstrin homology superfamily (PHg) (Li et al., 2016b). This domain is found in many eukaryotes and is predicted to function in protein or lipid binding (Doerks et al., 2000). They also have transmembrane domains, a key aspect of lipid transport proteins since they generally function in close proximity to membranes (Gatta et al., 2015). There are also genes in Arabidopsis that have GRAM domains. These genes are *AtBAGP1, AtVAD1, AT1G03370, and AT5G50170.* I now refer to *AT1G03370* as *AtLAM2* and *AT5G50170* as *AtLAM4.* Lam5 and Lam6 in yeast are more closely related to each other than they are to the other Lam proteins.

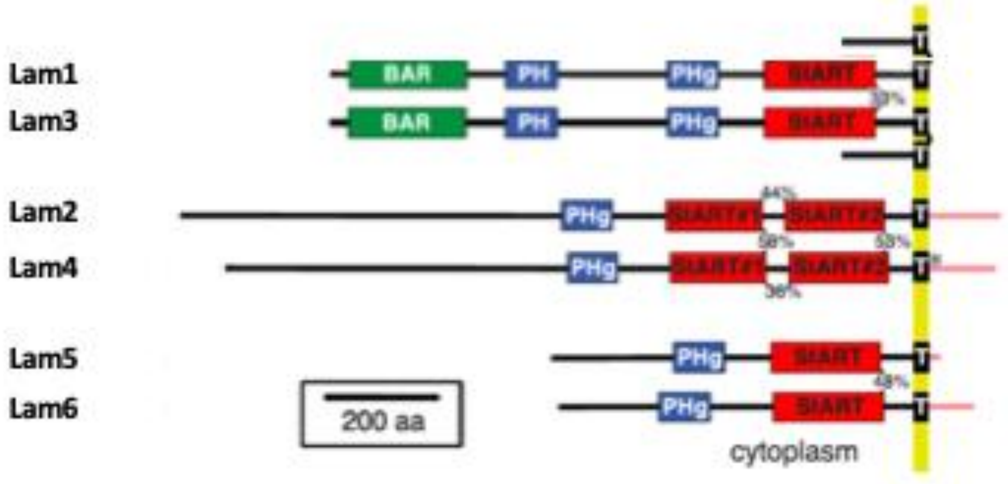

Figure 2.2: The Lam family in yeast. Out of the six members, Lam5 is most closely related to Lam6. Image adapted from (Gatta et al., 2015).

This family was also found to affect TORC1 and TORC2 signaling in yeast, indicating a possible role in cell growth, proliferation, and autophagy. Interestingly, overexpressing LAM6 leads to expansion of membrane contact sites, while loss of function of LAM6 disrupts communication between membrane contact sites (Elbaz-Alon et al., 2015). Membrane contact sites are very important areas within the cell, having functions such as lipid signaling, ion transport, autophagy, membrane formation, and crosstalk between organelles (Prinz et al., 2020).

I report here that Lam5 is used by BMV for its replication by redistributing Lam5 from MCS to the viral replication site. Testing yeast mutants that have loss of function in each of the LAM genes, I found that BMV replication is drastically reduced in the Δ*lam5* mutant. However, VRCs are formed in this mutant, suggesting a functional defect yet remains to be determined.

2.3 Methods

2.3.1 Yeast strain

The Saccharomyces cerevisiae strain 4741 was used in all experiments. Yeast cells were grown at 30°C in a synthetic medium with 2% galactose as the carbon source and with appropriate amino acids for plasmid selection. These cells were initially cultured overnight, then subcultured, and then were harvested at log phase.

2.3.2 Plasmids

BMV 1a and 2a were expressed from pB12VG1-L (Kushner et al., 2003). BMV RNA3 was expressed using pB3VG128-U (Zhang et al., 2012). GFP-tagged Sec63 served as the ER membrane marker. pB1YT3-mC, which expresses BMV 1a tagged with mCherry, was used to detect localization of BMV 1a. To observe localization of Lam proteins, the LAM genes were tagged with GFP and were expressed under the control of the *NOP1* promoter. For electron microscopy, BMV 1a was expressed from pB1YT3-H and BMV 2a was expressed from pB2YT5-L.

2.3.3 Fluorescence microscopy

Cells were grown to log phase at 30°C in the appropriate synthetic selection media for plasmid maintenance that contained 2% galactose as a carbon source. For staining of lipid droplets, cells were stained with 2 µm BODIPY 493/503 for 30 minutes and then washed with phosphate buffered saline (pH 7.4) twice. Cells were then concentrated by centrifugation and immobilized between a glass slide and coverslip, and visualized on a confocal microscope (Zeiss LSM 710) at room temperature.

2.3.4 RNA extraction and northern blotting

Total RNA was extracted from yeast cells that were harvested at OD_{600} values of 0.6 to 1.0 by a hot phenol method (Köhrer and Domdey, 1991). Equal amounts of total RNA were used for agarose-formaldehyde electrophoresis and then transferred to Nytran membranes. ^{32}P-labeled probes specific to BMV positive-strand RNA, or BMV negative-strand RNA, or 18S rRNA were used for hybridization. 18S served as the loading control to eliminate loading variations and to normalize the signals. Radioactive signals were scanned using a Typhoon FLA 7000 phosphoimager and intensities of the bands were quantified by using ImageJ.

2.3.5 Protein extraction and western blotting

Total protein was extracted from yeast cells that were harvested at OD_{600} values of 0.6 to 1.0 as previously described (Sathanantham et al., 2022). Equal volumes of extracted total proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Expression of target proteins were detected using the following antibodies and dilutions: rabbit anti-BMV 1a (from Dr. Paul Ahlquist at the University of Wisconsin-Madison) at 1:10,000, mouse anti-BMV $2a^{pol}$ (from Dr. Paul Ahlquist) at 1:3000, mouse anti-PGK1p (Thermo Fisher Scientific) at 1:10,000, and mouse anti-Dpm1 (Thermo Fisher Scientific) at 1:3000. Detection then used horseradish peroxidase-conjugated secondary antibodies (1:7500 dilution, Thermo Fisher Scientific) and then Supersignal West Femto Substrate (Thermo Fisher Scientific). Protein bands were then visualized using Azure c4000 (Azure biosystems) and quantified using ImageJ.

2.3.6 Membrane flotation assays

Ten OD_{600} units of yeast cells were resuspended in TNE buffer containing a 1:100 dilution of yeast/fungal protease arrest (G biosciences). Lysates were either prepared from whole cells or spheroplasts that were lysed via a 22-gauge, 4cm long needle. The resulting lysates were centrifuged for 5 min at 500 x g at 4°C to remove cell debris. The resulting supernatant was adjusted to 40% (vol/vol) iodixanol (Sigma) and was subjected to a density gradient at 201,000 x g at 4°C. The gradients were divided into six fractions and analyzed by western blotting.

2.3.7 Electron microscopy

Fixation, dehydration, and embedding were performed as previously described (Zhang et al., 2012). Briefly, yeast cells were fixed with 4% paraformaldehyde and 2% glutaraldehyde first and then fixed with 1% osmium tetroxide. Dehydration was then performed using a gradient of ethanol. Samples were then embedded using Spurr's resin from Electron Microscopy Sciences. Thin sections were imaged using a transmission electron microscope at Hope College.

2.4 Results

2.4.1 Lam5 is re-localized by and co-localizes with BMV 1a

To test if BMV recruits Lam proteins for its replication, I first performed a redistribution assay where LAM genes were tagged with GFP and their localization was assessed with or without BMV replication protein 1a. Tagging with GFP or other fluorophores is a common technique in which a protein of interest is attached to a fluorophore by cloning and then the localization of the resulting fused protein is detected by fluorescence microscopy. According to previous studies, BMV 1a localizes in a ring pattern at the perinuclear ER (Diaz et al., 2010; Li et al., 2016a). Consistent with other literature (Gatta et al., 2015), I observed a punctate localization for Lam5 and Lam6 when no viral proteins were expressed (Fig. 2.2). When Lam6-GFP was expressed along with1a-mCherry, the localization of Lam6-GFP still appeared as dots throughout the cell. However, when Lam5-GFP was expressed with 1a-mCherry, a drastic re-localization of Lam5-GFP was observed from punctate structures to a ring pattern. Furthermore, Lam5-GFP co-localizes with 1a-mCherry (Fig. 2.2).

