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Analysis of Host Proteome Interacting With *Brome Mosaic Virus* Encoded Replicase and Coat Proteins.

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Microbiology

by

Areeje Almasary

December 2019

Dissertation Committee:
Dr. A. L. N. Rao, Chairperson
Dr. Shou-wei Ding
Dr. Wenbo Ma

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2019

The Dissertation of Areeje Almasary is approved:

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Committee Chairperson

University of California, Riverside

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DEDICATION

To my loving parents, siblings, husband and my children.

ABSTRACT OF THE DISSERTATION

Analysis of Host Proteome Interacting With *Brome Mosaic Virus* Encoded Replicase and Coat Proteins

by

Areeje Almasary

Doctor of Philosophy, Graduate Program in Microbiology
University of California, Riverside, December 2019
Dr. A. L. N. Rao, Chairperson

Viruses with positive-sense single stranded RNA genomes are pathogenic to humans, animals and plants. Some of these include Poliovirus, West Nile Virus, Equine arteritis virus and Cucumber Mosaic Virus. *Brome Mosaic Virus* (BMV) belongs to the family Bromoviridae and is the type member of the family. In this thesis, BMV is used as a model to study replication, assembly, packaging and recombination of positive-sense RNA viruses. BMV is a tripartite virus with three RNA molecules packaged independently. For a successful infection, all three particles must infect the same cell for efficient genome replication, packaging and cell-to-cell spread. Chapter one of this dissertation will focus on proteome analysis of *Nicotiana benthamiana* infected with BMV to study host protein complexes that interact with three virally encoded proteins: the replicase proteins p1a and p2a, and

the coat protein. *N. benthamiana* is a model species where BMV cause local and systemic infection and is widely used in plant research. To this end, co-immunoprecipitation was performed to recover host proteins that interact with virally encoded proteins. Bioinformatics analyses to predict the subcellular localization and functions of the interacting proteins is discussed. Chapter two will focus on proteome analysis of the natural host for BMV, *Hordeum vulgare*, to study protein-protein interactions with the three virally encoded proteins: p1a, p2a, and the coat protein. In Chapter three the effect of silencing one of the proteins that interacted with BMV replicase protein on BMV accumulation is examined. Specifically, the protein S-phase kinase related protein-1 (SKP1) was silenced via Virus-Induced Gene Silencing (VIGS) in *N. benthamiana* and further infected with BMV to examine if SKP1 is important for BMV replication.

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INTRODUCTION

The positive single-strand RNA viruses group include most of the medically and agriculturally important viruses including *Brome Mosaic Virus* (BMV). BMV which is pathogenic to monocot plants like Barley and dicot plants like *N. benthamiana*, is used to model several stages of viral replication for this group of viruses. Viruses exploit host proteins and subcellular factors to promote their replication. Thus, studying host proteome that interacts with virally encoded proteins will give us an understanding of virus-host interaction. In this dissertation, the proteome of two plant hosts of BMV that interact with three virally encoded proteins are analyzed.

Brome Mosaic Virus

BMV belongs to the bromoviridae family of icosahedral positive sense RNA viruses. Bromoviridae is part of the alphavirus-like superfamily and it is the representative member for this family of viruses. It is non-enveloped, and its genome is divided into three capped RNAs, each is packaged independently in viral particles. RNA1 and RNA2 encode the replicase proteins p1a (a helicase-like protein) and p2a (polymerase-like protein) respectively. RNA3 encodes two proteins that are not required for BMV genome replication but are required for its short distance (cell-to-cell) and long distance (systemic) movement in infected plants. One of the proteins that is encoded from RNA3 is the movement protein, The other proteins is the capsid protein which is encoded from sub-genomic

RNA4 that is synthesized by internal initiation mechanism from the minus-strand of RNA3 (Figure 1) (Ahlquist 1992) (Miller, Bujarski et al. 1986).

BMV was first isolated from Bromegrass (*Bromus inermis*), it infects monocots like Barley (*Hordeum vulgare*) and dicots like *Nicotiana benthamiana*. It is used as a model to study replication, assembly and recombination among other stages of the replication cycle of plus-strand RNA viruses which include most of plant pathogenic viruses and many medically important viruses.

Replication *of plus strand-positive sense RNA viruses*

One-third of viruses have positive-sense RNA genomes, including *Cucumber Mosaic Virus*, Polio, hepatitis C virus, West Nile Virus among many others. Even though there are differences in structure and genome organization, plus-strand RNA viruses share similarities in RNA replication. RNA replication in these viruses depends on interactions between virally encoded proteins and host proteins, and depends on subcellular changes induced by the virus in specific intracellular membranes (Denison 2008).

Positive sense RNA viruses replicate their genome through a negative-strand intermediate. After entry, the plus-strand RNA1 and RNA2 are immediately translated to make the replicase proteins (p1a and p2a) which form replication complexes with host proteins (Quadt, Kao et al. 1993) (Srividhya and Savithri 1996). After translation of viral replicase proteins, the virus switches from protein expression to genome replication (Gamarnik and Andino 1998). Genome

replication takes place in membrane-derived virus replication complexes (Tao and Ye 2010). These replication complexes can form from several different intracellular membranes such as late endosome (Rubella virus), ER (Dengue virus, Poliovirus, BMV), and Golgi (Hepatitis C virus) (Denison 2008). Inside the viral replication complexes the plus-strand RNA is used to make the negative-strand which is then used as a template to make new plus-strand progeny RNA that will be packaged inside newly synthesized capsid proteins to infect a new host or be transported to a neighboring cell (Noueiry and Ahlquist 2003) (Schwartz, Chen et al. 2002). RNA replication is asymmetric, meaning the plus-strand is synthesized about 100 times more than the negative-strand (Tao and Ye 2010). Each step of the replication cycle the virus recruits different host proteins to support its replication.

Different roles host proteins play in the replication of Plant viruses

Proteins are macromolecules performing vital functions with organisms including catalyzing metabolic reactions, DNA replication and transporting molecules from one location to another. The proteome is a complex set of proteins in a given organism at a given time in a given environment. The proteome can undergo rapid changes due to a stress or infection by viral, bacterial or fungal pathogens. In the context of virology, the majority of plant viruses' genome is single-stranded, positive sense RNA. Positive-sense RNA viruses have smaller genomes relative to other viruses and encode limited numbers of proteins, so they exploit host factors to infect cells and spread

throughout the host. Host factors can include intra-cellular membranes, host proteins and metabolites (Xu and Nagy 2010). Viral replication is catalyzed by a replicase complex consisting of proteins encoded from their genome. However, a functional viral replicase is a mixture of virus-encoded proteins complexed with several host proteins. Therefore, a large-scale studying of proteins, referred to as proteomics, by a variety of techniques, to study changes in the proteome induced by viral infection or the host proteins that interact with virally encoded proteins, could provide a valuable insight into the molecular mechanisms of viral replication, plant defense, and promote development of targets for disease control.

Host proteins have two roles in viral infection: they are either used by the virus to promote a successful infection or are triggered by the host to defend against the virus. A group of proteins that were found to be differentially expressed during *Tobacco mosaic virus* (TMV) infection in *N. benthamiana* are linked to the innate immune response. When disulfide isomerase proteins NbERp57 and NbP5 and Calreticulins NbCRT2 and NbCRT3 are downregulated, the plants carry the N-gene (which confers resistance against TMV) became susceptible to TMV infection (Xu and Nagy 2010) (Caplan, Zhu et al. 2009). Proteins involved in Cellular metabolic processes were identified to have different expression levels during *Rice yellow mottle virus* (RYMV) compared with uninfected rice plants (Ventelon, Delalande et al. 2004). Heat shock proteins are a group of proteins that are induced from abiotic triggers. However, some were shown to be used by

viruses to promote their replication (Xu and Nagy 2010). Studies in yeast as a model system showed that Hsp70 is essential for the formation of tombusvirus replicase complex (Serva and Nagy 2006) (Wang, Stork et al. 2009) (Pogany, Stork et al. 2008). Another heat shock protein, Hsp90, was reported in a study done with *Drosophila* cells to be involved in translation of protein-A of flock house virus (Castorena, Weeks et al. 2007). Additionally, salt-stress-induced proteins were shown to be induced in viral replication, specifically the protein dehydrin was induced in RYMV infection in rice (Ventelon, Delalande et al. 2004). Proteins in the translation machinery also have differential expression levels during viral infection (Ventelon, Delalande et al. 2004). Examples include translation elongation factor 1A that was shown to be associated with the replicase proteins of tobamovirus (Serva and Nagy 2006) (Wang, Stork et al. 2009) (Pogany, Stork et al. 2008) and tombusvirus (Li, Pogany et al. 2009). Studies done with BMV and TMV show that virally encoded polymerase protein of these viruses are associated with the elongation factor 3 (eIF-3) (Quadt, Kao et al. 1993). Drought-stress related protein expression also is induced with the infection of some viruses. Beta-1, 3-Endoglucanase and Chitinase, which are usually used by plants to defend against fungi by causing the digestion of fungal cell walls, were induced in tomato fruits infected with TMV (Margis-Pinheiro, Martin et al. 1993, Kunze, Kunze et al. 1998).

Some proteome studies focused on virus-host protein-protein interactions based on cellular organelles or fractions. From such studies done with thylakoid membranes purified from *Pepper mild mottle virus* (PMMoV) infected *N. benthamiana* it was shown that photo-system II electron transport proteins are differentially expressed in infected hosts. The PsbP isoforms are inhibited, while PsbO was slightly reduced in infected plants (Rahoutei, García-Luque et al. 2000) which indicates that the virus is changing the expression of these proteins to favor its replication. Another study identified six proteins from purified nuclei isolated from TMV infected hot pepper plants: 14-3-3- protein, Rab11 GTPase, a hypothetical protein, mRNA-binding protein, ubiquitin extension protein and 26 proteasome subunit RPN7, which is believed to be involved in programmed cell death, were identified. Increase in RPN7 expression levels correlate with resistance of plants to a TMV strain vs another strain (Lee, Kwon et al. 2006).

Host proteins that interact with virally encoded proteins could be isolated after purifying viral proteins. For example, purification of viral replication complexes can result in the identification of viral and host proteins involved is viral genome replication. Using this method, two viral replicase proteins and some host proteins were identified by purifying the replication complex of *Cucumber necrosis virus* followed by 2D gel electrophoresis and mass spectrometry. The identified host proteins are: GAPDH, pyruvate decarboxylase (pdc1p), Hsp70, Cdc34p and Tef1/2p (Li, Barajas et al. 2008) (Li, Pogany et al. 2009). Hsp70 was also identified to be associated with tombusvirus replicase

complex. Additional studies showed that the viral proteins p33 and $p92^{pol}$ recruit Hsp70 from the cytosol to the site of viral replication complex (peroxisome membrane) and that Hsp70 is important for the recruitment of p33 to peroxisome membranes (Wang, Stork et al. 2009) (Wang, Stork et al. 2009), replicase proteins insertion into the cellular membranes (Wang, Stork et al. 2009) and replicase complex assembly for tombusvirus (Pogany, Stork et al. 2008). GAPDH is involved in Tombusvirus genome replication and is recruited from the cytosol to the peroxisome membranes to help in retaining the genome replication intermediate (minus-strand RNA) inside the replication complex (Wang and Nagy 2008). Studies done with *Turnip mosaic virus* (TuMV) identified three proteins associated with the viral polymerase in *Arabidopsis thaliana*: AtHsc70-3 (heat shock cognate 70-3), eEF1A (translation elongation factor) and PABP (poly(A)-binding proteins) (Thivierge, Cotton et al. 2008, Seay, Hayward et al. 2009) (Dufresne, Thivierge et al. 2008).

Another technique that is used to study protein-protein interaction and is used to explore host-virus interaction is yeast-two-hybrid screens. This technique has its advantage and disadvantages; one advantage is the interaction is happening in the cell where the conditions are best for cellular processes and interactions. However, the technique can give false-positives due to the protein being overexpressed in the yeast (Xu and Nagy 2010). Yeast-two-hybrid was utilized to identify *N. benthamiana* host proteins that interact with *Bamboo mosaic virus* (BaMV) viral RNA-dependent RNA polymerase. A putative

methyltransferase protein (PNbMTS1) was shown to interact with the polymerase and is thought to be involved in the host defense against BaMV (Cheng, Hsiao et al. 2009). Also, studies done to identify Arabidopsis proteins that interact with helicase domain of TMV replicase protein concluded that the host protein PAP1 (Aux/IAA protein) plays an important role in symptom manifestation by modulating PAP1 function (Padmanabhan, Kramer et al. 2008) (Padmanabhan, Shiferaw et al. 2006) (Padmanabhan, Goregaoker et al. 2005) (Wang, Goregaoker et al. 2009). Furthermore, the same technique was used to study host proteins that interact with *Potato virus X* (PVX) movement protein p12. The study showed that the proteins TIP1, TIP2, and TIP3 interact specifically with P12 and are believed to recruit the movement protein to plasmodesmata and assist in regulation of plasmodesmata size (Tamai and Meshi 2001).

The above techniques studied protein-protein interaction between host and virally encoded proteins. Another approach to study virus-host interaction looks at virus-protein interaction. This approach sheds light on the host proteins recruited by the virus particle which is accomplished by incubating purified virus with host protein extract (Brizard, Carapito et al. 2006). Proteins that were identified with a study done with RYMV have identified metabolism related enzymes that are involved in glycolysis, malate and citrate cycles, other proteins involved in defense like proteins involved in detoxification and reactive oxygen

species, other identified proteins are involved in protein synthesis like elongation factors, protein-disulfide isomerase and translation related proteins (Brizard, Carapito et al. 2006).