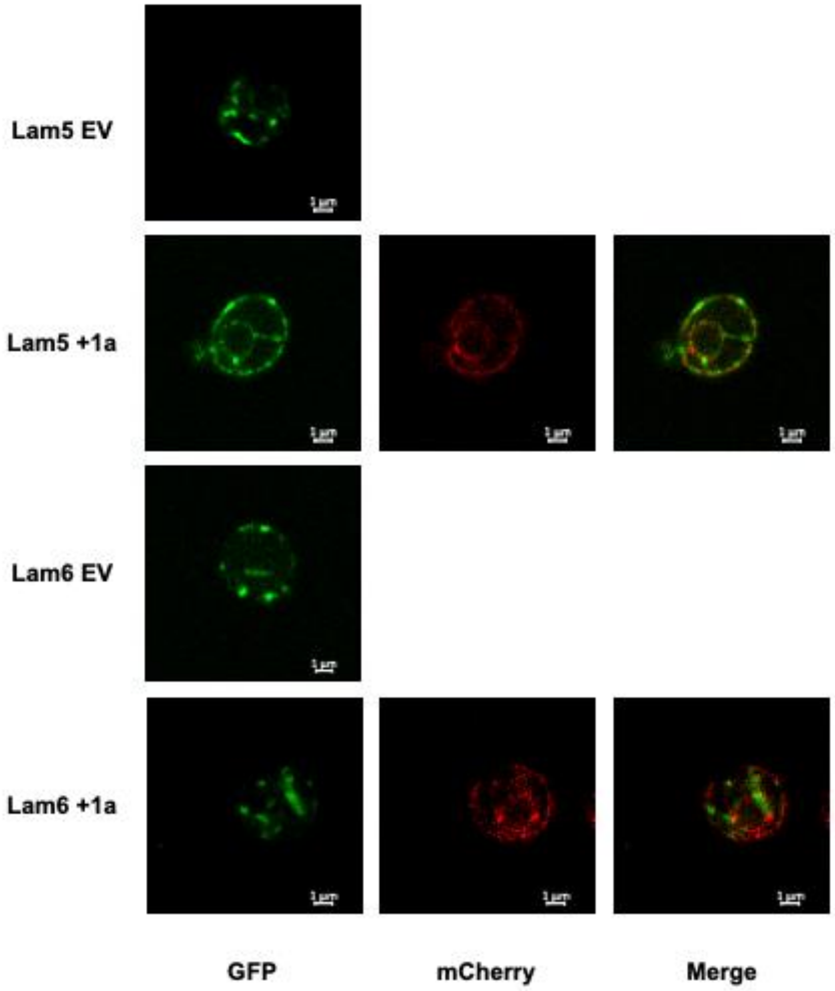

Figure 3.2. Re-localization of Lam5 by BMV 1a: Expression of either Lam5-GFP or Lam6-GFP co-expressed with either empty vectors or BMV 1a-mCherry in WT yeast. Yeast cultures were grown at 30° C until exponential phase and then visualized by confocal microscopy. Lam5 displayed a punctate distribution in absence of 1a, but when 1a-mCherry was expressed Lam5 displayed a ring localization pattern. This ring pattern colocalized with that of BMV 1a, as shown by the orange color in the merged image.

2.4.2 BMV replication is inhibited in the ∆lam5 mutant

To test if any members of the Lam family are involved in BMV genomic replication, I employed yeast mutants harboring single deletions in each of the six LAM genes. I then used plasmids to express BMV 1a, 2a^{pol} and genomic RNA3 transcript in each of those strains. BMV RNA-specific probes detected strong signals for both (+)RNA3 and (+)RNA4 from WT yeast cells. This indicates strong BMV replication because (+)RNA4 can only be synthesized after a full round of BMV replication. However, in *⊿lam5* cells especially, I saw very little accumulation of these molecules: only about 19% of (+)RNA4 was detected compared to WT (Fig. 2.3A), indicating low BMV replication in the mutant. Lack of LAM3 inhibited BMV replication by 65% and the other four mutations affect BMV replication mildly, ranging from 15-30% (Fig. 2.3A).

Since *⊿lam5* gave such a drastic viral replication phenotype, I next measured accumulated levels of 1a and 2a^{pol} protein in that mutant. I found that, by deleting LAM5, accumulation of BMV 1a was not significantly affected, but accumulation of BMV 2a^{pol} was slightly decreased (Fig. 2.3B). This rules out the possibility that the decrease in BMV RNA replication in *⊿lam5* was due to destabilizing effects on 1a.

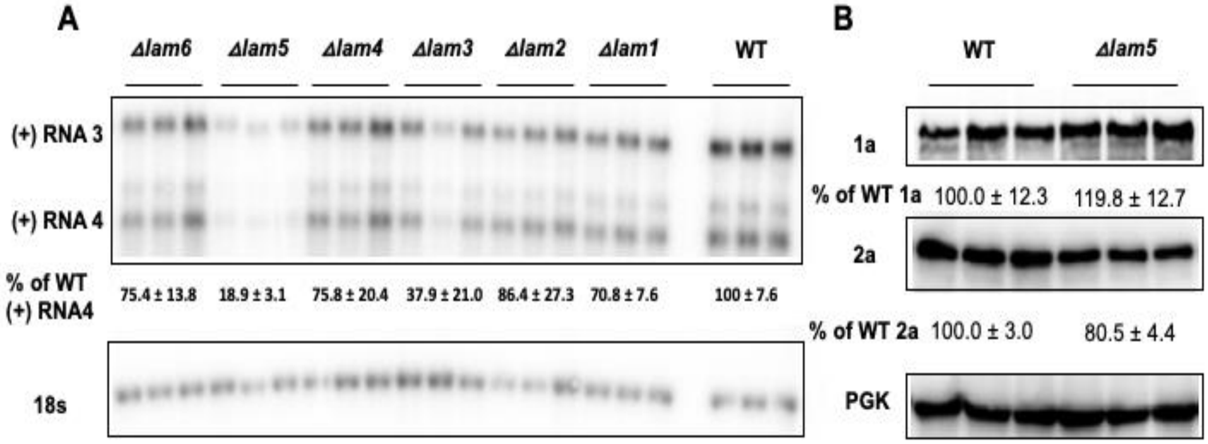

Figure 2.4. Deletion of LAM5 disrupts BMV replication: (A) WT yeast or yeast harboring single gene deletions of each LAM family member were tested for BMV replication by northern blotting. BMV RNA replication and subgenomic RNA synthesis was launched using a cDNA derivative of RNA3. Initial transcription produces (+)RNA which then serves as a template for 1a- and $2a^{pol}$ -dependent RNA3 replication. Radioactive probes specific to either BMV (+)RNA3 or (+)RNA4 detected less signal for Δ*lam5* yeast compared to WT. 18s rRNA served as the loading control. (B) Accumulation of BMV 1a and $2a^{pol}$ were analyzed by western blotting. Total proteins were extracted from equivalent numbers of yeast cells that expressed all BMV components and then these protein samples were analyzed by SDS/PAGE and anti-1a or $2a^{pol}$ antibody. PGK was used as a loading control to ensure equal loading of total protein.

2.4.3 BMV 1a is membrane-associated in Δlam5 cells

The impact of deleting LAM5 on 1a's membrane association was subsequently assessed. BMV viral replication takes place inside VRCs, which are formed at the perinuclear ER membranes. BMV 1a plays central roles in orchestrating the VRC formation once it is targeted to the perinuclear membrane: recruiting $2a^{pol}$ and proviral host proteins, modulating lipid synthesis/modification/transport, and remodeling membranes (Schwartz et al., 2002; Wang et al., 2005). To test whether 1a is membrane associated, I performed a membrane flotation assay. This assay uses an iodixanol density gradient to distinguish membrane-associated proteins from cytoplasmic soluble proteins. Under such experimental conditions, membranes and membrane-associated proteins float to the upper layers of the gradient, while soluble fractions will be at the bottom (den Boon et al., 2001). My results showed that both 1a and the membrane marker, dolichol-phosphate mannosyltransferase (Dpm1), were found in the membrane layers as expected in WT cells. I also found that both of these proteins were still in the membrane fractions in Δ*lam5* mutant cells. In fact, I saw about a 23% increase of 1a in the membrane fraction in the mutant (Fig. 2.4). Membrane association of 1a or Dpm1 was determined as the percentage of protein present in the top fraction, where membrane-associated proteins are present. This shows there is no disruption of 1a membrane association in the mutant.

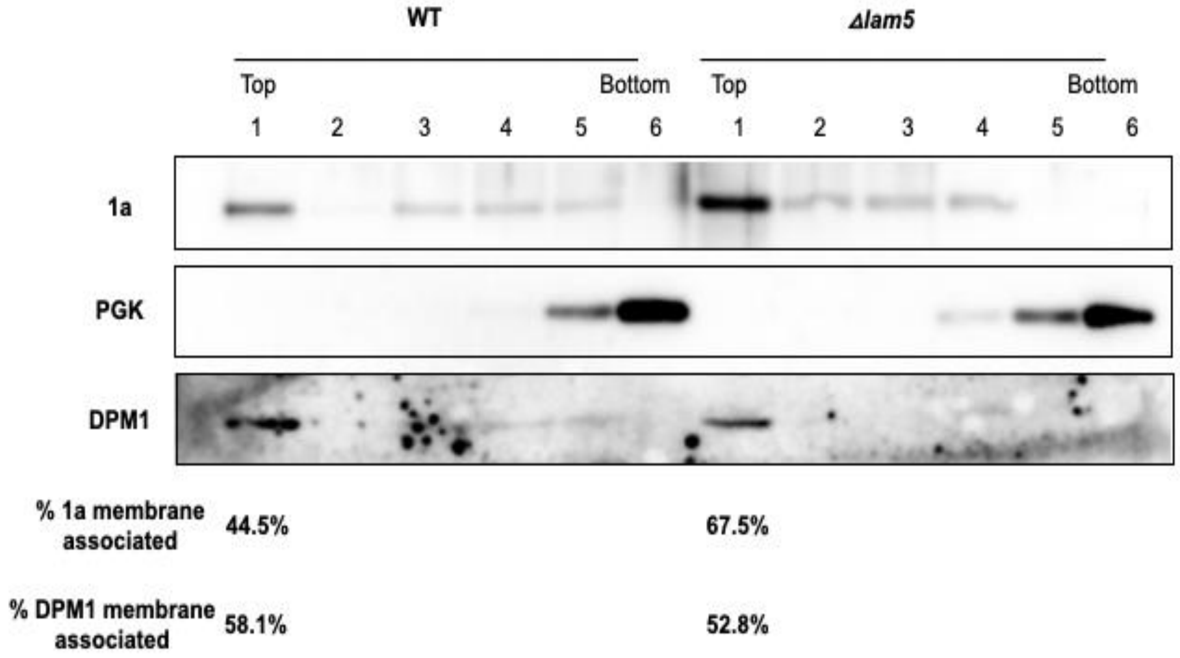

Figure 2.5. Membrane flotation assay comparing WT to Δ*lam5*: Cells were separated into six fractions using an iodixanol gradient and ultracentrifugation. Total protein was then extracted from each fraction and separated by SDS-PAGE. Proteins of interest were detected by western blotting with antibodies against BMV 1a, cytosolic control PGK, and membrane control Dpm1. Percentage of membrane-associated 1a or Dpm1 was calculated by measuring the signal intensity of each protein in the top-most fraction compared to the sum of all six fractions.