In addition to virus-host protein-protein interactions, host proteins can interact with viral RNAs. For example, yeast protein microarray was used to identify proteins that interact with tombusvirus RNA. Some of the identified proteins are: translation initiation factor eIF2B delta subunit (Gcd2p), ATP-dependent RNA helicase (Dbp2p) and translation elongation factor eEF1A (Tef2p) which was found to bind to a cis-acting element in the 3' untranslated region of TBSV RNA (the replication silencer element) (Li, Pogany et al. 2009).

Good progress was made in the field of virus-host interactions, but a lot still need to be done. In this dissertation, a global proteome study is conducted to study host-virus protein-protein interaction to identify plant proteins that interact with BMV encoded proteins.

Replication of Brome Mosaic Virus

BMV's p1a protein plays several functions during the replication of BMV. At its N-terminus is a domain required for capping, at the C-terminal there is an RNA helicase homology domain (Noueiry and Ahlquist 2003). It induces the formation of viral replication complexes on the ER where it recruits more p1a and p2a proteins to replicate the genome of the virus. p1a interacts with the viral RNAs which is important step for the specificity of viral RNA replication (Schwartz, Chen et al. 2002). The other replicase protein, p2a, has a central

domain that is conserved in RNA dependent RNA polymerase proteins. The two viral replicase proteins interact to form the replicase complex (Quadt and Jaspars 1990) (Quadt, Verbeek et al. 1988) to synthesize the negative strand RNA which would be used as a template for the generation of the positive-strand genome of BMV. p1a can function as a replicase only when bound to p2a, however, p2a can synthesize the subgenomic RNA4 from the (-) strand of RNA3 via an internal promoter (Noueiry and Ahlquist 2003) (Rao, Chaturvedi et al. 2014). The replicase proteins are vital for specificity of viral RNA packaging rather than cellular RNA being packaged (Annamalai and Rao 2005, Rao, Chaturvedi et al. 2014). Unlike the RNA replicase protein of *Turnip Yellow Mosaic virus* (TYMV) that was shown to synthesize RNA from TYMV RNA, and also from another tymovirus, *Eggplant Mosaic Virus*, RNA (Mouches, Candresse et al. 1984), BMV replicase protein p2a is specific for BMV RNAs (Quadt and Jaspars 1990).

Efficient RNA replication is controlled by cis-acting elements. The 3' terminal of BMV RNAs have a tRNA-like structure which is important for the initiation of negative-strand synthesis and all BMV RNAs 3' ends have a region between 135 to 200 bases that function as promoters and are important for replication initiation (Miller, Bujarski et al. 1986). Additionally, the 5' end has some regulatory functions. RNA2's 5' end non-coding region regulates the translation of p2a in relation to other BMV proteins' expression. Furthermore, the 5' end of both RNA1 and 2 and the intergenic region of RNA3 has a role in

selectivity and recruitment of the RNAs from translation sites to the RNA replication complexes on ER membranes (Schwartz, Chen et al. 2002).

The coat protein (CP) is the structural protein encoded from sub-genomic RNA4 that is synthesized from the minus-strand of RNA3. BMV coat protein not only serve to package newly synthesized RNA progeny but is a multifunctional protein. Progression of BMV infection depends on the amount of coat protein expressed (Ivanov and Makinen 2012) (Kao, Ni et al. 2011). When CP levels are low at the beginning of infection CP binds to an RNA element at the 3' tRNA like structure and promotes replication. At later stages of infection CP's role inhibits RNA replication and promotes translation (Ivanov and Makinen 2012) (Chapman and Kao 1999) (Ivanov and Makinen 2012) This concentration-dependent regulation is achieved via binding of CP to the B-box in the RNAs: an RNA element at the 5' untranslated region of RNA 1 and 2 (Ivanov and Makinen 2012). BMV CP was also shown to induce ER rearrangement and spherule production that are essential for BMV replication. Spherules contain viral replicase proteins and template viral RNAs (Ivanov and Makinen 2012). BMV CP was shown to co-localize with the site of BMV replication complexes which suggests that viral RNA replication is coupled with packaging (Ivanov and Makinen 2012).

Live imaging and Bimolecular Flourescence Complementation (Bi-FC) assays to identify interactions between BMV encoded proteins showed that the

replicase protein p2a but not p1a interacts with CP, and self-interactions for p1a, p2a and CP were also shown. Movement protein was not included in the study (Chaturvedi and Rao 2014). It is believed that the interaction between CP:p2a is involved in the upregulation of the (+) strand synthesis versus the (-) strand RNA (Rao, Chaturvedi et al. 2014).

Additionally, replication of BMV depends on host proteins to complete each of its replication steps. For the important role host factors play in viral replication it is important to understand what host proteins are involved in viral replication and characterizing the function they play in the replication cycle.

Interactions between BMV encoded proteins and host proteins in yeast

Host proteins play several roles in viral replication; the assembly of a functional replicase complex which includes p1a and p2a, regulation of RNA translation and BMV gene expression, recruitment of viral RNA to the replication complexes, activation of the replication complex or lipid modification on the endoplasmic reticulum membrane to support replication complexes formation (Noueiry and Ahlquist 2003). Host proteins can also associate with viral RNA (Andino, Rieckhof et al. 1990) (Mouches, Candresse et al. 1984).

From a study done in barley cells a host protein was identified to be associated with BMV's p2a protein (Quadt, Kao et al. 1993). The protein was identified to be an analog of eIF-3 subunit p41. The addition of p41 stimulates minus-strand synthesis three times more than un-supplemented barley cells.

BMV RNA replication steps including synthesis of the sub-genomic RNA4 and encapsidation of viral RNAs were shown to occur in yeast. Yeast was used to study replication of BMV and several host proteins were identified to play different roles in BMV replication using the yeast system. Ded1p, a general yeast initiation factor was shown to have an important role in p2a translation initiation, when mutated it caused p2a translation to decrease about 20-fold which did not affect translation of p1a or yeast cellular protein translation. This regulation involves a 31 nucleotide sequence in the 5' non-coding region (NCR) of RNA2 that is not present in the 5' NCR of RNA1 (Noueiry, Chen J Fau - Ahlquist et al. 2000).

When BMV replicates, it must recruit RNAs 1, 2 and 3 from translation to replication sites in viral replication complexes on ER membranes. This process is selective in that the virus must recruit its RNAs and exclude cellular RNAs, and BMV also excludes recruiting subgenomic RNA4. Studies have shown that the yeast Lsm1-7p/pat1p complex is required for repressing translation by p1a. Lsm1-7p/pat1p/Dhh1p complex, which is a deadenylation-dependent decapping complex that switches cellular mRNAs from translation to degradation, is used by BMV for recruitment of BMV RNAs from translation to genome replication (Noueiry and Ahlquist 2003, Mas, Alves-Rodrigues et al. 2006).

Viruses with positive single-strand RNA genomes replicate on cellular membranes which is essential for anchoring the viral replication complex,

activation of capping enzymes which was shown for Semliki Forest alphavirus, and synthesis of positive sense RNA of picornaviruses (Noueiry and Ahlquist 2003). Ole1p (Δ9 fatty acid desaturase) is a yeast protein that synthesizes unsaturated fatty acids (UFA) which are important for the fluidity and physical characteristics of cellular membranes. When the gene OLE1 is mutated in yeast it inhibited BMV RNA replication which was rescued when the cells were provided with UFA (Noueiry and Ahlquist 2003). These studies showed that BMV required UFA levels that are five-times higher than what yeast cells need for growth. This inhibition is independent of recruitment of RNA3 or p2a to the replication sites, but it acts on a step just before negative strand synthesis.

Another yeast protein important for negative strand synthesis is Ydj1p which assists the protein chaperons Hsp70 and Hsp90 in protein folding, protein transport across membranes and macromolecule complexes formations. It is believed that Ydj1p is involved in the formation of functional replication complexes with the polymerase p2a to support negative strand synthesis (Noueiry and Ahlquist 2003).

SCOPE OF THESIS

Biological processes depend on molecular interactions between proteins and other molecules. Viruses, especially RNA viruses, have very small genomes and encode only few proteins; these viruses depend heavily on protein-protein interactions between their own encoded proteins and host proteins.

BMV-host interaction studies to identify host proteins involved in BMV replication used a surrogate host: yeast. Other studies identified host proteins that interact with BMV RNAs (Xu and Nagy 2010). To accomplish the latter, yeast protein microarray was used to identify yeast proteins that interact with the 3' untranslated region of RNA3. Among the twelve proteins identified from the screen three were validated for their role in BMV replication in plants: pseudouridine synthase Pus4p and actin patch binding protein which slightly caused the reduction of positive-strand RNA accumulation. Additionally, pus4p inhibited the packaging of BMV RNAs. The other protein that was identified is UDP-N-acetylglucosamine pyrophosphorylase Qri1p (Xu and Nagy 2010).

A method that was used in the past to study virus-host protein-protein interactions is 2D-gel electrophoresis combined with mass spectrometry analyses. This method can identify proteins that are abundant and limit the molecular weight of identifiable proteins to 10 to 100 kDa (Xu and Nagy 2010). Another approach is co-immunoprecipitation combined with Mud-pit analysis which will can identify proteins that are in low quantities and could play a role in viral replication and infection (Xu and Nagy 2010). This method will be used in this study to identify plant proteins that interact with BMV encoded replicase proteins p1a and p2a and the coat protein. In the past, co-immunoprecipitation was used to identify host proteins associated with the replicase protein using

partially purified replicase complex (Xu and Nagy 2010). Here, a genome-wide approach is used to precipitate plant proteins from the proteomes of BMV hosts: *N. benthamiana* and Barley.

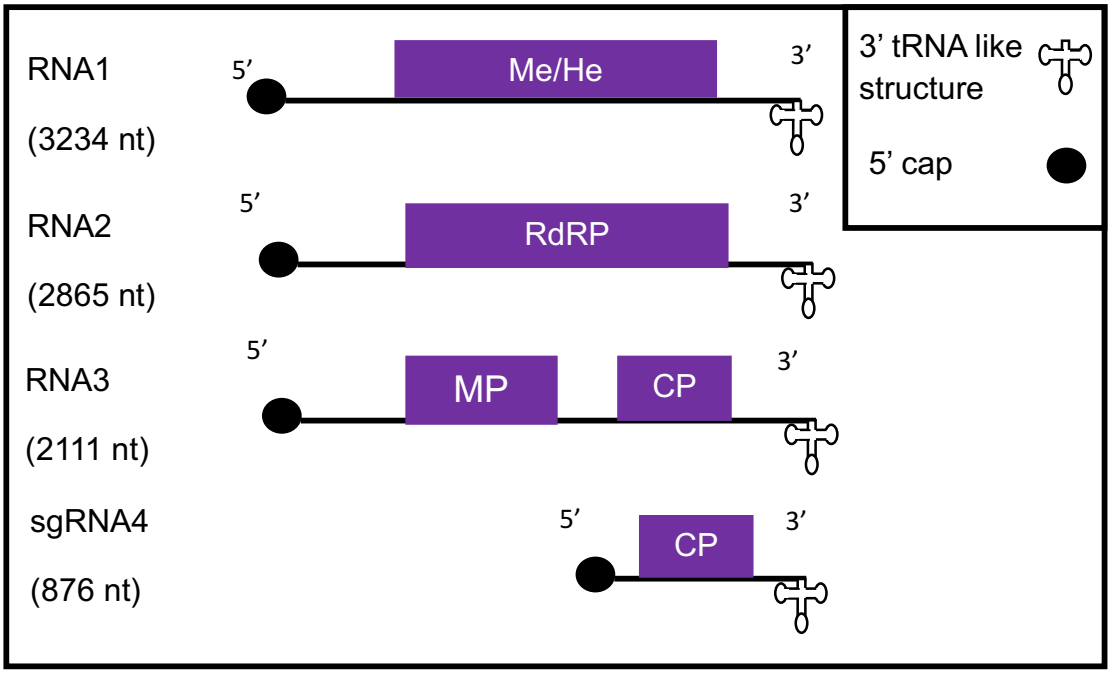


Figure 0.1 Genome Organization of *Brome Mosaic Virus.*

CHAPTER 1

Nicotiana benthamiana proteome interacting with *Brome Mosaic Virus* encoded replicase and coat proteins

Abstract

Viruses replicate using their virally encoded replicase proteins in addition to utilizing several host proteins to fulfill different roles in the viral replication cycle. *Brome Mosaic Virus* (BMV) has a single stranded positive-sense RNA genome and is used as a model to study the replication cycle of other viruses with similar genomes. Previous studies to identify host proteins involved in BMV replication were done in a surrogate host, yeast. In this study I isolated plant proteome, specifically *Nicotiana benthamiana,* that interact with virally encoded replicase proteins and coat protein using a co-immunoprecipitation assay. To shed light on relevance of recovered proteins to BMV replication, bioinformatics analyses were employed to predict the subcellular localization and function of the proteins. The subcellular localization analysis revealed that host proteins predicted to localize in cytoplasm, chloroplast and nucleus are the most abundant among the pulled-down proteins, and regarding the predicted functions it is evident that hydrolase, transferase and oxidoreductases are the most abundant among the pulled-down proteins that interact with either p1a, p2a or CP. Furthermore, putative orthologues for yeast protein previously identified to be important for BMV replication in *N. benthamiana* are discussed. This analysis

showed that 14 out of the 28 analyzed yeast proteins have putative orthologues in *N. benthamiana* as determined by the criteria set in this study.

Introduction

The host proteome plays an important role in the infection cycle of pathogens, this is especially true for viruses. Because viruses have relatively smaller genomes, they depend on host factors and proteins for a successful infection, from genome replication, packaging and cell-to-cell and systemic movement. Host proteins can play direct (protein-protein interactions with virally encoded proteins) or indirect role in the replication cycle. Identifying host proteins that are utilized by the virus for its replication can lead to development of novel and effective therapeutics against pathogens.