2.4.4 BMV is still capable of protecting viral replication template RNA3 and forming layer VRCs in Δ*lam5* cells

BMV 1a with low expression of 2a^{pol} induces formation of spherules while with high expression of 2a^{pol} induces layer-format VRCs (Schwartz et al., 2004). Layer-format VRCs consist of 2-7 karmellae-like layers of double membranes, which are from ER membranes and are separated by regular 50-60 nm spaces. These layer-VRCs support BMV replication as effectively as spherules (Schwartz et al., 2004).

Expression of 1a alone can induce spherule formation and recruit RNA3 into spherules. RNA3 is unstable when expressed alone, with a half-life of 3 minutes (Janda and Ahlquist, 1998). When coexpressed with BMV 1a, RNA3's half-life increased to more than 3 hours and as a result, accumulated levels of RNA3 dramatically increased, termed as 1a-mediated RNA3 protection (Janda and Ahlquist, 1998). This protection is due to RNA3 being in a membrane-associated, RNase-resistant state, suggesting its presence inside of VRCs, which can be in the form of spherules or layers. BMV spherules are vesicle-like invaginations of the ER, while BMV layers are stacks of appressed double membranes (Schwartz et al., 2004). I found that when 1a is expressed in Δ*lam5*, 1a-mediated protection of RNA3 still occurs, but at a lower rate than that of WT (Fig 2.5A). Consistent with this result, when I expressed BMV 1a along with high levels of BMV 2a^{pol} in Δ*lam5*, I was able to detect layer formation in yeast cells with electron microscopy (Fig. 2.5B).

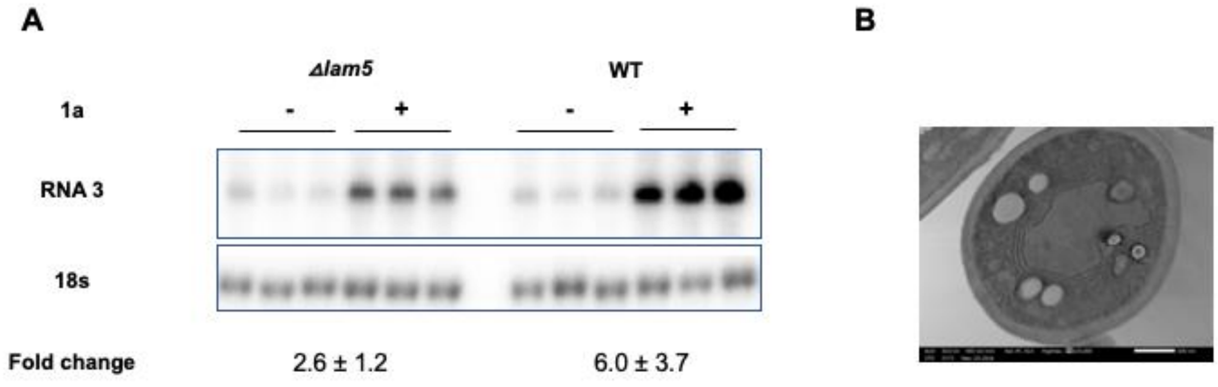

Figure 2.6. BMV RNA3 protection in Δ*lam5*: (A) Total RNA was extracted from WT or Δ*lam5* yeast cells that either did or did not express BMV 1a. BMV 1a is known to induce

formation of VRCs which serve to protect BMV RNA. (+)RNA3 accumulation in the samples was then detected by northern blotting using a specific probe to detect viral RNA3. 18s rRNA served as the loading control. (B) EM image of Δ*lam5* yeast cells expressing BMV-induced layer-format VRCs. These VRCs consist of continuous sheets of double-membrane ER and are typically capable of supporting BMV replication as effectively as spherules. Scale bar represents 500 nm.

2.4.5 Deletion of Lam5 affects localization of neutral lipids

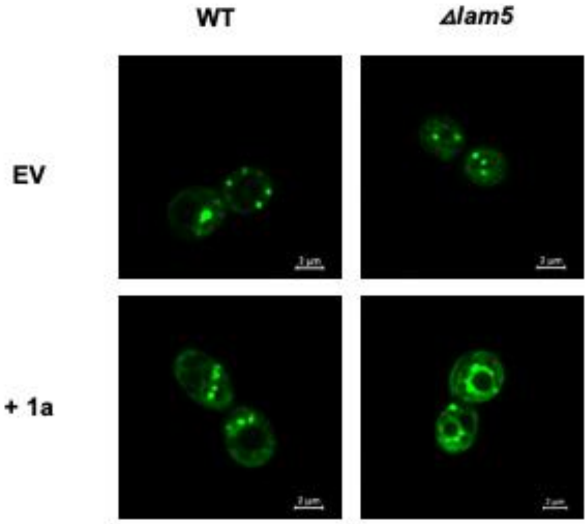

Figure 2.7. Effect of LAM5 deletion on lipid droplet distribution: WT and Δ*lam5* were grown to exponential phase and then stained with BODIPY 493/503. After a brief incubation period and PBS washes, cells were visualized by confocal microscopy. BODIPY 493/503 is a stain for neutral lipids, which are usually found in lipid droplets throughout the cell.

Since Lam5 is a lipid transport protein and because the lipid environment is so crucial for (+)RNA virus replication, I stained WT and Δ*lam5* yeast cells with BODIPY 493/503. This fluorescent dye stains neutral lipids such as cholesteryl ester or triacylglycerol. It can thus be used as a detector of lipid droplets. I found that in both WT and Δ*lam5*, lipid droplets appeared as dots throughout the cell, consistent with previous reports (Han and Carman, 2017). When 1a was expressed in these cells, fluorescent microscopy still showed primarily dot structures in WT: 92% of cells (n=300) displayed a punctate pattern. However, in Δ*lam5* cells, there were clear ring structures and also puncta associated with ring structures (Fig. 2.6). In Δ*lam5*, 65% of cells (n=300) displayed punctate localization of GFP, while the other 35% displayed the ring pattern. However, whether the ring structure represents the perinuclear ER membranes or vacuole membranes needs further clarification.

2.4.6 AtLam2 is re-localized by BMV 1a in yeast cells

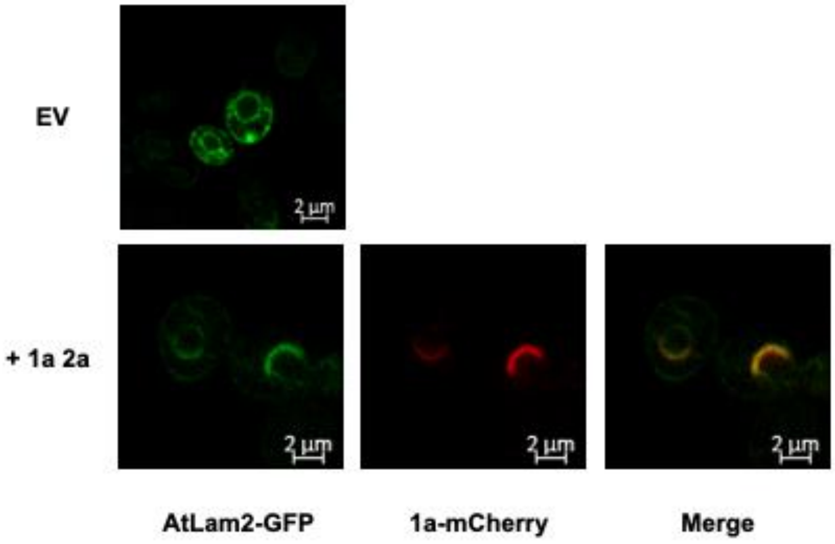

Figure 2.8. Re-localization of AtLam2 in yeast cells by BMV replication proteins: AtLam2 was cloned from Arabidopsis and tagged with GFP. This resulting construct was then expressed in yeast cells. Images depict WT yeast cells either expressing AtLam2-GFP alone or with both 1a-mCherry and $2a^{pol}$.

To test whether LAM homologs in plants are similarly involved in BMV replication, I cloned LAM homologs, which have GRAM domain, from Arabidopsis. When I expressed GFP-tagged AtLam2 in yeast cells, GFP signal was found as two ring structures of ER membranes. The inner ring likely represents the perinuclear ER membrane, and the outer ring is the peripheral ER membrane, which is quite different from the localization patterns of the six yeast LAM members. BMV 1a and $2a^{pol}$ are known to localize to full-ring or half-ring structures at the perinuclear ER. When both 1a and $2a^{pol}$ were expressed along with AtLam2-GFP, GFP signal was predominantly in half-rings pattern instead of two full rings and coincided with localization of 1a-mCherry. This re-distribution of AtLam2-GFP suggests that AtLam2 may have a role in BMV replication in plants.

2.5 Discussion

Viruses use host cellular membranes as sites for viral replication. These membranes play critical roles, including serving as scaffolds for VRCs and protection from host immune responses. Membrane contact sites are especially important areas within cells that allow for important communication and signaling between different organelles. I have found that deletion of Lam5, a sterol transfer protein at membrane contact sites, drastically reduces BMV replication in yeast. LAM5 belongs to a family of lipid transfer genes that all possess GRAM domains.

My data show that Lam5 is redistributed upon expression of BMV 1a and that deletion of LAM5 disrupts BMV replication. Although deletion of LAM5 in a WT background does not affect lipid droplet localization, deletion of Lam5 together with expression of BMV 1a causes lipid droplet localization to shift from punctate structures to ring-structures (Fig 2.7). Taken together, these data indicate a connection between BMV 1a and Lam5 to affect localization of lipids. Since BMV relies on lipids for its replication, I speculate that BMV uses Lam5 to adjust lipid environment throughout the cell. Perhaps sterols are involved in BMV replication and thus BMV re-distributes sterols for its own benefit via Lam5. However, Lam5 does not seem to be essential for BMV layer-VRC formation.