BMV is a plant pathogenic virus with single positive-sense RNA genome. The genome is divided into three segments each is packaged individually. BMV encodes four proteins: two replicase proteins p1a and p2a are encoded from RNA 1 and RNA 2 respectively. Movement protein is encoded from RNA3 which is disistronic and also encodes the coat proteins from sub-genomic RNA4. RNA4 is transcribed from the negative sense RNA3. p1a and p2a form the replicase complex which is critical for the replication of BMV RNA. Movement and coat proteins are important for the spread of BMV throughout the host.

The replication of BMV was extensively studied in the past, however, host proteins involvement in BMV replication was studied using a surrogate host,

yeast system. For example, a study has shown that a yeast general translation initiator factor, Ded1p, is important for the translation initiation of BMV p2a (Noueiry, Chen J Fau - Ahlquist et al. 2000). The yeast deadenylation-dependent decapping complex is important to switch from translating viral proteins to genome replication (Mas, Alves-Rodrigues et al. 2006). Yeast protein Ole1p plays an indirect role in BMV replication in yeast as shown in a study that unsaturated fatty acids made by the Δ9 fatty acid desaturase, Ole1p, is important for BMV RNA replication (Lee, Ishikawa et al. 2001) among other proteins. Additionally, most of the studies done on yeast were not validated in plants and there is no global proteome study that attempts to identify plant host proteins that could be involved in BMV infection. Thus, I initiated a project to identify plant host proteins that interact with BMV encoded replicase proteins p1a and p2a and coat protein (CP) via Co-immunoprecipitation (Co-IP) followed by MudPIT analysis to identify plant proteins that interact with BMV encoded replicase and coat proteins.

Materials and Methods

Plant infection and protein extraction

Eight-week old *Nicotiana benthamiana* (here after referred to as NB) plants were infected via *Agrobacterium*-based T-DNA constructs corresponding to BMV RNAs as described previously (Annamalai and Rao 2006) (Chaturvedi, Kalantidis et al. 2014). Four-days post infiltration, BMV infection in symptomless

NB leaves was confirmed by Western blot analysis using anti-BMV CP. Total proteins in BMV infected leaves were isolated by grinding NB leaves in liquid nitrogen followed by the addition of three volumes of extraction buffer (20 mM Tris–Cl [pH 7.5], 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1% plant protease inhibitor). Resulting extracts from each sample were centrifuged at 12,000 rpm for 15 mins at 4 °C, and the supernatants were collected and subsequently used for co-immunoprecipitation experiments.

Co-immunoprecipitation (Co-IP)

To identify the host proteome of NB interacting with BMV encoded proteins, Co-IP was performed by adding 25µl of either anti- p1a, p2a or CP and 20µl of anti-rabbit agarose beads to every gram of infected leaf material. Similar co-immunoprecipitation performed with pre-immune serum served as a negative control. The mixture containing the leaf extract and the desired antibody was incubated at 4 °C for 3 hours with gentle shaking. Then, the incubated mixture was centrifuged for 15 min at 4°C to collect the precipitate. The collected agarose beads were washed three times with extraction buffer, followed by a short spin at 4°C. Proteins were eluted from the agarose beads and subjected to Liquid chromatography tandem mass spectrometry (LC–MS/MS) at The Proteomics Center, University of California Riverside.

Bioinformatics analyses

Proteins recovered from the Co-IP assay were identified using Proteome Discoverer 2.1 software (Thermo Scientific) for NB plants. Subcellular localization of proteins interacting with BMV encoded proteins was identified by WoLF-PSORT (Horton, Park et al. 2007) and putative functions of pulled-down proteins were predicted by INGA (Piovesan, Giollo et al. 2015). NCBI BLASTp was used to identify the host proteins of NB plants as putative orthologues of yeast proteins having a query coverage >= 70%, identity percentages >= 30%, and an e-value of 1e-4 or lower. Functional protein domains present in a desired set of proteins were predicted using NCBI and aligned using CLUSTAWL2.

Results

Host proteins recovered against anti-p1a, p2a and CP

The procedure used to recover host proteins from BMV infected *N.benthamiana* following Co-IP with either replicase proteins p1a, p2a or CP or pre-immune serum used as a negative control is summarized in figure 1.1. After subtracting proteins precipitated with pre-immune serum, a total of 583, 761 and 595 host proteins were respectively precipitated with p1a, p2a and CP. A venn diagram representing the number of proteins commonly shared among all three is shown in Fig. 1.2. A total of 147 host proteins are specifically interacting with p1a, 55 with p2a and 101 with CP. A pair wise comparison revealed that 144 are

shared between p1a and p2a, 34 between p2a and CP and 120 between p1a and CP. Finally, 340 host proteins are commonly shared among the three virus-encoded protein.

Host proteins recovered from each Co-IP sample were subjected to bioinformatic analysis as summarized below they are classified based on either function or subcellular localization.

(a) Classification based on function

Using the web bioinformatic webtool INGA (Piovesan, Giollo et al. 2015), the functions of the co-immunoprecipitated proteins from BMV infected *N. benthamiana* were predicted and grouped (Figs. 1.3 and Table 1.1). Among 25 predicted groups of proteins, four functional groups oxidoreductase, hydrolase, transferase and transport appear to be more prevalent among the proteins recovered from BMV infected *N. benthamiana.*

(b) Classification based on subcellular localization

Viruses are known to replicate on specific locations within a cell such as chloroplasts, endoplasmic reticulum (ER), mitochondria etc. Therefore, gaining some insight on the subcellular localization of the recovered proteins is important to understand the functional relationship between BMV replication and proteins associated with a specific subcellular site. Consequently, subcellular localization of recovered proteins was predicted using WoLF-PSORT (Horton, Park et al.

2007). Results are summarized in Fig. 1.4. It was observed that the majority of the proteins recovered were predicted to localize in the cytoplasm, chloroplast and nucleus (Fig. 1.4).

Host proteins associated with ER

As mentioned above the ER is the preferred site of RNA replication for BMV, there it assembles active replicase complexes involving p1a and p2a and host factors (Restrepo-Hartwig and Ahlquist 1996, Restrepo-Hartwig and Ahlquist 1999, Noueiry and Ahlquist 2003). Although p1a is involved in the induction of ER-derived spherules in a surrogate, non-host yeast system (Diaz, Wang et al. 2010), CP has been shown to induce ER-derived vesicles in plant hosts (Bamunusinghe, Chaturvedi et al. 2013). Therefore, we further analyzed the recovered plant host proteins predicted to localize in the ER.

Among 941 host proteins recovered from *N. benthamiana*, 23 predicted to localized in the ER (Table 1.2). Among these proteins, 9 proteins are commonly shared among p1a, p2a and CP, one to four proteins were found to be shared between two of the three factors (e.g. p1a:p2a and p2a: CP etc.) (Figure 1.5). One protein that is particularly interesting is Bip5 that belongs to the family of Heat Shock Protein 70 (HSP70). HSP70 or its isoform has been shown play an important role in the life cycle of RNA viruses such as viral replication, virus

assembly, disassembly and cell-to-cell transport (Serva and Nagy 2006, Pogany, Stork et al. 2008); (Mayer 2005, Wang, Stork et al. 2009, Wang, Stork et al. 2009, Verchot 2012).

Using NCBI, BLASTp orthologues for recovered *N. benthamiana* putative ER proteins in barley were predicted. 62% of the *N. benthamiana* proteins have putative orthologues in barley (Figure 1.7) and could be good candidates for future functional analyses.

Plant vs. yeast

BMV is a plant virus. Results of this study represent the first report of plant proteins that specifically interact with replicase proteins (p1a and p2a) and CP. Prior to this study, 28 host proteins were reported to be intimately involved in BMV replication in a yeast (Carbonell, García et al. 2001) (Table 1.3). 50% of these yeast proteins have putative orthologues in *N. benthamiana* (Figure 1.7).

Out of the 28 yeast proteins, only two proteins Acb1p and Rpt6p have putative orthologues in *N. benthamiana* that were recovered by anti-p1a and p2a. Host proteins RPT6 and Snf7p were classified as orthologues of the yeast proteins based on a previously described cut-off used for this study (Table 1.3). BMV replicates on plant ER (Bamunusinghe, Chaturvedi et al. 2013) but neither of the *N. benthamiana* putative orthologues of the two yeast proteins Acb1p nor Rpt6p are predicted to localize on the ER (Acb1p putative orthologue predicted to localize in the mitochondria, Rpt6p putative orthologue predicted to localizes in

the cytoplasm in *N. benthamiana*) (Table 1.3), the involvement of these two proteins in BMV replication in plants needs further verification. Therefore, our results accentuate that it is imperative to establish the functionality of yeast host proteins in plant systems.

When looking at the amino acid level to compare the yeast proteins with their closest orthologues in *N.* benthamiana, Out of the 28 yeast proteins examined in this study I found 8 of the proteins were studied and mutations were made in them to show the importance of mutated amino acids for the protein to function in BMV replication in yeast; 5 out of the 8 proteins had the functional amino acids conserved in *N. Benthamiana* (Table 1.3).

Discussion

Subcellular localization of recovered N. benthamiana proteins

Although BMV is not known to replicate either in the chloroplast or nucleus, a relatively high number of host proteins related to these subcellular sites have been recovered in our Co-IP experiments (Figure. 1.4). Since RNA capping is a nuclear event (Ramanathan, Robb et al. 2016), it is possible that proteins related to the nucleus might play an indirect role by transiently transporting the BMV RNA to the nucleus for capping.

The chloroplast play different roles in viral infection from involvement in viral infection, manifestation of symptoms and host defense (Zhao, Zhang et al. 2016). Thus, it is not surprising that proteins that are predicted to localize in the

chloroplast comprise a large percentage of the recovered proteins (Figure 1.4). For example, the chloroplast aid in uncoating of Tombusvirus particle, and the chloroplast factor Tsip1 interacts with CMV's 1a and 2a to promote replication. Additionally alfamovirus AMV and TuMV among other viruses replicate in association with chloroplast membranes probably to escape the host anti-viral RNA silencing defense response (Zhao, Zhang et al. 2016). The coat protein of several viruses including *Tobacco Mosaic Virus*, CMV and *Potato Virus X* affect chloroplast ultrastructure and are involved in symptom development (Zhao, Zhang et al. 2016). The chloroplast is also involved in anti-viral defense; it is the site where Jasmonic acid and salicylic acids, hormones involved in plant defense, are synthesized. Jasmonic acid is involved in defense against viruses in susceptible hosts (Zhao, Zhang et al. 2016).

We are specifically interested in host proteins related to the ER since BMV is known to replicate on the ER derived vesicles (Diaz, Wang et al. 2010, Bamunusinghe, Seo et al. 2011, Bamunusinghe, Chaturvedi et al. 2013). Consequently, the 23 proteins that were predicted to localize in ER were subjected to further analysis.

One of the recovered *N. benthamiana* putative ER proteins is protein-disulfide isomerase-like 1-4. This protein has a putative orthologue in barley (query cover 84%, identity percentage 62 and e-value 0) that was recovered in a similar Co-IP experiment done with BMV infected barley (see chapter 1). This

protein was identified to interact and recruited by viral particles of *Rice Yellow Mottle Virus* (Brizard, Carapito et al. 2006). Additionally, it was shown to be important for *N. benthamiana* N-gene mediated defense against TMV (Yang, Lüpken et al. 2014).

Plant vs. Yeast

BMV replicates on ER membranes which are perinuclear in yeast but distinctly organized in plants. 64% of the 28 yeast proteins that were identified to be involved in BMV replication (Carbonell, García et al. 2016) are nuclear, however only 14 have possible orthologs in *N. benthamiana*. Three out of the 14 putative *N. benthamiana* orthologues are predicted to be nuclear in *N. benthamiana*. Because the ER is in the close proximity to the nucleus in yeast cells it could be expected that some nuclear proteins are involved in BMV replication in these cells. However, because of the subcellular differences between the two cells, the nuclear proteins that are involved in BMV replication in yeast could be replaced by ER or cytoplasmic proteins that are in the vicinity where BMV replicate in plant cells.