Lam6, the closest homolog of Lam5, has important functions for membrane contact sites. A study in yeast showed that Lam6 can function to expand membrane contact sites when overexpressed and Lam6 has been shown to be important for cross-talk between membrane contact sites (Elbaz-Alon et al., 2015). However, to our knowledge, nothing like this has been reported for Lam5. My data shows Lam6 did not relocalize by BMV and deletion of Lam6 did not give a strong decrease in BMV replication. It will be interesting in the future to follow up on Lam5's cellular functions and if BMV subverts those functions for its own gain.

The four genes with the GRAM domain from Arabidopsis are very exciting. They all are predicted to have lipid-binding activity. One of them, BAGP1, plays a role in autophagy and in defense against the fungus *Botrytis cinerea* (Li et al., 2016b). Another, VAD1, helps regulate the hypersensitive response cell death (Lorrain et al., 2004). The *vad1* Arabidopsis mutant displays stunting and has necrotic lesions, much like the Arabidopsis *cpr5* mutant (Lorrain et al., 2004). The VASt (VAD1 analog of StAR-related lipid transfer) domain was shown to be critical for VAD1's function in suppressing the HR response (Khafif et al., 2017). In fact, several proteins that suppress cell death in plants contain hypothesized lipid-binding domains (Khafif et al., 2014). Three of these plant GRAM domain genes significantly change expression in response to

Geminivirus infection (Ascencio-Ibáñez et al., 2008). Expression of AtLam2 changes in response to brassinosteroid treatment (Goda et al., 2004). My data shows AtLam2, when expressed in yeast, is redistributed by BMV. This suggests that BMV may be capable of subverting AtLam2 for its own viral replication. However, more work needs to be done to confirm this in plants.

This study adds Lam5 to a growing list of several lipid-related host factors in yeast that are critical for BMV replication. These factors include Ole1, a fatty acid desaturase, Acbp, a protein that binds and transports acyl-CoAs, and Cho2, an enzyme functioning in phosphatidylcholine synthesis (Lee and Ahlquist, 2003; Zhang et al., 2012; Zhang et al., 2016). In fact, it was found that BMV and other (+)RNA viruses induce PC synthesis at their viral replication complexes (Zhang et al., 2016). The reticulons, a family of membrane-shaping proteins, have been implicated in BMV replication as well. Lam5, just like Cho2 was re-localized by and co-localized with BMV 1a. It is unclear if or how Lam5 interacts with these other BMV-related host factors and why BMV is able to subvert these host factors in particular. Nevertheless, it is clear that BMV modifies aspects of host lipid transfer and metabolism for its own gain. These processes and host factors could be used as targets to develop antiviral therapies in the future. Understanding more about the liposome at viral replication complexes will be useful to understanding more about positive-strand RNA virus replication.

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Chapter 3: Identification of host genes restricting the infection of brome mosaic virus in dicotyledonous plants

3.1 Abstract

Brome mosaic virus (BMV) is a positive-strand RNA virus that primarily infects monocot crops, such as rice, wheat, and barley. Although much knowledge in gene expression, genome replication mechanisms, and virus-host interactions has been gained by using the engineered BMV-yeast system, there remains much to be learned about BMV-plant interactions. Wild-type *Arabidopsis thaliana*, which is a model dicotyledonous plant, is not a systemic host for BMV, but Arabidopsis mutants with a dysfunctional *CPR5* gene allow for BMV infection. I developed a genetic screen to identify additional Arabidopsis mutants that allow for BMV infection. This screen used western blotting and leaf press-blotting to quickly check for accumulated BMV coat protein and thereby identify Arabidopsis genes that restrict BMV growth in wild-type Arabidopsis. I have identified four such genes that are critical for blocking BMV infection in Arabidopsis. My results also indicate a strong connection of ROS (reactive oxygen species) metabolism to BMV replication. This project aids in our understanding of how dicot plants defend themselves from BMV infection and identified potential host factors for virus control in monocot crops.

3.2 Introduction

Plants possess a sophisticated immune system to combat pathogens. This system can roughly be separated into two components: PTI (PAMP triggered immunity) and ETI (Effector triggered immunity). PTI is based on PRRs (pattern recognition receptors) that recognizes PAMPs (pathogen associated molecular patterns) (DeFalco and Zipfel, 2021). PRRs are specialized proteins on the cell surface that serve to detect foreign structures in the plant extracellular environment (Bentham et al., 2020). PRR recognition of the pathogen activates basal defense responses, including reactive oxygen species, calcium ion influxes, and production of defense hormones (Majumdar et al., 2023). However, many pathogens have ways to suppress PTI by secreting molecules called effectors. Plants have receptors (typically NB-LRRs) that can detect pathogen effectors (Bentham et al., 2020), leading to ETI, where NB-LRR activation can activate a potent immune response, involving the hypersensitive response and SAR (Peng et al., 2022). Plant R genes can recognize viral proteins such as coat proteins or replicases (de Ronde et al., 2014).

RNA interference (RNAi) is a major form of defense used by plants against viruses. RNAi occurs when plant dicer-like proteins recognize and cleave double-stranded RNA (which are intermediates in viral replication). This cleavage results in production of short duplex RNAs that are loaded onto the host RNA-induced silencing complex (RISC) to produce short interfering RNAs (siRNAs). RISC is then able to bind to and degrade viral RNA molecules that have complementary sequences to siRNAs (Lopez-Gomollon and Baulcombe, 2022). Another form of RNAi is host-produced microRNAs (miRNAs), which can get processed and loaded onto RISC complexes, resulting in cleavage of target mRNA or a halt of translation (Yu et al., 2017). However, many viruses counter RNAi by encoding viral suppressors of RNAi. It is also possible for viruses to take advantage of the host RNAi system to silence host defense genes (Leonetti et al., 2021).

Arabidopsis thaliana is a model plant organism that is not a host for BMV. This is an example of nonhost resistance. Plants are constantly exposed to an array of microbes, yet plants are nonhosts for the majority of these microbes. Nonhost resistance is what makes an entire species incompatible to infection by a certain pathogen. This is a very broad and durable type of resistance (Lee et al., 2017). Both PTI and ETI can contribute to nonhost resistance. Recessive resistance to viruses is also a component of nonhost resistance (Panstruga and Moscou, 2020). For example, viral replication may depend on the virus using a certain allele of a host factor (such as eIF4E). If that host factor is not present or a mutant allele of the host factor is present in a plant species, then that plant would be nonhost to that virus. Since nonhost resistance is very potent and durable, elucidating the molecular components of nonhost resistance can be very valuable for transferring resistance to susceptible crops.

Although WT Arabidopsis is not a host for BMV, BMV has been shown to systemically infect Arabidopsis *cpr5* mutants (Fujisaki et al., 2009). CPR5 (constitutive expresser of PR genes) is a nucleoporin that is reported to have roles in both PTI and ETI. The *cpr5* mutant has enhanced resistance to multiple pathogens and increased programmed cell death, but also stunted growth (Bowling et al., 1997). CPR5 protein typically exists as a dimer, functioning to restrict transport of ETI signaling molecules into the nucleus (Wang et al., 2014). Upon NB-LRR activation, this CPR5 complex is disrupted and allows for the entrance of signaling molecules into the nucleus to activate the expression of defense genes, release of immune signals, and programed cell death (Gu et al., 2016). CPR5 also localizes to nuclear speckles and is an RNA binding protein that functions in RNA processing (Peng et al., 2022). In contrast to its role in suppressing ETI, CPR5 promotes PTI responses. In fact, the *cpr5* Arabidopsis mutant has impaired ROS bursts (Ma et al., 2023).

In this study I replicated the result of (Fujisaki et al., 2009) by detecting BMV CP (coat protein) in *cpr5* Arabidopsis. I then screened 103 other Arabidopsis mutants which were selected by analyzing transcriptomic data on *CPR5*, or were related to the spliceosome or ROS. These screens were an attempt to find additional genes that contribute to defense against BMV. I identified four such genes: *MUG13.4, AT1G52000, AT3G10770, and AOX1A.* MUG13.4 and AT3G10770 are RNA-binding proteins and homologous genes that belong to the same family. AT1G52000 belongs to the lectin-like superfamily and AOX1A is an alternative oxidase that lowers ROS levels in the cell. Heterologous expression of these Arabidopsis genes in *Nicotiana benthamiana* resulted in decreased BMV replication, while expression of another ROS-related gene *APX1* promoted BMV replication. These findings contribute to our knowledge of BMV-plant interactions and plant immunity.