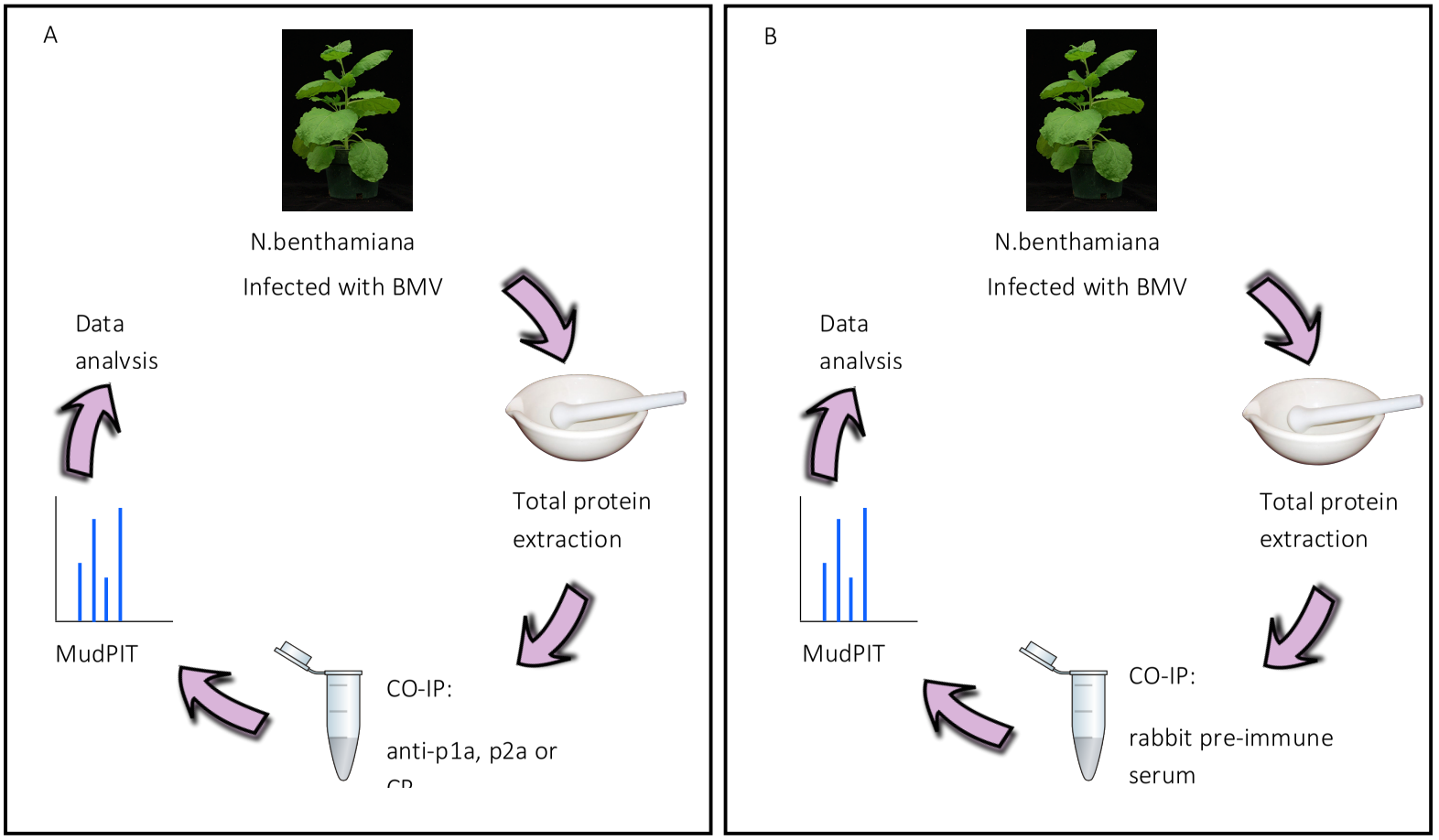

A
N.benthamiana
Infected with BMV
Total protein
extraction
CO-IP:
anti-p1a, p2a or
MudPIT
Data
analysis
B
N.benthamiana
Infected with BMV
Total protein
extraction
CO-IP:
rabbit pre-immune
serum
MudPIT
Data
analysis

Figure 1.1 Experimental design to identify NB proteins that interact with BMV coat protein and replicase proteins p1a and p2a (A) and using pre-immune serum for the negative control (B).

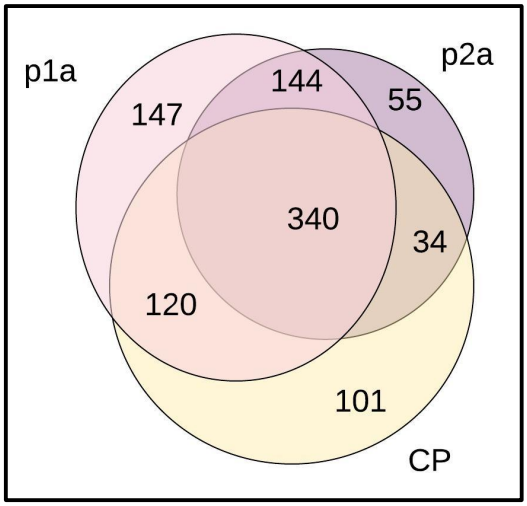
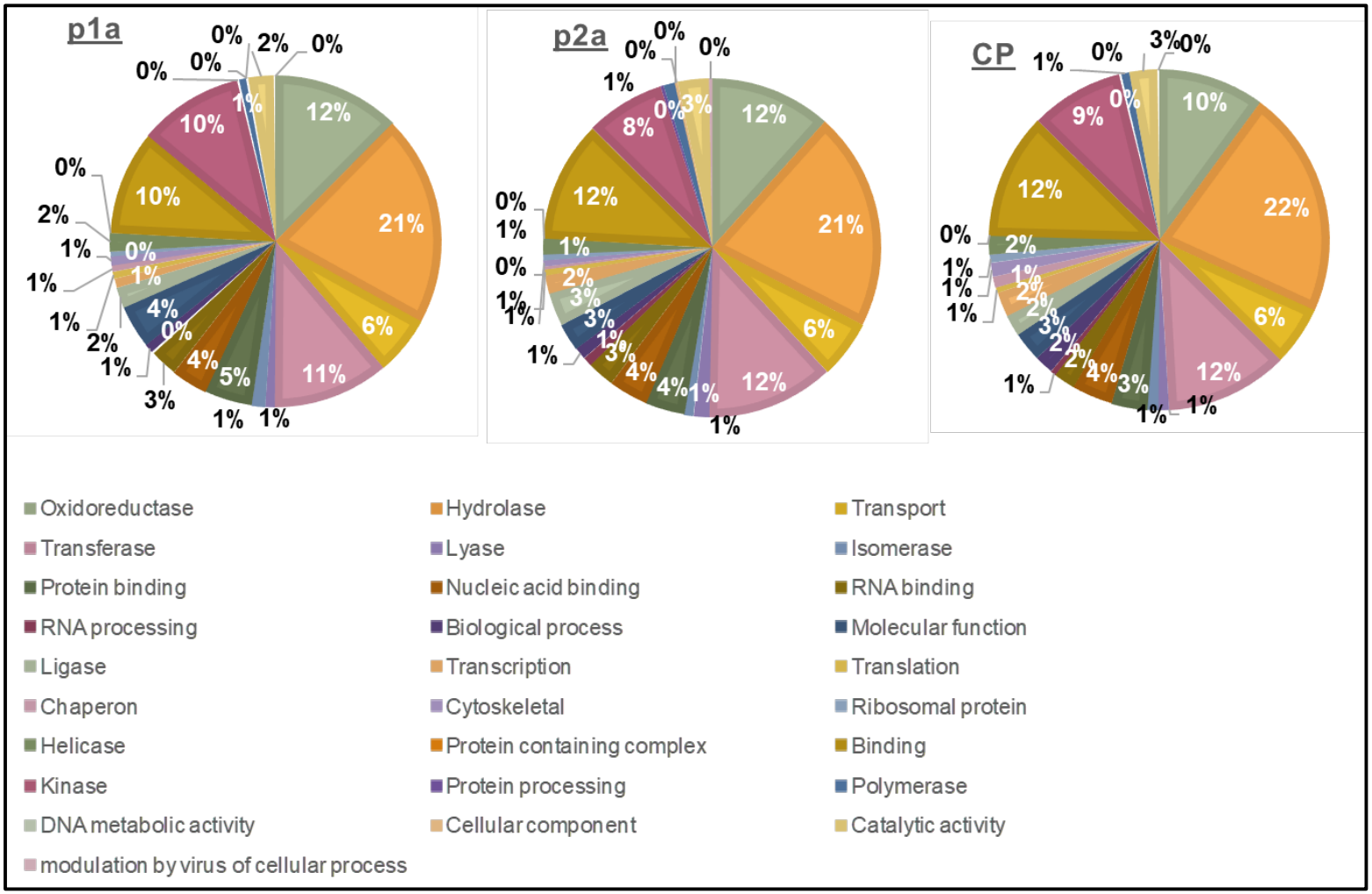


Figure 1.2 Venn diagram shows the number of plant host proteins that are recovered from the Co-IP assay done with NB with each of the antibodies (antibodies against p1a, p2a or CP).


p1a
0%
2%
0%
0%
0%
1%
12%
10%
0%
10%
21%
2%
1%
0%
1%
1%
4%
0%
1%
2%
4%
1%
5%
3%
11%
1%
1%
6%
p2a
0%
0%
0%
1%
8%
0%
3%
12%
0%
12%
21%
1%
0%
1%
2%
3%
1%
3%
1%
4%
4%
1%
12%
1%
6%
CP
1%
0%
3%
0%
9%
0%
10%
12%
22%
0%
2%
1%
1%
1%
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2%
3%
2%
2%
4%
1%
3%
1%
12%
1%
6%
Oxidoreductase
Hydrolase
Transport
Transferase
Lyase
Isomerase
Protein binding
Nucleic acid binding
RNA binding
RNA processing
Biological process
Molecular function
Ligase
Transcription
Translation
Chaperon
Cytoskeletal
Ribosomal protein
Helicase
Protein containing complex
Binding
Kinase
Protein processing
Polymerase
DNA metabolic activity
Cellular component
Catalytic activity
modulation by virus of cellular process

Figure 1.3 Functional prediction of proteins recovered from NB Co-IP assay performed using anti p1a, p2a or CP.

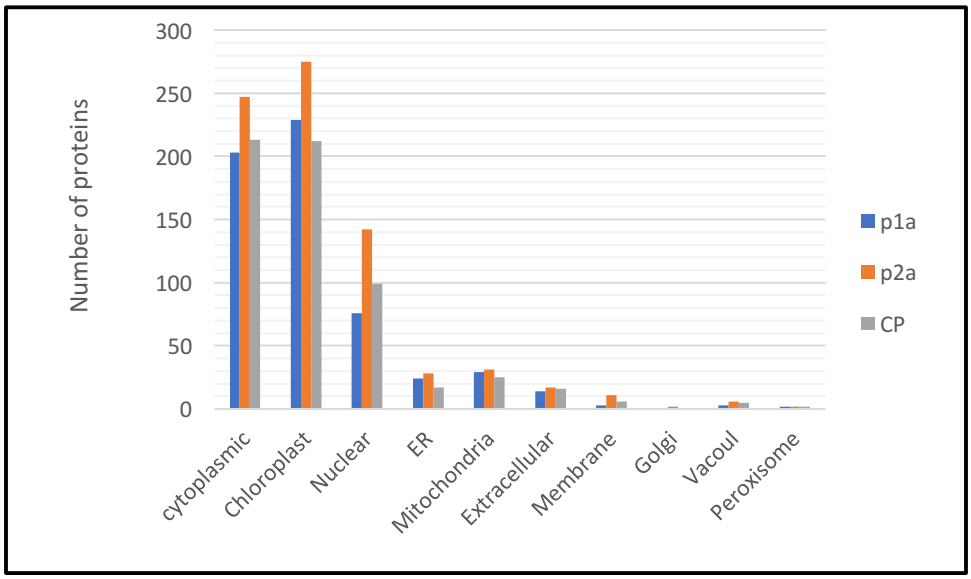

Figure 1.4 Subcellular localization of plant host proteins NB interacting with BMV encoded proteins p1a, p2a and CP predicted based on the amino acid sequences of the recovered proteins.

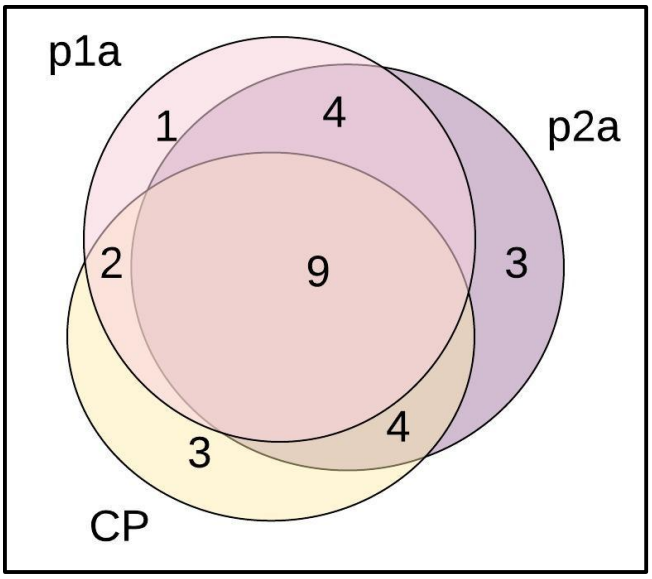

Figure 1.5 Venn diagram showing the number of recovered N. benthamiana proteins that were predicted to localize in the ER, ER membrane or ER-cytoplasm. The venn diagram shows the number of proteins that interact with p1a, p2a or CP, or with more than one of the viral proteins.

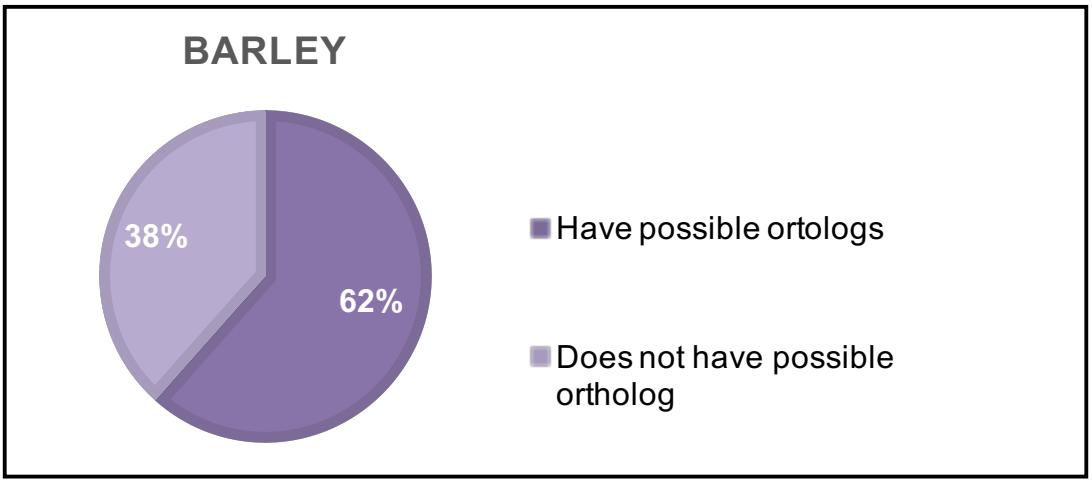

Figure 1.6. BLASTp was used to find orthologues for recovered putative *N. benthamiana* ER proteins in Barley (total number of recovered putative *N. benthamiana* ER proteins is 26).

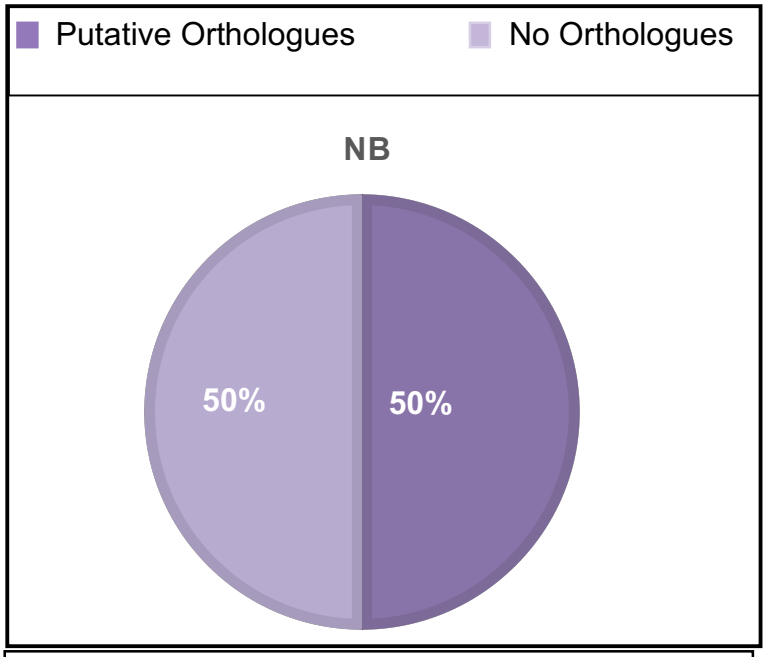

Figure 1.7 Pie chart showing percentage of yeast proteins that were shown important for BMV replication that have putative orthologues in *N. benthamiana*.

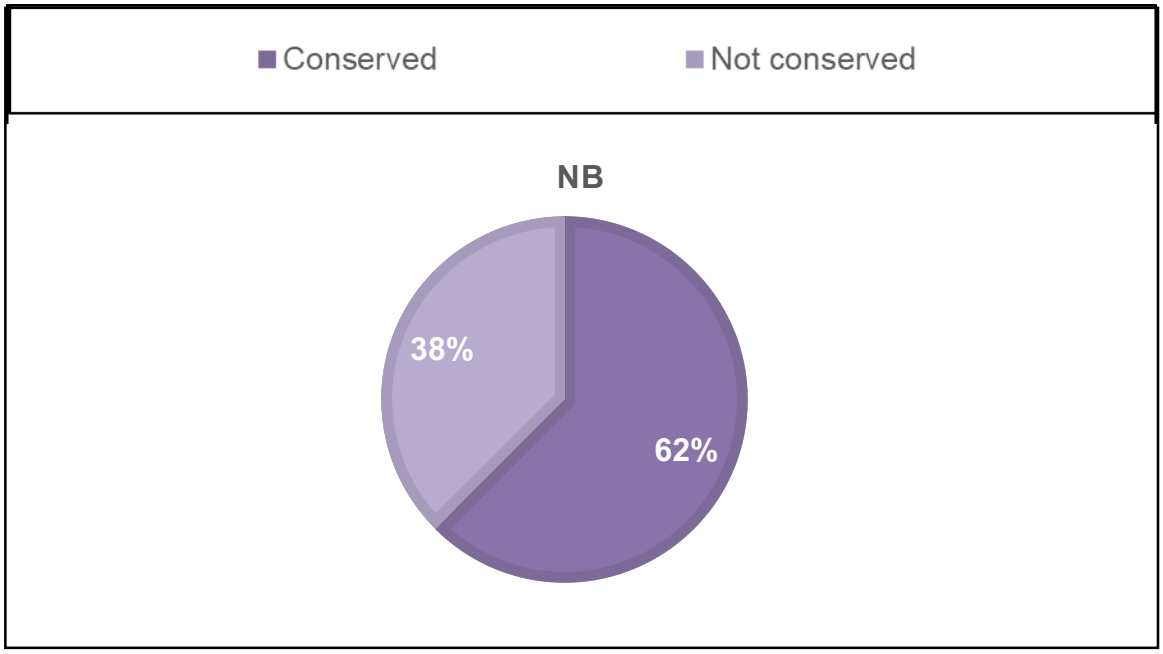

Figure 1.8. Pie chart showing percentage of functional amino acids that were shown in literature important for BMV replication in yeast that are conserved in *N. benthamiana.*

	Function Category	Example for: *N.benthamiana*
	Oxidoreductase	Thioredoxin
	Hydrolase	Vacoular ATP synthase subunit C
	Transport	Exocyst complex component 7
	Transfer	Vacuolar protein sorting-associated protein 54
	Lyase	Isopenicillin N epierase
	Isomerase	Protein disulfide isomerase-like 1-4
	Protein binding	protein transport protein SEC61 subunit gamma
	RNA binding	U3 small nuclear RNA-associated protein 18 homolog
	Ligase	E3 ubiquitin-protein ligase UPL3-like isoform 1
	Transcription	CCR4-NOT transcription complex subunit 7
	Translation	Translation initiation factor eIF-2B subunit beta
	Chaperon	Class II small heat shock protein Le-HSP17.6
	Cytoskeletal	Actin-4
	Ribosomal protein	30S ribosomal protein S1
	Helicase	DEAD-Box ATP dependent RNA helicase 3
	Kinase	MAP kinase

Table 1.1 Example proteins from each functional category that were recovered from N. benthamiana

Protein ID	Protein Name	Interaction	Putative function
NbS00040865g0006.1	Bip5 (Heat shock 70 protein)	p1a, p2a	Adenyl nucleotide binding
NbS00020366g0018.1	SUMO-activating enzyme subunit 2-like	P1a, p2a	adenyl nucleotide binding
NbS00020409g0009.1	Serpin-ZX	P1a, p2a	endopeptidase inhibitor
NbS00013071g0001.1	Peroxidase N1	P1a	oxidoreductase
NbS00010399g0124.1	5-oxoprolinase	P2a	hydrolase
NbS00040816g0010.1	Methionine S-methyltransferase	CP-p2a	S-adenosylmethionine-dependent methyltransferase activity
NbS00007488g0017.1	Tubulin-specific chaperone D	CP, p2a	beta-tubulin binding
NbS00038999g0004.1	Pleiotropic drug resistance protein 2	CP, p1a	ion transport
NbS00024101g0016.1	histocompatibility antigen H13	P1a, p2a	Endopeptidase
NbS00014205g0122.1	protein disulfide isomerase-like 1-4	P1a, p2a, CP	disulfide oxidoreductase
NbS00014369g0008.1	E3 ubiquitin-protein ligase	p2a	ubiquitin-protein transferase activity
NbS00009603g0006.1	N/A	p2a	N/A
NbS00017517g0004.1	TIP1: S-acyltransferase	CP	protein-cysteine S-acyltransferase activity
NbS00043545g0007.1	coatomer subunit epsilon-1-like	CP	vesicle-mediated transport
NbS00010366g0317.1	Coatomer beta subunit	CP, p2a	receptor serine/threonine kinase binding
NbS00000261g0008.1	Calreticulin-3	CP, p1a, p2a	calcium binding
NbS00002039g0009.1	26S proteasome regulatory subunit S5A	CP, p1a, p2a	polyubiqiotin binding
NbS00010066g0030.1	Molybdopterin biosynthesis protein CNX1	CP, p1a, p2a	molybdenum ion binding
NbS00021848g0002.1	endo-beta-1,4-D-glucanase precursor	CP, p1a, p2a	N/A
NbS00004592g0014.1	somatic embryogenesis receptor-like kinase 1	CP, p1a, p2a	protein serine/threonine kinase activity
NbS00022745g0019.1	Phosphorylated carbohydrates phosphatase TM_1254	CP, p1a, p2a	phosphoric ester hydrolase activity
NbS00011798g0007.1	Transmembrane and coiled-coil domain-containing protein 4	CP, p1a, p2a	organelle membrane
NbS00020610g0009.1	Protein WAX2	CP, p1a, p2a	transition metal ion binding

Table 1.2 List of proteins predicted to localize in ER recovered from BMV infected N. benthamiana showing which viral proteins they interact with and predicted function, names of the proteins were inferred by similarity

Yeast protein		*N. benthamiana*					
Protein	Subcellular localization	Ortholog	IP[A]	Query cover %	Identity %	Functional amino acids[B]	Subcellular localization
ACB1	Nuclear	No	1a, 2a	10	65	Yes	Mitochondria
BRO1	Nuclear	No	No	93	20	N/A	Nuclear
DOA4	Nuclear	No	No	36	37	No	Nuclear
DED1	Nuclear	Yes	No	86	52	Yes	Nuclear
DBP2	Nuclear	yes	No	86	52	N/A	Chloroplast
VPS23	Nuclear	No	No	94	24	N/A	Cytoplasmic
VPS20	Nuclear	yes	No	72	33	No	Peroxisome
SNF7	Nuclear	No	No	65	31	Yes	Nuclear
VPS24	Nuclear	No	No	64	33	N/A	Cytoplasmic
VPS2	Nuclear	Yes	No	97	47	N/A	Mitochondria
VPS4	Cytoplasmic	Yes	No	98	55	N/A	Nuclear
HSF1	Nuclear	No	No	29	45	N/A	Nuclear
LSM1	Nuclear	No	No	44	47	9 out of 13	Cytoplasmic
LSM2	Nuclear	Yes	No	94	64	N/A	Cytoplasmic
LSM3	Nuclear	Yes	No	85	44	N/A	Cytoplasmic
LSM4	Nuclear	No	No	52	38	N/A	Cytoplasmic
LSM5	Cytoskeletal	Yes	No	88	45	N/A	Cytoplasmic
LSM6	Cytoplasmic	Yes	No	87	43	N/A	Chloroplast
LSM7	Nuclear	Yes	No	75	47	N/A	Cytoplasmic
PAT1	Mitochondria	No	No	22	27	N/A	Nuclear
DHH1	Nuclear	Yes	No	75	65	3 out of 5	Nuclear
OLE1	Plasma membrane	No	No	44	29	No	Plasma membrane
PRE1	Cytoplasmic	yes	No	96	38	N/A	Cytoplasmic
RPT6	Plasma membrane	Yes	1a, 2a	95	75	N/A	Cytoplasmic

RTN1	Plasma membrane	No	No	51	25	N/A	Plasma membrane
RTN2	Nuclear	No	No	39	30	N/A	Plasma membrane
YOP1	Plasma membrane	No	No	53	42	N/A	Extracellular
YDJ1	Cytoplasmic	Yes	No	90		N/A	Nuclear

Table 1.3 *N. benthamiana* putative orthologs for yeast proteins shown in literature important for BMV replication (Carbonell et al., 2016). A: this column shows list of BMV proteins that interact with the putative ortholog and was recovered from the BMV infected plant host, no indicates that the putative ortholog was not recovered from BMV infected plant host. Cut-off used to identify the putative orthologs area as follows: query coverage 70%, identity 30%. B: this column shows if the amino acids that were shown in the literature to be important for BMV replication cycle or the protein function that is important for BMV replication are conserved between the yeast and *N. benthamiana* proteins, NA: no literature was found on protein mutations, numbers indicates how many of studied amino acids are conserved.

CHAPTER 2

Hordeum vulgare proteome interacting with *Brome Mosaic Virus* encoded replicase and coat proteins

Abstract

Viruses replicate using their virally encoded replicase proteins in addition to utilizing several host proteins to fulfill different roles in the viral replication cycle. *Brome Mosaic Virus* (BMV) has a single stranded positive-sense RNA genome and is used as a model to study the replication cycle of other viruses with similar genomes. Previous studies to identify host proteins involved in BMV replication were done in a surrogate host, yeast. In this study I isolated plant proteome, specifically *Hordeum vulgar* (Barley), that interact with virally encoded replicase proteins and coat protein using a co-immunoprecipitation assay. To shed light on relevance of recovered proteins to BMV replication, bioinformatics analyses were employed to predict the subcellular localization and function of the proteins. The subcellular localization analysis revealed that host proteins predicted to localize in cytoplasm, chloroplast and nucleus are the most abundant among the pulled-down proteins, and regarding the predicted functions it is evident that hydrolase, Transferase and oxidoreductases are the most abundant among the pulled-down proteins that interact with either p1a, p2a or CP. Furthermore, putative orthologues for yeast protein previously identified to

be important for BMV replication in Barley are discussed. This analysis showed that 54% of the 28 analyzed yeast proteins have putative orthologues in Barley as determined by the criteria set in this study.

Introduction

The host proteome plays an important role in the infection cycle of pathogens, this is especially true for viruses. Because viruses have relatively smaller genomes they depend on host factors and proteins for a successful infection, from genome replication, packaging and cell-to-cell and systemic movement. Host proteins can play direct (protein-protein interactions with virally encoded proteins) or indirect role in the replication cycle. Identifying host proteins that are utilized by the virus for its replication can lead to development of novel and effective therapeutics against pathogens.