Group	**Gene name**	**Group**	**Gene name**
1	*AT2G26800*	3	*AT5G11650*
1	*AT3G26000*	3	*AT5G22540*
1	*SEIPIN2*	3	*AT5G36930*
1	*ATG16*	3	*AT5G43450*
1	*CDF2*	3	*BI1*
1	*SRFR1*	3	*WRKY48*
1	*CTL07*	3	*AT5G24810*

1	***MUG13.4***	3	*MES7*
1	*AT4G28260*	3	*BAT1*
1	*AT1G68440*	3	*GLC*
2	*AT1G18710*	3	*ZIP7*
2	***AT1G52000***	3	*AT1G03020*
2	*AT1G74430*	3	*AT1G78450*
2	*AT3G28220*	3	*AT3G21670*
2	*CYP74B2*	3	*AT1G13650*
2	*AT5G52320*	3	*AT4G33960*
2	*EAT3*	3	*FLA13*
2	*MYB75*	3	*MES18*
2	*AFL1*	3	*AT5G62360*
2	*COR15A*	3	*AT1G51090*
2	*GOLS2*	3	*HAT1*
3	*AT2G41090*	3	*APS3*
3	*AT1G55450*	4	*PRL1*
3	*AT1G03400*	4	*FIP1*
3	*AT1G22930*	4	*MAC3A*
3	*AT1G65490*	4	*MAC3B*
3	*UCP5*	4	*CDC5*
3	*HB1*	4	*CPSF30*
3	*PEN3*	5	*ADR1*
3	*AT1G16260*	5	*IRE1A*

3	*RAP2.4*	5	*RIN4*
3	*SNRK2-8*	5	*ADR-L1*
3	*CRK3*	5	*LORE*
3	*SUC1*	5	*BZIP28*
3	*BZIP60*	5	*ADR1-L2*
3	*AT1G15670*	5	*CAT3*
3	*AT1G19020*	5	*APX1*
3	*AT1G02360*	5	*APX2*
3	*SYP121*	5	*CAT2*
3	***AOX1A***	5	*RBOHA*
3	*MKK4*	5	*RBOHB*
3	*ARO4*	5	*RBOHC*
3	*AMT1;1*	5	*RBOHD*
3	*AT4G18425*	5	*RBOHE*
3	*NPR4*	5	*RBOHF*
3	*AT4G34150*	6	*AT2G40960*
3	*AT4G38550*	6	***AT3G10770***
3	*EP3*	6	*AGO1*
3	*AT3G59660*	6	*AGO2*
3	*PMT6*	6	*MBP1*
3	*ACD6*	6	*GST21*
3	*SUFE2*		

Table 3.1 Summary of genes tested in Arabidopsis screens. All the genes tested in this project are listed and sorted into groups. The group number is listed to the left of the gene name. Group numbers represent the following: Group 1 genes are co-expressed with *CPR5*. Group 2 genes are co-expressed with *MYB47*. Group 3 genes' expression is significantly changed upon deletion of *CPR5*. Group 4 genes are related to the spliceosome. Group 5 genes are related to ROS. Group 6 is miscellaneous. Bolded genes indicate loss of function mutants that allow for BMV infection.

3.3 Methods:

3.3.1 Plant and virus materials

The majority of Arabidopsis mutant and transgenic seeds used in this project were ordered from Arabidopsis Biological Resource Center (ABRC) at Ohio State University. The others are described here: *cpr5*, cpr*5/sim/smr1*, *pAPX1::APX1*/Col-0, *pAPX1::APX1*/*cpr5*.
Arabidopsis seeds were stratified in water at 4°C in the dark for 2 days, and then grown in growth chambers at 20°C - 23°C.

To perform the overexpression experiments done in this study, the respective gene was cloned into the pAG2PB vector with a HA tag and driven by the 35S promoter from *Cauliflower mosaic virus* (CaMV). Full length cDNA clones of BMV RNA1, RNA2, and RNA3 were used for *N. benthamiana* virus inoculations. Constructs were introduced into *Agrobacterium tumafaciens* strain GV3101 by heat-shock transformation.

3.3.2 Plant inoculations and infiltrations

Arabidopsis experiments in this study used BMV viral particles diluted in a phosphate inoculation buffer (Bujarski, 1998). Final concentration of viral particles used was 100 ng/uL. 15-day old Arabidopsis plants were treated with carborundum and then inoculated via rubbing infection of BMV viral particles (Dzianott and Bujarski, 2004). Care was taken to rub the top and bottom of two leaves per plant thoroughly, but to not rub the plant too strongly. Then the Arabidopsis plants were incubated in the growth chamber overnight with a lid to maintain humid conditions. BMV replication was assessed two weeks after inoculation.

To test BMV replication in *N. benthamiana*, 4-week old *N. benthamiana* plants were infiltrated with *A. tumafaciens* harboring designated overexpression constructs. 48 hours afterward, the plants were infiltrated with *A. tumafaciens* harboring the BMV cDNA clones. BMV replication was assessed 48 hours after.

3.3.3 Detection of viral infection

BMV replication was determined by analyzing accumulation of BMV CP. Systemic leaves were ground and protein was extracted using Laemmli's sample buffer (Laemmli, 1970). These total protein samples were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Expression of BMV CP was detected using a 1:4000 dilution of anti-BMV CP antibody, a gift from Dr. Rodrigo Valverde at Louisiana State University. Detection then used horse-radish peroxidase-conjugated secondary antibodies (1:7500 dilution, Thermo Fisher Scientific) and then Supersignal West Femto substrate (Thermo Fisher Scientific). Protein bands were then visualized using Azure c4000 (Azure biosystems) and quantified using ImageJ. In Arabidopsis mutants that were able to support BMV replication, press-blotting (also referred to as leaf tissue printing) was used to confirm presence of brome mosaic virus coat protein as performed in (Fujisaki et al., 2009).

3.4 Results:

3.4.1 *cpr5* mutants support BMV infection

To our knowledge, Arabidopsis mutants lacking functional CPR5 were the only previously known Arabidopsis mutants capable of supporting BMV systemic infection (Fujisaki et al., 2009). I started this study by attempting to replicate this result. I was able to successfully detect BMV replication in the *cpr5* mutant, as shown by western blotting which detected BMV coat protein (Fig. 3.1). Most *cpr5* mutants, either single or high-order mutants, are dwarfed (Fujisaki et al., 2009). The growth defect not only makes plant inoculation difficult but also suggests that the allowance of BMV infection is possibly related to growth defects. To address these two issues, the *cpr5/sim/smr1* triple mutant and the *sim/smr1* double mutant were used. These mutants were obtained from Dr. Yangnan Gu. These two Arabidopsis lines grew similar to WT, as previously reported (Gu et al., 2016). These lines were inoculated with BMV viral particles and then tested for BMV replication. BMV CP was clearly present in the triple mutant, at a similar level to that of the *cpr5* mutant (Fig. 3.1). However, no BMV CP was detected in the *sim/smr1* mutant, indicating that those *SIM* (*SIAMESE*) and *SMR1* (*SIAMESE-related 1*) genes are not involved but the lack of CPR5 function in *cpr5/sim/smr1* is what allows for BMV infection.

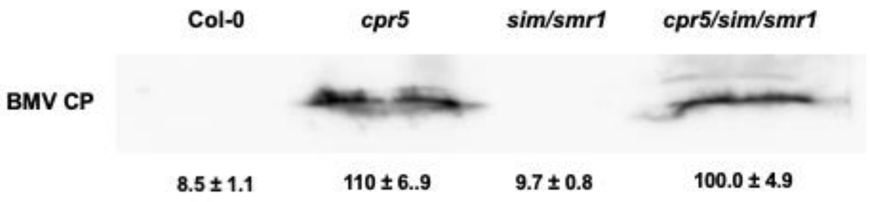
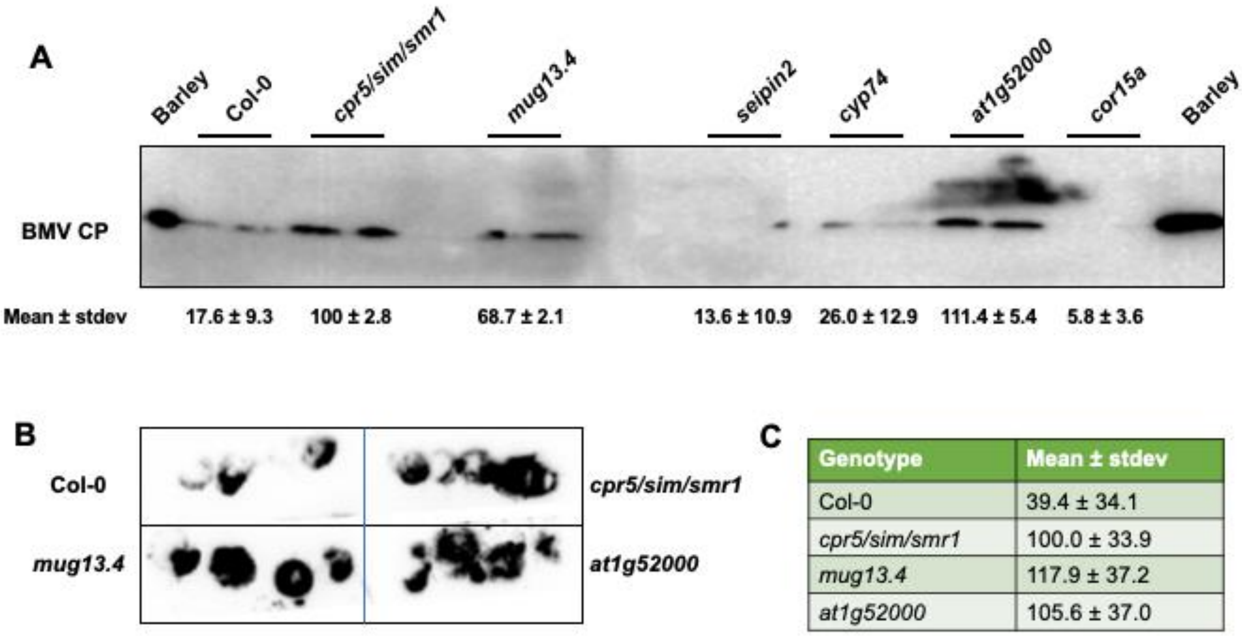


Figure 3.1. BMV infection is blocked in wild-type Arabidopsis, but occurs in Arabidopsis mutants that lack a functional CPR5. Arabidopsis lines were inoculated with BMV and then western blotting was performed to detect BMV CP (coat protein) in Col-0 and respective mutants. BMV CP is a measure of BMV replication.