BMV is a plant pathogenic virus with single positive-sense RNA genome. The genome is divided into three segments each is packaged individually. BMV encodes four proteins: two replicase proteins p1a and p2a are encoded from RNA 1 and RNA 2 respectively. Movement protein is encoded from RNA3 which is disistronic and also encodes the coat proteins from sub-genomic RNA4. RNA4 is transcribed from the negative sense RNA3. P1a and p2a form the replicase complex which is critical for the replication of BMV RNA. Movement and coat proteins are important for the spread of BMV throughout the host.

The replication of BMV was extensively studied in the past, however, host proteins involvement in BMV replication was studied using a surrogate host, yeast system. For example, a study has shown the that a yeast general translation initiator factor, Ded1p, is important for the translation initiation of BMV p2a (Noueiry, Chen J Fau - Ahlquist et al. 2000). The yeast deadenylation-dependent decapping complex is important to switch from translating viral proteins to genome replication (Mas, Alves-Rodrigues et al. 2006). Yeast protein Ole1p plays an indirect role in BMV replication in yeast. A study has shown that unsaturated fatty acids made by the Δ9 fatty acid desaturase, Ole1p, is important for BMV RNA replication (Noueiry and Ahlquist 2003) among other proteins. Thus, I initiated a project to identify plant host proteins that interact with BMV encoded replicase proteins p1a and p2a and coat protein (CP).

Most of the studies done on yeast were not validated in plants. Additionally, there is no global proteome study that attempts to identify plant host proteins that could be involved in BMV infection. Thus, in this study I took a global approach to identify Barley proteome that interacts with BMV encoded replicase proteins and CP via Co-immunoprecipitation followed by MudPIT analysis to identify plant proteins that interact with BMV encoded replicase and coat proteins.

Materials and Methods

Plant infection and protein extraction

Approximately six-day old barley (*Hordium vulgare*) plants were mechanically inoculated with sap from BMV infected leaves. Symptomatic barley leaves were collected 10-15 days post-inoculations. Total proteins in BMV infected plants were isolated by grinding barley leaves with liquid nitrogen followed by the addition of three volumes of extraction buffer (20 mM Tris–Cl [pH 7.5], 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1% plant protease inhibitor). Resulting extracts from each sample were centrifuged at 12,000 rpm for 15 mins at 4°C, and the supernatants were collected and subsequently used for co-immunoprecipitation experiments.

Co-immunoprecipitation (Co-IP)

To identify the host proteome of Barley interacting with BMV encoded proteins, Co-IP was performed by adding 25µl of either anti- p1a or p2a or CP and 20µl of anti-rabbit agarose beads to every gram of infected leaf material. Similar co-immunoprecipitations performed with pre-immune serum served as a negative control. The mixture containing the leaf extract and the desired antibody was incubated at 4°C for 3 hours with gentle shaking. Then, the incubated mixture was centrifuged for 15 min at 4°C to collect the precipitate. The collected agarose beads were washed three times with extraction buffer, followed by a short spin at

4°C. Proteins were eluted from the agarose beads and subjected to Liquid chromatography tandem mass spectrometry (LC–MS/MS) at The Proteomics Center, University of California Riverside.

Bioinformatics analyses

Proteins recovered from the Co-IP assay were identified using NCBI BAST for barley plants. Subcellular localization of proteins interacting with BMV encoded proteins was identified using the WoLF-PSORT (Horton, Park et al. 2007). NCBI BLASTp was used to identify the host proteins of barley as putative orthologs of yeast proteins having a query coverage >= 70%, identity percentages >= 30%, and an e-value of 1e-4 or lower. Functional protein domains present in a desired set of proteins were predicted using NCBI and aligned using CLUSTAWL2.

Results

Host proteins recovered against anti-p1a, p2a and CP

The procedure used to recover host proteins from BMV infected barley following Co-IP with either replicase proteins p1a, p2a or CP or pre-immune serum used as a negative control is summarized in figure 2.1. After subtracting proteins precipitated with pre-immune serum, the following number of host proteins specifically precipitated for each virus-encoded protein: a total of 505, 387 and 434 host proteins were respectively precipitated with p1a, p2a and CP. A venn diagram representing the number of proteins commonly shared among all

the three p1a, p2a and CP is shown in Figure. 2.2. In Figure. 2.2, a total of 116 host proteins are specifically interacting with p1a, 43 are with p2a and 66 are with CP. A pair wise comparison revealed that 52 are shared between p1a and p2a, 31 between p2a and CP and 76 between p1a and CP. Finally, 261 host proteins are commonly shared among the three virus-encoded protein.

Host proteins recovered from each Co-IP sample were subjected to bioinformatic analysis and as summarized below they are classified based on either function or subcellular localization.

(a) Classification based on the function

Using the web bioinformatic tool INGA (Piovesan, Giollo et al. 2015), the functions of the co-immunoprecipitated proteins from BMV infected barley were predicted and grouped (Figure. 2.3 and Table 2.1). Among 28 predicted groups of proteins, four functional groups such as oxidoreductase, hydrolase, transferase and transport appear to be more prevalent.

(b) Classification based on subcellular localization

Viruses are known to replicate on specific location of the cell such as chloroplasts, endoplasmic reticulum (ER), mitochondria etc. Therefore, gaining some insight on the subcellular localization of the recovered proteins can shed light on the functional relationship between BMV replication and proteins associated with a specific subcellular site. Consequently, subcellular localization of recovered proteins was predicted using WoLF-PSORT (Horton, Park et al.

2007). Results are summarized in Figure. 2.4. It was observed that the majority of the recovered proteins are predicted to localize in the cytoplasm, chloroplast, and nucleus (Figure. 2.4) which confirms with previous observations from *N. benthamiana* (chapter 1 Figure 1.4).

Host proteins associated with ER

As mentioned above the ER is the preferred site of RNA replication for BMV by assembling active replicase complex involving p1a and p2a and host factors (Restrepo-Hartwig and Ahlquist 1996, Restrepo-Hartwig and Ahlquist 1999, Noueiry and Ahlquist 2003). Although p1a is involved in the induction of ER-derived spherules in a surrogate, non-host yeast system (Diaz, Wang et al. 2010), CP has been shown to induce ER-derived vesicles in plant hosts (Bamunusinghe, Chaturvedi et al. 2013). Therefore, we further analyzed host proteins predicted to localize in the ER.

Among 645 host proteins recovered from barley, 34 predicted to localized in the ER (Table 2.2). Among the 34 host proteins of Barley, 12 proteins are commonly shared among p1a, p2a and CP and the remaining proteins shared in pair-wise combination (e.g. p1a:p2a and p2a: CP etc.) ranged between 0-7 (Figure. 2.5). One protein from this list protein-disulfide isomerase-like 1-4 (Table 2.2). Protein-disulfide isomerase has a function in protein synthesis and was shown to interact with *Rice Yellow Mottle Virus* (RYMV) and is thought to aid the virus in translating its proteins as soon as uncoating occurs (Brizard, Carapito

et al. 2006). protein-disulfide isomerase-like 1-4 was also recovered from *N. benthamiana* (See chapter 1 Table 1.2). However, protein disulfide isomerease like 5-1 was shown to have a role in N-mediated resistance against TMV in *N. benthamiana* plants (Yang, Lüpken et al. 2014).

Plant vs. yeast

BMV replicate on ER membranes which is perinuclear in yeast but has a separate structure in plants. 64% out of the 28 yeast proteins that were identified to be involved in BMV replication (Carbonell, García et al. 2016) are nuclear, however only 13 have possible orthologs in Barley (Figure 2.6), out of which 5 proteins are predicted to be nuclear in Barley. Because the ER is in close proximity to the nucleus in yeast cells, it could be expected that some nuclear proteins are involved in BMV replication in these cells. However, because of the subcellular differences between the two cell types (i.e. plant vs. yeast), the nuclear proteins that are involved in BMV replication in yeast could be replaced by ER or cytoplasmic proteins that are in the vicinity where BMV replicate in plant cells.

Prior to this study, 28 host proteins were reported to be intimately involved in BMV replication in a yeast as a model, a non-host surrogate system (Carbonell, García et al. 2001) (Table 2.3). Out of the 28 yeast proteins, 6 were shown to interact with BMV p1a protein (Díez, Ishikawa et al. 2000, Diaz, Wang et al. 2010, Diaz, Zhang et al. 2015, Jungfleisch, Chowdhury et al. 2015). only

one protein, Snf7p, from barley was recovered by anti-p1a and p2a. Host protein Snf7p was classified as an orthologue of the yeast protein based on cut-off used for this study (Table 2.3). The fact that BMV replicates on plant ER (Bamunusinghe, Seo et al. 2011, Bamunusinghe, Chaturvedi et al. 2013) and the putative barley orthologue of yeast Snf7p was predicted to localizes in the nucleus not on ER (Table 2.3), the involvement of this protein in BMV replication in plants is questionable. Therefore, these results accentuate it is imperative to establish the functionality host proteins of yeast in plants system.

Out of the 28 yeast proteins examined in this study we found 8 of the proteins were studied and mutations were made in them; 6 out of the 8 proteins had conserved amino acids in Barley (Table 2.3).

Discussion

Subcellular localization of recovered Barley proteins

Although BMV is not known to replicate in either the chloroplast or nucleus, a relatively high number of host proteins related to these subcellular sites have been recovered in our Co-IP experiments (Figure. 2.4). Since RNA capping is nuclear event (Furuichi 2015), it is likely that proteins related to the nucleus might have an indirect role by transiently transporting the BMV RNA to the nucleus for capping.

The chloroplast play different roles in viral infection from involvement in viral infection, manifestation of symptoms and host defense (Zhao, Zhang et al.

2016). Thus, it is not surprising that proteins that are predicted to localize in the chloroplast comprise a large percentage of the recovered proteins (Figure 2.4). For example, the chloroplast aid in uncoating of Tombusvirus, and the chloroplast factor Tsip1 interacts with CMV's 1a and 2a to promote replication. Additionally alfamovirus AMV and TuMV among other viruses replicate in association with chloroplast membranes probably to escape the host anti-viral RNA silencing defense response (Zhao, Zhang et al. 2016). The coat protein of several viruses including *Tobacco Mosaic Virus*, CMV and *Potato Virus X* affect chloroplast ultrastructure and are involved in symptom development (Zhao, Zhang et al. 2016). The chloroplast is also involved in anti-viral defense; it is the site where Jasmonic and salicylic acids, plant hormones involved in plant defense, are synthesized. Jasmonic acid is involved in defense against viruses in susceptible hosts (Zhao, Zhang et al. 2016).

Plant vs. Yeast

BMV replicate on ER membranes which is perinuclear in yeast but has a separate structure in plants. 64% out of the 28 yeast proteins that were identified to be involved in BMV replication (Carbonell, García et al. 2016) are nuclear, however only 14 and 13 have possible orthologs in *N. benthamiana* and Barley, respectively, out of which 3 and 5 proteins are predicted to be nuclear in *N. benthamiana* and Barley, respectively. Because the ER is in close proximity to the nucleus in yeast cells it could be expected that some nuclear proteins are involved in BMV replication in these cells. However, because of the subcellular

differences between the two cells, the nuclear proteins that are involved in BMV replication in yeast could be replaced by ER or cytoplasmic proteins that are in the vicinity where BMV replicate in plant cells.

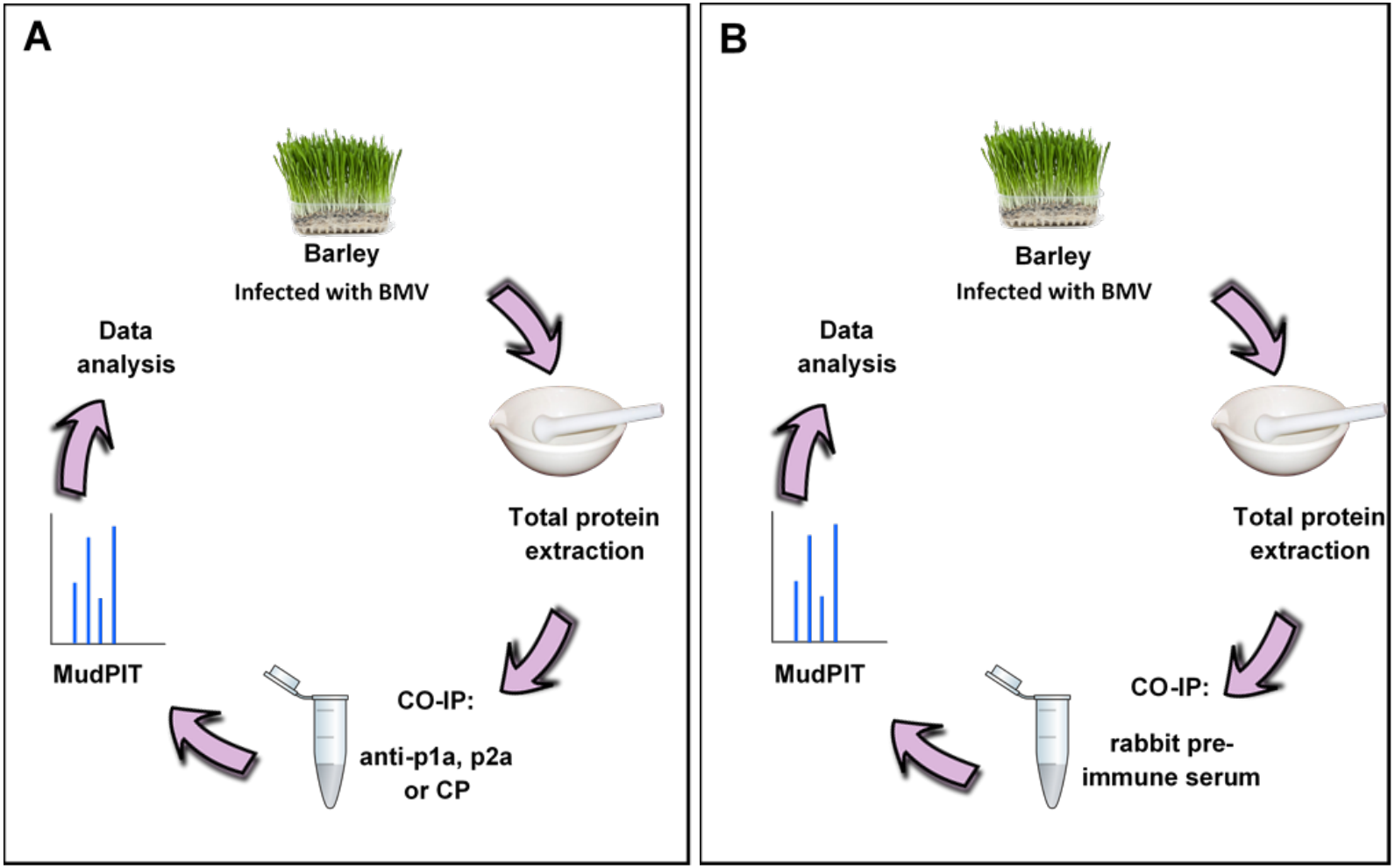
A
Barley
Infected with BMV
Total protein extraction
CO-IP:
anti-p1a, p2a or CP
MudPIT
Data analysis
B
Barley
Infected with BMV
Total protein extraction
CO-IP:
rabbit pre-immune serum
MudPIT
Data analysis

Figure 2.1 Experimental design to identify Barley proteins that interact with BMV coat protein and replicase proteins p1a and p2a (A) and using pre-immune serum for the negative control (B).

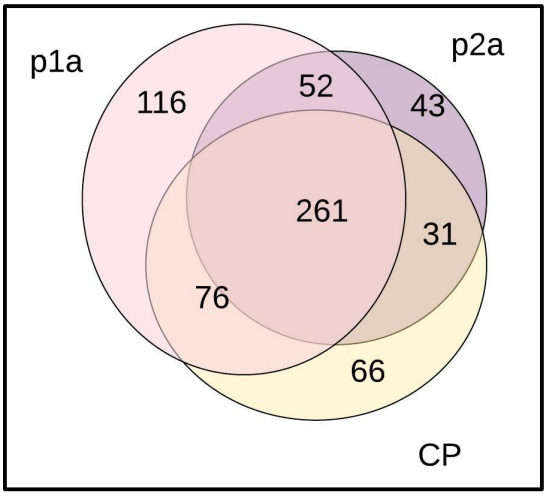

Figure 2.2 Venn diagram shows the number of plant host proteins that are recovered from the Co-IP assay done with Barley with each of the antibodies (antibodies against p1a, p2a or CP).

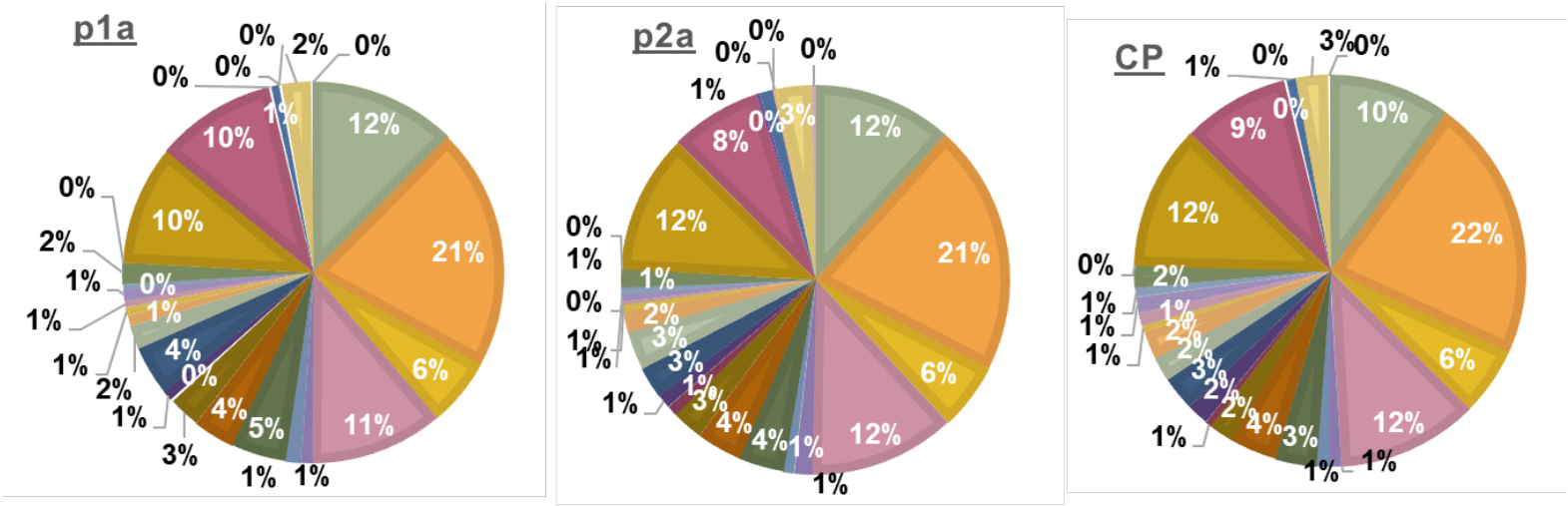
p1a
12%
21%
6%
11%
1%
1%
5%
4%
3%
1%
2%
1%
1%
4%
0%
1%
2%
0%
10%
10%
0%
0%
0%
2%
0%
p2a
12%
21%
6%
12%
1%
4%
4%
3%
3%
1%
3%
2%
1%
1%
0%
1%
0%
12%
8%
0%
3%
1%
0%
0%
0%
CP
10%
22%
6%
12%
1%
1%
3%
2%
4%
2%
2%
2%
3%
2%
1%
1%
1%
2%
12%
9%
0%
1%
0%
3%
0%
Oxidoreductase
Hydrolase
Transport
Transferase
Lyase
Isomerase
Protein binding
Nucleic acid binding
RNA binding
RNA processing
Biological process
Molecular function
Ligase
Transcription
Translation
Chaperon
Cytoskeletal
Ribosomal protein
Helicase
Protein containing complex
Binding
Kinase
Protein processing
Polymerase
DNA metabolic activity
Cellular component
Catalytic activity
modulation by virus of cellular process

Figure 2.3 Functional prediction of proteins recovered from Barley Co-IP assay performed using anti p1a, p2a or CP

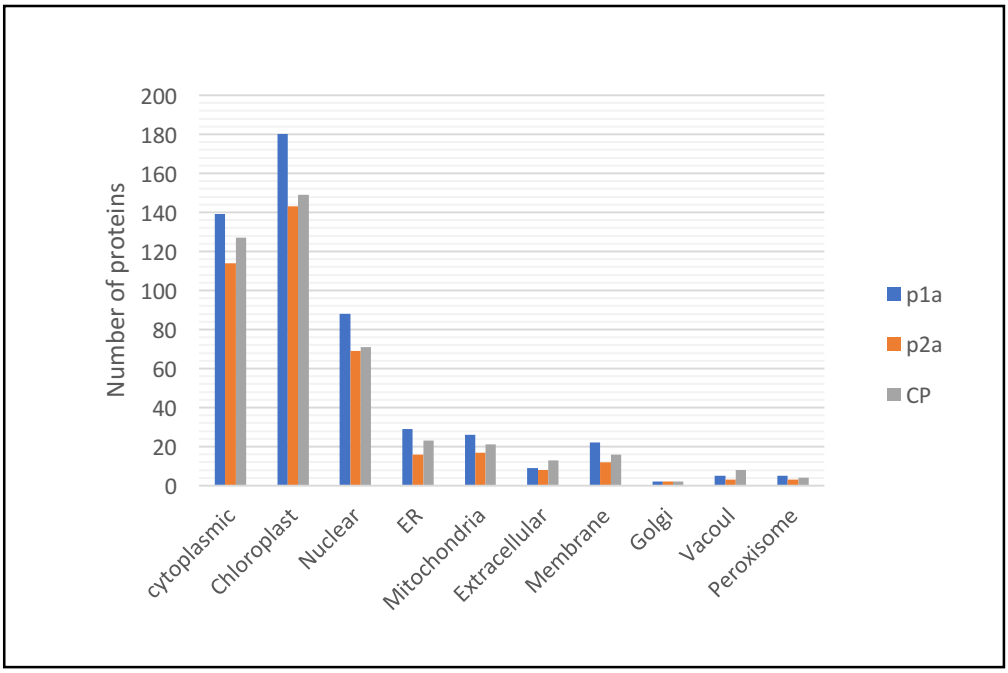

Figure 2.4 Subcellular localization of Barley proteins interacting with BMV encoded proteins p1a, p2a and CP predicted based on the amino acid sequences of the recovered proteins

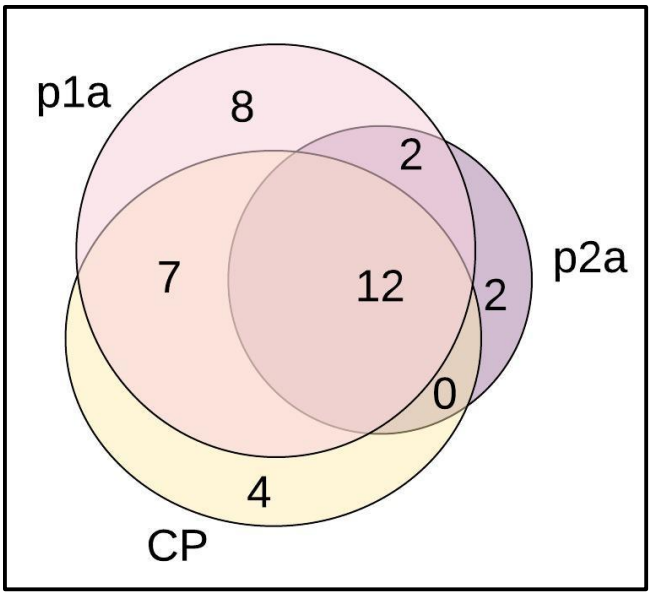

Figure 2.5 Venn diagram showing the number of recovered proteins from Barley that were predicted to localize in the ER, ER membrane or ER-cytoplasm. The venn diagram shows the number of proteins that interact with p1a, p2a or CP, or with more than one of the viral proteins.

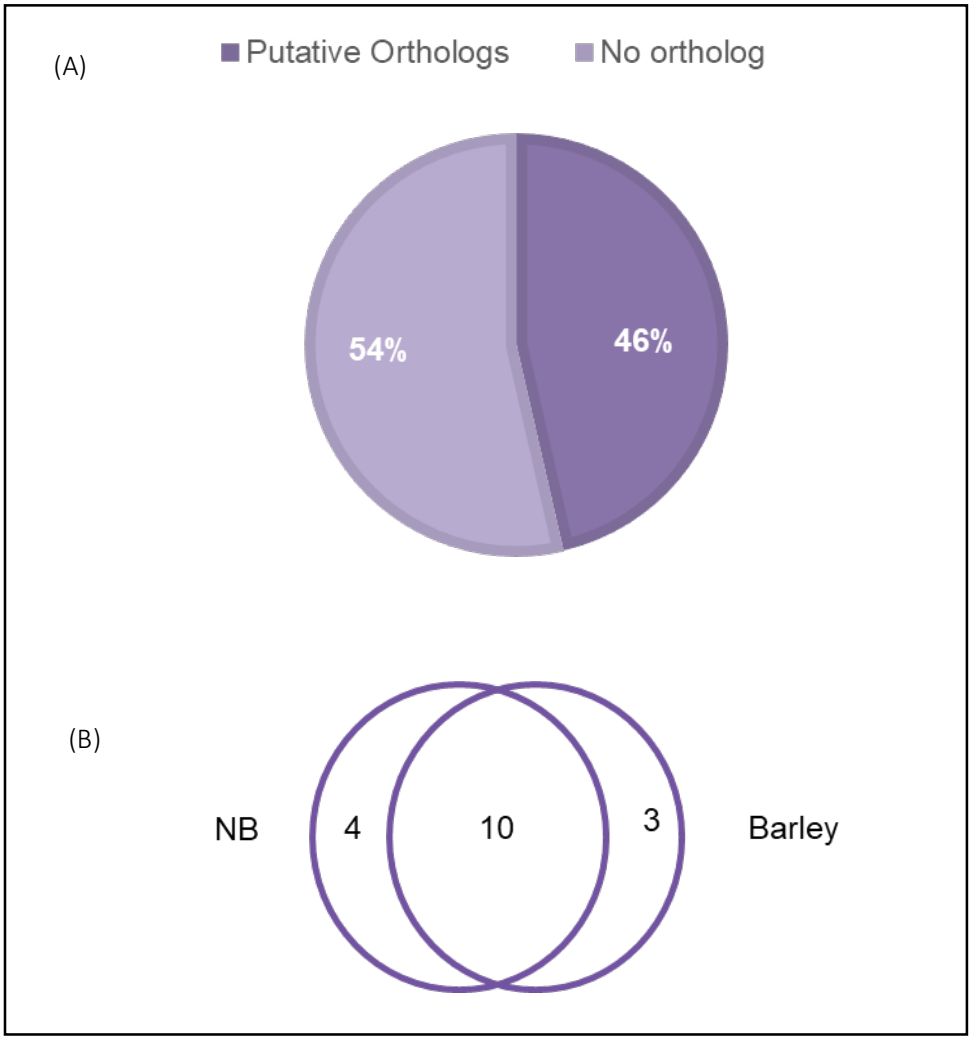

Figure 2.6 Yeast proteins shown to be important for BMV replication putative orthologues in plants. (A) Pie chart showing percentage of yeast proteins in barley. (B) Venn diagram showing number of yeast proteins that have putative orthologues in *N. benthamiana* (NB) and barley and the number of yeast proteins that have putative orthologues in both plants