3.4.2 Screen of genes that co-express with CPR5 or MYB47

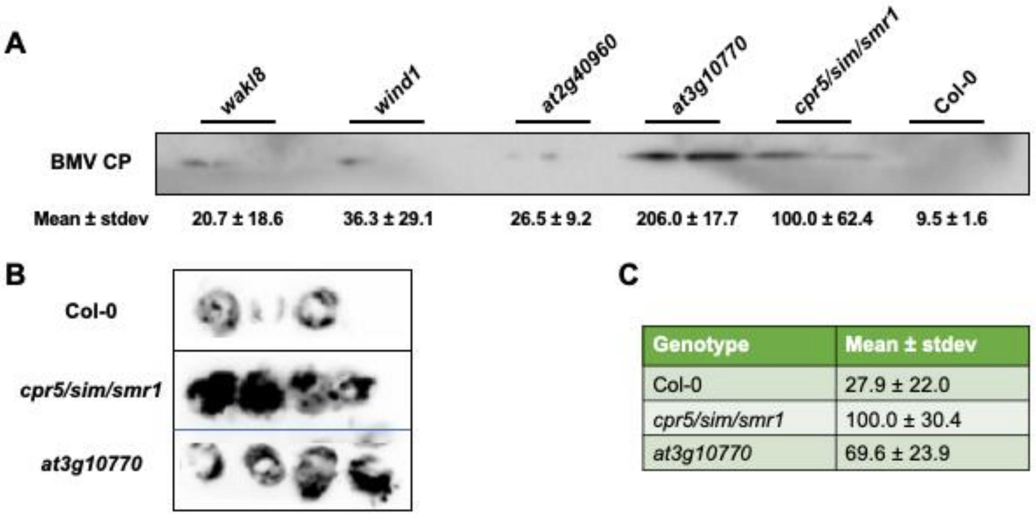
The goal of this study was to identify genes beyond *CPR5* that contribute to resistance against BMV. To do this, I tested a large number of Arabidopsis mutants to see if they could support BMV replication. Since *CPR5* is the only Arabidopsis resistance gene to BMV identified so far (Fujisaki et al., 2009), the first group I decided to screen were genes that co-express with *CPR5*. In other words, I screened loss-of-function mutants of genes that are turned on/off in the same conditions as *CPR5*~~.~~ I also included genes that co-express with *MYB47*, which is a transcription factor that is down-regulated in the *cpr5* mutant. These Arabidopsis mutants were inoculated using BMV viral particles and BMV infection was assessed 14 days afterwards.



Genotype	Mean ± stdev
Col-0	39.4 ± 34.1
cpr5/sim/smr1	100.0 ± 33.9
mug13.4	117.9 ± 37.2
at1g52000	105.6 ± 37.0

Figure 3.2. Mutants with dysfunctional MUG13.4 or AT1G52000 allow for BMV replication: (A) Western blotting to detect BMV CP in Arabidopsis mutants lacking function in genes that co-express with either *CPR5* or *MYB47*. (B) Press-blotting to detect BMV CP to confirm strong BMV infection in *mug13.4* and *at1g52000* Arabidopsis lines. (C) Quantification of press-blotting results. All data was quantified using ImageJ and normalized with *cpr5/sim/smr1* set to 100.

Immunoblotting results indicated that BMV was able to efficiently infect *cpr5/sim/smr1* and in barley, as shown previously. WT plants displayed a low accumulation of BMV CP as expected (Fujisaki et al., 2009). I was able to detect BMV CP in two other Arabidopsis mutants: *mug13.4* and *at1g52000* (Fig. 3.2A). *MUG13.4* co-expresses with *CPR5* and *AT1G52000* co-expresses with *MYB47*. Press-blot analysis supported these results (Fig. 3.2B). Quantifications of the press-blotting and western blotting showed that *mug13.4*, *at1g52000*, and *cpr5/sim/smr1* all support two- to three-fold more BMV replication than Col-0 (Fig. 3.2C).



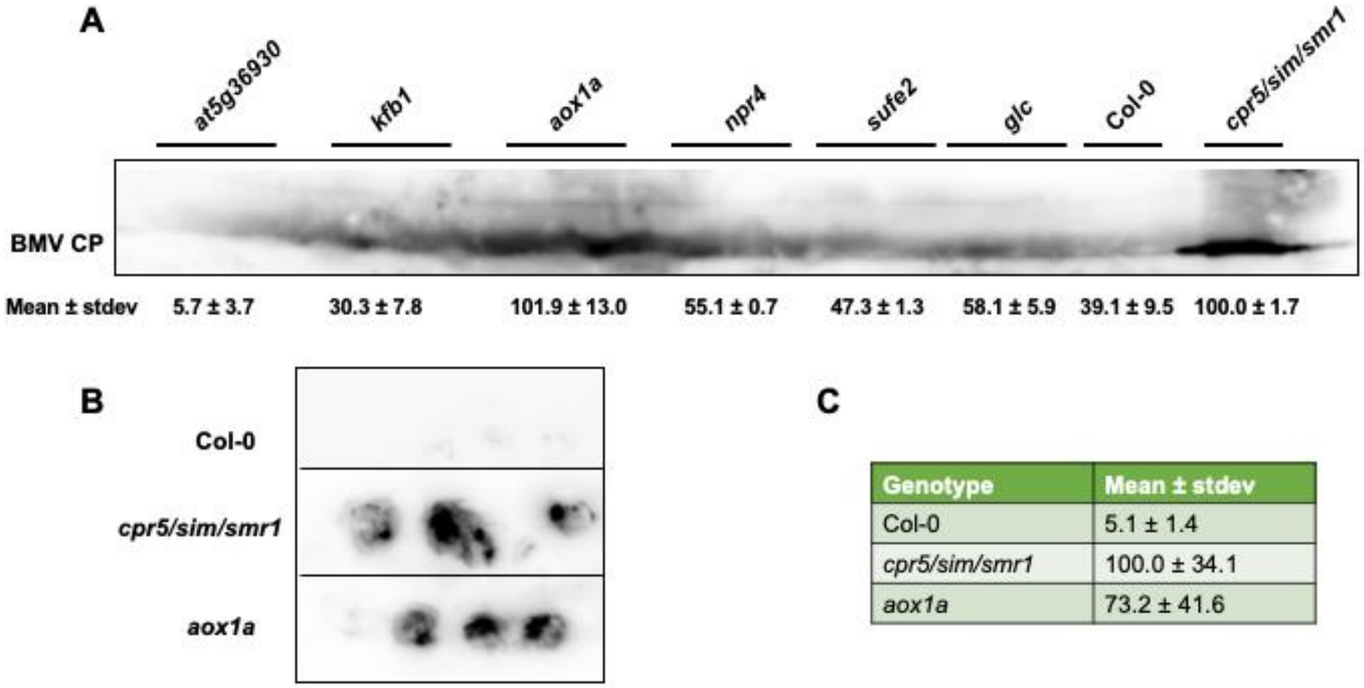
Genotype	Mean ± stdev
Col-0	27.9 ± 22.0
cpr5/sim/smr1	100.0 ± 30.4
at3g10770	69.6 ± 23.9

Figure 3.3. Mutant with dysfunctional AT3G10770 allows for BMV replication: (A) Western blotting to detect BMV CP in several mutants including two homologs of MUG13.4. (B) Press-blotting to detect BMV CP in *at3g10770*. (C) Quantification of press-blotting results. All data was quantified using ImageJ and normalized with *cpr5/sim/smr1* set to 100.

I also tested two homologs of MUG13.4: AT2G40960 and AT3G10770. While *at2g40960* supported very little BMV replication, *at3g10770* supported significantly more BMV replication than even *cpr5/sim/smr1* based on western blotting (Fig. 3.3A). Press blot analysis still detected BMV CP in *at3g10770*, but it was lower compared to *cpr5/sim/smr1* (Fig. 3.3B).

3.4.3 Screen of genes whose expression is affected when CPR5 is disrupted

CPR5 is a nucleoporin that gates the entry of immunity-related signaling molecules into the nucleus. I hypothesized that deletion of *CPR5* could allow virus-related immunity genes to be upregulated or downregulated. Therefore, the next group of Arabidopsis mutants I tested was loss-of-function mutants in genes whose expression is significantly changed when *CPR5* is disrupted. Transcriptomic analysis from Dr. Yangnan Gu at University of California Berkeley identified 54 genes whose expression was affected by the disruption of *CPR5* (Gu et al., 2016). These were genes that were differentially expressed in the *cpr5* mutant compared to wild-type. Among the 54 Arabidopsis mutants tested, one mutant was able to support BMV infection based on the accumulated CP by western blotting. This mutant had loss of function in the gene *AOX1a* (Alternative oxidase 1a). Alternative oxidases are located in the mitochondria and serve to reduce oxygen without using the electron transport chain. My results showed roughly equal BMV infection in *aox1a* and *cpr5/sim/smr1* plants as shown by western blotting (Fig. 3.4A) and press blotting (Fig. 3.4B).



Genotype	Mean ± stdev
Col-0	5.1 ± 1.4
cpr5/sim/smr1	100.0 ± 34.1
aox1a	73.2 ± 41.6

Figure 3.4. Mutant with dysfunctional AOX1a allows for BMV replication. (A) Western blotting to detect BMV CP in several mutants that lack function in genes whose expression was altered by deletion of CPR5. (B) Press blotting to detect BMV CP in *aox1a*. (C) Quantification of press-blotting results. All data was quantified using ImageJ and normalized with *cpr5/sim/smr1* set to 100.

3.4.4 Genes identified in Arabidopsis screens contribute to defense against BMV in *N. benthamiana*

I next wanted to see if my results in Arabidopsis could translate to *N. benthamiana*. Unlike Arabidopsis, *N. benthamiana* is susceptible to BMV and the majority of plant viruses, and serves as a universal host for plant viruses (Goodin et al., 2008). This is partially because the RdRp1

(RNA-dependent RNA polymerase 1) of *N. benthamiana* is not induced by salicylic acid and/or virus infection. Thus, this RdRP cannot confer *N. benthamiana* resistance to several viruses (Yang et al., 2004). I cloned genes of interest in an expression vector under the control of the CaMV (cauliflower mosaic virus) 35S promoter. These constructs were then transformed into Agrobacterium, which were then infiltrated into *N. benthamiana* leaves. Two days after agroinfiltration, BMV infection was then launched in the same leaves where targeted genes were infiltrated. Two days post BMV launching, leaves were harvested and ground. Heterologous expression of MUG13.4, AT3G10770, or AOX1a led to at least 2-fold decrease in BMV replication (Fig. 3.5). In particular, AT3G10770 gave the strongest phenotype; *N. benthamiana* leaves that heterologously expressed AT3G10770 only exhibited about 30% of BMV replication as the control leaves that expressed YFP (Yellow fluorescent protein). YFP is known to not have any effect on viral replication, so it was used as a positive control. Heterologous expression of AtCPR5, which is known to strongly decrease BMV replication, was used as a negative control.

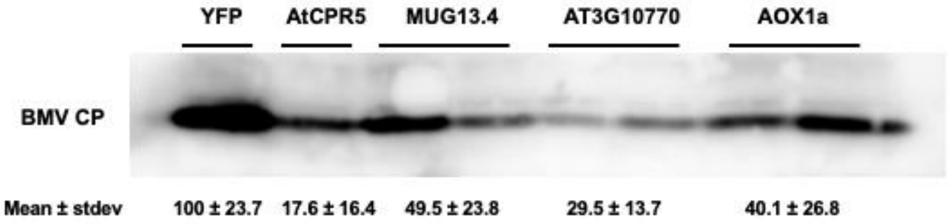


Figure 3.5. Heterologous expression of MUG13.4, AT3G10770, or AOX1a decreases BMV replication in *N. benthamiana*: Four-week old *N. benthamiana* plants were infiltrated with agrobacterium harboring constructs to overexpress genes identified in the Arabidopsis screens.

Two days later the same leaves were inoculated with BMV. Protein samples were extracted from the leaves and western blotting was performed to detect BMV CP. Data was quantified using ImageJ and normalized to YFP set to 100.

3.4.5 APX1, a key gene related to ROS, affects BMV replication

Since AOX1a has roles in ROS signaling, I was interested to see how other genes related to ROS could impact BMV replication. For instance, APX1 (ascorbate peroxidase 1) is a protein that functions to remove ROS in the cytoplasm (Davletova et al., 2005). In the *cpr5* mutant, RNA and protein levels of *AtAPX1* are lower than WT and overexpression of *AtAPX1* in the *cpr5* mutant complemented the growth defect, indicating that decreased expression of *AtAPX1* was responsible for the growth defect (Qi et al., 2023).

AtAPX1 was then expressed in *N. benthamiana* leaves, followed by launching of BMV. Heterologous expression of *AtAPX1* caused an increase of BMV replication compared to leaves infiltrated with YFP (Fig. 3.6A). Two APX1 mutants APX1^{W41F} and APX1^{R38H} were also included. APX1^{W41F} mimics horse-radish peroxidase (HRP) and has higher catalytic activity than WT, while APX1^{R38H} is HRP-dead and has lower catalytic activity (Hong et al., 2023). The expression of APX1^{W41F} blocked BMV replication while APX1^{R38H} had no significant effect (Fig. 3.6A). Given that expression of AtAPX1 promoted BMV replication, I tested whether AtAPX1 may enhance BMV replication when co-expressed with AtCPR5, whose expression substantially inhibits BMV replication. None of the APX1 isoforms were able to override the role of AtCPR5; when co-infiltrated with an APX1 isoform and AtCPR5, little BMV replication was observed. Consistent with our results in *N. benthamiana,* overexpression of *AtAPX1* in WT

Arabidopsis did not promote BMV infection due to the presence of *AtCPR5* (Fig. 3.6B, APX1/Col-0). However, to our surprise, no increase in BMV infection was observed in the *cpr5* mutant that overexpressed AtAPX1, differing from our work in *N. benthamiana* (Fig. 3.6B, APX1*/cpr5).*

Given my results regarding AOX1a and APX1, I decided to screen Arabidopsis T-DNA insertional mutants with dysfunctional genes related to ROS production and/or signaling. This included *APX2*, an isoform of *APX1*, and genes in the RBOH (respiratory burst oxidase homolog) family, which is involved in ROS production. The RBOH family was of particular interest because RBOHB has been shown to be necessary for replication of BMV and red clover necrotic mosaic virus (RCNMV) in *N. benthamiana* (Hyodo et al., 2017). As shown in Fig. 3.6C, none of these dysfunctional mutants supported BMV replication. These results imply that, although ROS-related genes such as APX1 and RBOHB can clearly affect BMV replication in *N. benthamiana*, these are not sufficient to modulate BMV replication in Arabidopsis. Therefore, there must be other mechanisms at work in Arabidopsis that contribute to BMV resistance.

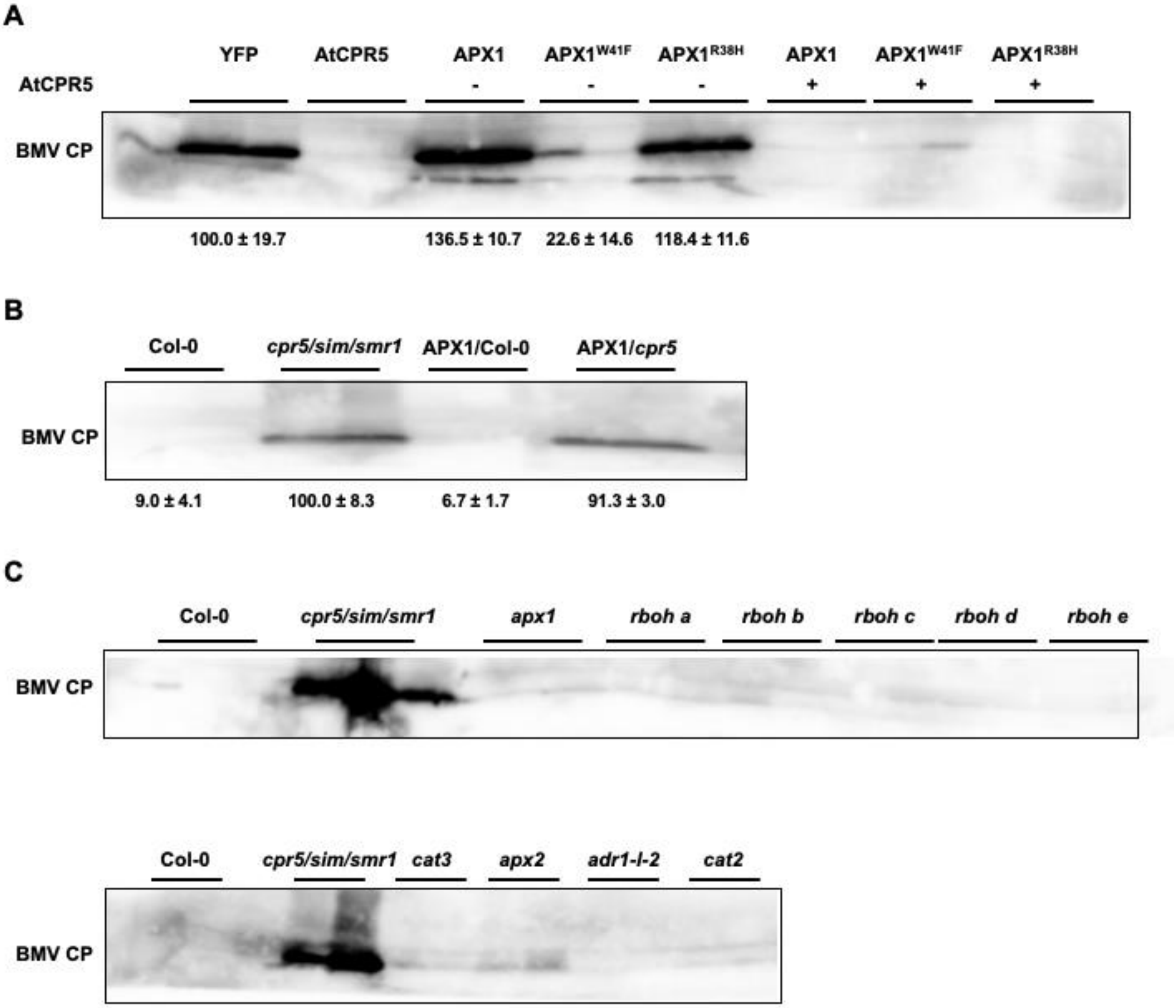


Figure 3.6. APX1 promotes BMV replication in *N. benthamiana* but not in Arabidopsis. (A) Effect of expressing WT AtAPX1 and two isoforms of AtAPX1 on BMV replication in *N. benthamiana*.. Heterologous expression of YFP and AtCPR5 was included as controls. Western blotting was performed to detect BMV CP. (B) Screen of Arabidopsis mutants that lack function

of key genes related to ROS production and/or signaling. Plants were inoculated with BMV viral particles and then tested for BMV replication by western blotting. (C) Western blotting to detect BMV CP in Arabidopsis lines overexpressing AtAPX1 either in a Col-0 or *cpr5* background.

3.5 Discussion

Arabidopsis is not a host to BMV and by that definition, possesses nonhost resistance to BMV. In this study, I have identified 4 Arabidopsis mutants supporting BMV infection, suggesting that these genes contribute to Arabidopsis immunity to BMV.

MUG13.4 is an RNA binding protein that contributes to salt tolerance. It is known to physically interact with AGO2 and microRNA (Wang et al., 2019). AT3G10770 is also an RNA binding protein (Simpson et al., 2008). It was recently shown that CPR5 also has RNA binding properties, functioning in RNA processing (Peng et al., 2022). It was hypothesized that CPR5 is involved in alternatively splicing or mRNA poly-adenylating tail of genes that are involved in plant immunity. This could provide an explanation of how plants can regulate immunity to several pathogens with only a limited number of immune receptors.

AT1G52000 contains a jasmonate-inducible domain. Jasmonates play important roles in plant defense against herbivores, such as insects. Wounding of leaves by insects causes production of jasmonic acid, which activates anti-herbivore defense genes including protease inhibitors. Since plant viruses typically enter plants via insect feeding or mechanical wounding, it is possible that *AT1G52000* could be a defense gene that is upregulated in response to wounding. AT1G52000 also belongs to the mannose-binding lectin superfamily. Lectins are proteins that bind carbohydrates. They are well known for their function in the human innate immune system, but less is known about them in plants.

ROS are an important component of plant immunity and are also regularly produced by the plant during normal conditions. Mitochondria, peroxisomes and chloroplasts are important sources of ROS, since cellular respiration and photosynthesis produce ROS. Specific ROS include superoxide, hydroxyl radicals, and hydrogen peroxide. ROS help plants combat pathogens by inducing stomatal closure to limit pathogen entry into the plant, fortifying the cell wall to hinder pathogen entry into plant cells, and modifying the expression of defense genes (Hyodo et al., 2017). ROS can damage intracellular components and thus lead to several symptoms which are characteristic of plant viral infection, including local lesions, necrosis, and mosaic symptoms (Xu et al., 2024).

Many plant mutants that exhibit disease resistance have high levels of ROS, chlorotic lesions, and lower growth (Qi et al., 2023). This includes the *cpr5* mutant that has increased resistance to most tested pathogens but became susceptible to BMV (Bowling et al., 1997; Fujisaki et al., 2009), suggesting that high levels of ROS could actually aid BMV replication. When AtAPX1 was expressed in *N. benthamiana*, an increase in BMV replication was observed (Fig. 3.6A). However, overexpression of APX1 in Arabidopsis, both in Col-0 or *cpr5* backgrounds, did not affect BMV replication.

In recent years, several (+)RNA viruses have been shown to rely on oxidizing conditions for their replication (Nishikiori and Ahlquist, 2021). For example, flaviviruses cause oxidative stress in the host cells and flavivirus replication is decreased by antioxidants (Gullberg et al., 2015). Another study has shown BMV replication in *N. benthamiana* depends on RBOHA and RBOHB. Furthermore, BMV 1a makes transmembrane pores in the ER to affect the ROS levels of its VRCs (Nishikiori and Ahlquist, 2018). BMV also makes use of the host factor ERO1 (ER luminal thiol oxidase 1), which generates ROS, for its replication (Nishikiori and Ahlquist, 2018).

Alternative oxidases provide an alternate pathway of cellular respiration. Instead of electrons going through the electron transport chain, they can go to a pathway that leads to alternative oxidase (Li et al., 2023). The alternative oxidase pathway, in comparison to the traditional electron transport chain pathway, results in lower yield of ATP, but also a lower amount of ROS produced (Maxwell et al., 1999; Selinski et al., 2018). AtAOX1a clearly plays critical roles in BMV infection as BMV infects Arabidopsis *aox1a* mutant plants (Fig. 3.4) and heterologous expression of AtAOX1a inhibited BMV replication in *N. benthamiana* (Fig. 3.5). This implies that AOX1a's function of lowering ROS hinders BMV replication, which would be consistent with the other literature.

ROS have traditionally been thought to be a tool for the plant immune system. Yet, it has been shown that BMV and other viruses actually utilize ROS for their replication. In the future it will be interesting to learn more about the exact connections between virus replication and ROS. Oxidizing conditions allow for BMV 1a's capping function and formation of 1a multimers using disulfide bonds (Nishikiori and Ahlquist, 2018). Perhaps a similar mechanism exists for other viruses as well. It is unclear how BMV 1a creates pores to modify the ROS levels. ROS could also regulate signal transduction within the cell, thus perhaps modifying host defenses. It will also be interesting to see if viruses target components of cellular respiration or photosynthesis, since these are cellular processes that produce high amounts of ROS.

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Chapter 4: Conclusion and Future Perspectives

(+)RNA viruses make up the largest class of viruses and share similar replication strategies. They all depend on host factors to aid in their viral genome replication and infection. Furthermore, they all cause remodeling of host intracellular membranes and thus have a reliance on cellular lipid metabolism and lipid composition at the viral replication complexes (VRCs) (Diaz and Wang, 2014). We use brome mosaic virus (BMV), a model virus which can replicate in yeast, to study (+)RNA virus replication mechanisms and the host factors involved in that process.

In this study, I found that Lam5 is necessary for proper BMV replication in yeast. Lam5 belongs to a six-member family in yeast that facilitate sterol transfer across host organelle membranes (Gatta et al., 2015). Expression of BMV 1a causes a drastic redistribution of Lam5 from punctate structures to ring structures and this new localization colocalizes with BMV 1a. I also found there are similar levels of BMV 1a in Δ*lam5* compared to wild-type, but there is a decrease of $2a^{pol}$ accumulation in the mutant. Furthermore, deletion of Lam5 does not disrupt BMV RNA3 protection nor layer-format VRC formation.

Much remains to be learned about how BMV uses lipids and how Lam5 is involved in this process. My data indicates there is some connection between Lam5, BMV 1a, and lipid droplet localization. When 1a is expressed in Δ*lam5,* neutral lipid localization changes from punctate structures throughout the cell to rings. Whether these rings co-localize with the ER or vacuole membrane remains to be tested. It could be that expression of 1a is a source of stress to the Δ*lam5* cells, and thus the cells mobilize the lipid droplets upon 1a expression. BMV replication has previously been shown to rely on phosphatidylcholine synthesis at the VRCs and the lipid composition of the VRCs is critical (Zhang et al., 2016). Perhaps certain sterols are somehow detrimental to optimal BMV replication, so the cell uses Lam5 to transport sterols away from BMV replication sites. A sterol-specific stain could be used to detect localization of sterols upon BMV 1a expression. Lipidomic analysis by mass spectrometry could also be useful to understanding how BMV 1a modulates host lipid composition with or without Lam5 present.

One particularly interesting result I had was from my membrane flotation assay. One cause of decreased BMV replication could be decreased membrane-association of BMV 1a. However, my membrane flotation assay actually showed an increase in BMV 1a levels at the membrane layer. I hypothesize that there may be too much BMV 1a in the VRCs and this could potentially be disrupting proper BMV replication. Perhaps there is too much BMV 1a at the necks of the VRCs and that is disrupting proper viral/host protein movement into or out of the VRCs.

It is also imperative that we translate the work on Lam5 from yeast to plants. Cloning LAM5 and AtLAM2, expressing them in *Nicotiana benthamiana* and testing for BMV replication would be a useful first step. More work on the four Arabidopsis homologs of Lam genes is also important. These genes are implicated in important functions including autophagy and pathogen resistance, so they may play a role in BMV replication in plants as well. The *vad1* Arabidopsis mutant is stunted and has necrotic lesions, much like the *cpr5* mutant (Bowling et al., 1997; Lorrain et al., 2004). It will be interesting to see if the Arabidopsis *cpr5* mutant has decreased expression of VAD1 or if *vad1* has abnormal ROS levels.

Although BMV serves as a useful model virus, mainly owing to its ability to replicate in yeast, there still remains much to be discovered about plant resistance to BMV. A significant barrier to more discoveries on this topic is that wild-type Arabidopsis is not a host for BMV. This means BMV researchers typically lose out on the genetic resources and ease associated with working with Arabidopsis, a model plant. Despite that, it was previously found that the Arabidopsis *cpr5* loss-of-function mutant can support BMV infection (Fujisaki et al., 2009). I performed screens in Arabidopsis in the hopes of identifying more mutants that could potentially support BMV infection.

In this study, I screened more than 100 Arabidopsis mutants and my data indicates I have found four genes that contribute to defense against BMV in Arabidopsis. These include two RNA-binding proteins which are homologs of each other: MUG13.4 and AT3G10770. Since CPR5 is known to regulate RNA via alternative splicing or poly-adenylation, I hypothesize these two proteins could have similar functions. Another gene I identified in my screens, *AT1G52000*, codes for a mannose-binding lectin superfamily protein. Some proteins in this superfamily have been shown to act as components of PTI (Lannoo and Van Damme, 2014).

AOX1a, another hit of my screens, is an alternative oxidase and has an impact on intracellular ROS levels (Maxwell et al., 1999). This is particularly of interest, since recent studies revealed ROS-producing genes are implicated in BMV replication; deleting or silencing yeast *ERO1* or *N. benthamiana RBOHB* leads to a decrease in BMV replication (Hyodo et al., 2017; Nishikiori and Ahlquist, 2018).

Although the genes that were hits in my screens have interesting known functions, it remains to be seen whether these functions are what allow them to contribute to resistance to BMV. More details on the mechanisms of how these genes has to be studied. It would also be interesting to check the expression of these genes by qRT-PCR both in WT and the *cpr5* mutant with or without BMV infection.

Furthermore, it is important that we translate the basic science gained from Arabidopsis studies to have an impact on agriculture. Wheat is a monocot that is susceptible to BMV and is able to be transformed via agrobacterium (Hayta et al., 2019). It would be great to transfer the Arabidopsis genes I identified in my screens to wheat and determine if those genes can confer resistance in wheat as well.

Overall, my research has expanded our knowledge of how BMV replicates in yeast and of how plants defend themselves against BMV. BMV may be using lipid-transport proteins and ROS to aid in its own replication. On the other hand, I hypothesize that plants may be using RNA-processing proteins or have ways to reduce ROS to combat against BMV replication. Since (+)RNA viruses often share similar replication strategies, the information gained from these studies may apply to other (+)RNA viruses as well. More needs to be learned about these genes, but I am hopeful the genes I studied in my research could be used in the future to provide broad-spectrum resistance to BMV and possibly other (+)RNA viruses.

4.1 References

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