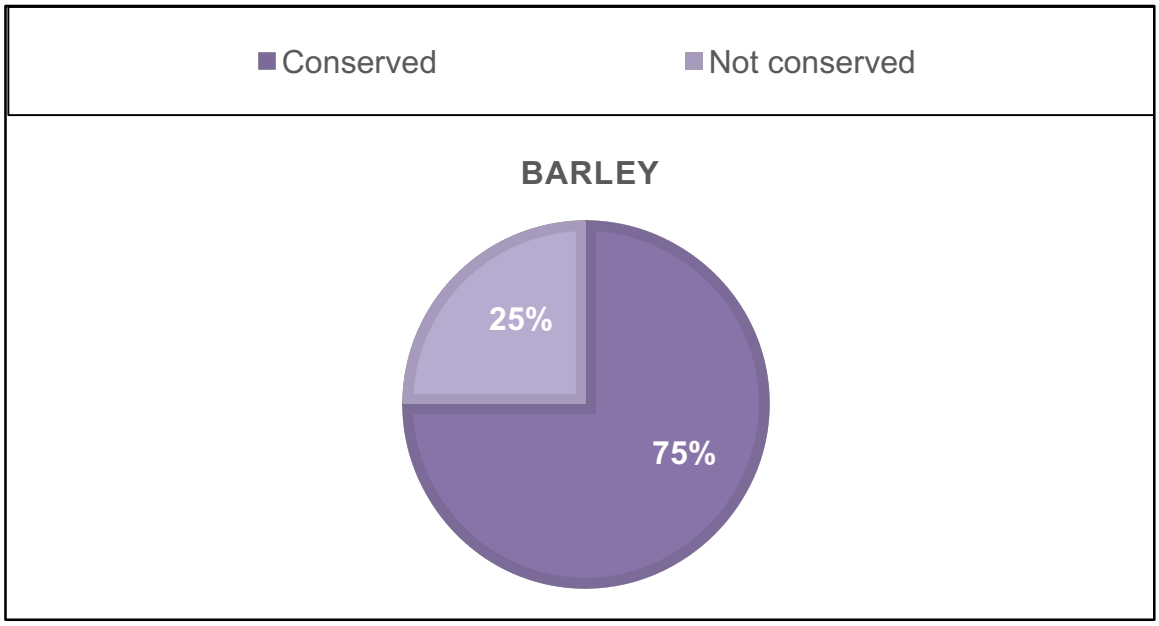

Figure 2.7 Pie chart showing percentage of functional amino acids that were shown in literature important for BMV replication in yeast that are conserved in Barley.

	Function Category	Example for: Barley
	Oxidoreductase	Thioreduxin-like 4
	Hydrolase	Vacoular ATPase subunit C
	Transport	Exocyst complex component 70B1
	Transfer	Vacuolar protein sorting-associated protein 42
	Lyase	Isopenicillin N epierase
	Isomerase	Protein disulfide isomerase-like
	Protein binding	protein transport protein Sec61 subunit alpha-like
	RNA binding	U3 small nuclear RNA-associated protein 21 homolog
	Ligase	E3 ubiquitin-protein ligase UPL3
	Transcription	CCR4-NOT transcription complex subunit 4
	Translation	Translation initiation factor eIF-2B subunit beta isoform X2
	Chaperon	Class II small heat shock protein Le-HSP17.8
	Cytoskeletal	Actin-2
	Ribosomal protein	30S ribosomal protein S1 homolog A-like isoform X5
	Helicase	DEAD-Box ATP dependent RNA helicase 40-like
	Kinase	MAP kinase

Table 2.1 Example proteins from each functional category that were recovered from barley

Protein ID	Protein Name	Interaction	Putative function
BAK01309.1	protein disulfide isomerase-like 1-4	P1a	Protein disulfide isomerase
BAJ99663.1	Pleiotropic drug resistance protein 4	CP	nucleoside-triphosphatase activity
BAJ84877.1	4-coumarate--CoA ligase-like 5	P1a	Fatty-acyl-CoA synthase
BAK07483.1	Reticulon-like protein B8	CP, p1a	protein complex binind
BAJ93964.1	Cysteine-rich receptor-like protein kinase 8	CP, p1a, p2a	protein kinsase
BAJ84938.1	triose phosphate translocator	P2a	carbohydrate derivative transporter
BAJ85388.1	U-box domain-containing protein 12	P1a, p2a	Ubiquitin-protein transferase
BAJ94616.1	lysine-specific histone demethylase 1 homolog 2	P1a	nucleic acid binding
BAV58149.1	cytochrome c oxidase subunit 2	P1a	Oxidoreductase activity
BAJ87595.1	riboflavin synthase	CP	Riboflavin synthase
BAJ88167.1	PREDICTED: cytochrome b561 and DOMON domain-containing protein At4g12980-like	p1a	positive regulator of cellular process
BAJ85974.1	PREDICTED: protein transport protein Sec61 subunit alpha-like	p1a	Ribonucleoprotein complex binding
BAK06468.1	Receptor-like protein kinase FERONIA	p1a	Protein kinase activity
BAJ92906.1	PREDICTED: probable envelope ADP,ATP carrier protein	p1a	Phosphate transmembrane transporter activity
BAJ96889.1	Calcium-transporting ATPase 1	p2a	calcium ion transmembrane transporter activity
AFP72240.1	sucrose: fructan 6-fructosyltransferase	CP	Hydrolase activity (alpha-glucosidase)
BAK03411.1	protein GPR107-like	CP	Positive regulator of molecular process
BAJ97945.1	CCR4-NOT transcription complex subunit 10	CP, p1a	deadenylation-dependent decay

BAJ88601.1	PREDICTED: splicing factor 3B subunit 1	Cp, p1a	Poly(A) RNA binding
BAK03651.1	UPF0261 protein	CP, p1a	molecular function
BAJ88105.1	D-xylose-proton symporter-like 2	CP, p1a	transmembrane transporter activity
BAJ88374.1	PREDICTED: probable signal peptidase complex subunit 2	CP, p1a	Hydrolase activity
BAJ96968.1	PREDICTED: DUF21 domain-containing protein At4g14240 isoform X2	CP, p1a	cellular manganese ion homeostasis
BAJ85829.1	uncharacterized	CP, p1a, p2a	lipase activity-phosphatidylcholine 1- acylhdrolase activity
BAK03385.1	PREDICTED: exportin-2	CP, p1a, p2a	importin-alpha export receptor activity
BAJ95494.1	PREDICTED: protein transport protein sec24	CP, p1a, p2a	ER to Golgi transport vesicle membrane
BAK01248.1	hypothetical protein TRIUR3_18907	CP, p1a, p2a	tubulin binding
BAK00448.1	PREDICTED: protein TIC 55, chloroplastic	CP, p1a, p2a	oxidoreducate activity
BAJ93231.1	PREDICTED: oxalate--CoA ligase	CP, p1a, p2a	acid-thiol ligase activity
BAK03852.1	PREDICTED: protein NRT1	CP, p1a, p2a	dipeptide transporter activity
BAJ89995.1	Aspartic proteinase nepenthesin-2	CP, p1a, p2a	endopeptidase activity
BAJ92029.1	PREDICTED: PRA1 family protein E-like	CP, p1a, p2a	Ras GTPase binding
BAJ93024.1	protein transport protein SFT2	CP, p1a, p2a	signal transducer activity
BAJ91083.1	PREDICTED: aspartic proteinase-like protein 1	CP, p1a, p2a	endopeptidase activity

Table 2.2 List of proteins predicted to localize in ER recovered from BMV infected Barley showing which viral proteins they interact with and predicted function, names of the proteins were inferred by similarity.

Yeast		Barley					
Protein	Subcellular localization	Orthologue	IP [A]	Query cover % [B]	Identity % [C]	Functional amino acids[D]	Subcellular localization
ACB1	Nuclear	No	No	69	39	Yes	ER
BRO1	Nuclear	No	No	10	34	N/A	Nuclear
DOA4	Nuclear	No	No	38	40	No	Chloroplast
DED1	Nuclear	Yes	No	91	50	Yes	Nuclear
DBP2	Nuclear	Yes	No	94	47	N/A	Nuclear
VPS23	Nuclear	No	No	87	25	N/A	Cytoplasmic
VPS20	Nuclear	Yes	No	77	31	Yes	Cytoplasmic
SNF7	Nuclear	Yes	1a -2a	99	31	Yes	Nuclear
VPS24	Nuclear	No	No	63	35	N/A	Cytoplasmic
VPS2	Nuclear	yes	No	96	36	N/A	Mitochondria
VPS4	Cytoplasmic	Yes	No	98	54	N/A	Nuclear
HSF1	Nuclear	No	No	13	44	N/A	Nuclear
LSM1	Nuclear	No	No	50	44	4 out of 13	Cytoplasmic
LSM2	Nuclear	Yes	No	93	64	N/A	Cytoplasmic
LSM3	Nuclear	yes	No	87	42	N/A	Cytoplasmic
LSM4	Nuclear	No	No	55	39	N/A	Cytoplasmic
LSM5	Cytoskeletal	no	No	41	41	N/A	Cytoplasmic
LSM6	Cytoplasmic	N/A	No	N/A	N/A	N/A	N/A
LSM7	Nuclear	No	No	67	40	N/A	Cytoplasmic
PAT1	Mitochondria	No	No	9	26	N/A	Vacuole
DHH1	Nuclear	Yes	No	80	71	5 out of 5	Nuclear
OLE1	Plasma membrane	yes	No	75	46	No	ER
PRE1	Cytoplasmic	No	No	36	27	N/A	Nuclear
RPT6	Cytoskeletal	Yes	No	95	75	N/A	Cytoplasmic

RTN1	Plasma membrane	no	No	55	24	N/A	ER
RTN2	Nuclear	no	No	38	24	N/A	Plasma membrane
YOP1	Plasma membrane	yes	No	79	38	N/A	Chloroplast
YDJ1	Cytoplasmic	Yes	No	98	44	N/A	Nuclear

Table 2.3 Barley putative orthologs for yeast proteins shown in literature important for BMV replication (Carbonell et al., 2016). A: this column shows list of BMV proteins that interact with the putative ortholog and was recovered from the BMV infected plant host, no indicates that the putative ortholog was not recovered from BMV infected plant host. Cut-off used to identify the putative orthologs area as follows: B: query coverage 70%, b: identity 30%. D: this column shows if the amino acids that were shown in the literature to be important for BMV replication cycle or the protein function that is important for BMV replication are conserved between the yeast, Barley proteins, NA: no literature was found on protein mutations, numbers indicates how many of studied amino acids are conserved

Chapter 3

The effect of S-phase kinase related protein 1 (SKP1) silencing on BMV accumulation in locally and systemically infected leaves

Abstract

RNA silencing is utilized by plants to defend against invading viruses. Dicer-like proteins recognize the double stranded RNAs formed during viral replication and cuts it into short fragments, one strand is incorporated into the RISC complex which targets the complimentary RNA for silencing. The RISC complex includes one protein from the argonaute family which has the slicing activity. To evade this immune response, viruses encode silencing suppressors that suppress the silencing pathway via different mechanisms. Poleroviruses encode the silencing suppressor P0 which targets argonaute proteins for degradation by interacting with SKP1. In this study, SKP1 was identified to interact with *Brome Mosaic Virus* (BMV) replicase proteins p1a and p2a. Silencing of SKP1 in *Nicotiana benthamiana* plants and further infected with BMV resulted in significant reduction in BMV accumulation in silenced plants compared to unsilenced plants.

Introduction

RNA silencing is a pathway used by plants to defend against viral infection among other functions like gene expression regulation and protection against transposons (Baulcombe 2004). RNA silencing is initiated by the recognition of double-stranded RNA (dsRNA) molecules by proteins that have RNase III

domains, Dicer-like protein (DCL) in plants. DCL proteins cut the dsRNA into short RNA fragments ranging in size between 21 to 26 nucleotides called small interfering RNAs (siRNA) or primary siRNA (Baulcombe 2004). These siRNAs are then amplified to produce more siRNAs which are called secondary siRNA. DCL2 and DCL4 are both thought to be important in anti-viral RNA silencing host defense response (Mallory and Vaucheret 2010) (Parent, Bouteiller et al. 2015). However, DCL2 is involved in primary siRNA synthesis while DCL4 is believed to synthesize secondary siRNAs (Parent, Bouteiller et al. 2015).

Another group of proteins that are involved in the silencing pathway is the Argonaute family. Argonaute proteins are part of the RISC complex (RNA-induced silencing complex) which bind to siRNA via the PAZ domain (piwi-argonaute-zwille) and has a ribonuclease function and acts as the slicer to cut the targeted RNA (Baulcombe 2004) (Zamore and Haley 2005). In Arabidopsis there are ten argonaute proteins. Arogonautes 1, 2, 5 and 7 were shown to be involved in antiviral defense response, AGO2 and AGO5 were shown to incorporate siRNAs of Cucumber Mosaic Virus (CMV) (Csorba, Lozsa et al. 2010).

Viruses can evade this immune response via several mechanisms: using their virally encoded silencing suppressing proteins, by having RNA secondary structures or the virus replicate inside inside cellular compartments to protect its RNA from detection by the host (Baulcombe 2004). The viral suppressor proteins

act by different mechanisms to suppress host silencing defense response; for example: the *Tomato bushy stunt virus* protein p19 and *Beet western yellow virus* p21 protein bind to the siRNAs preventing them from targeting the RISC complex to the corresponding viral RNA (Baulcombe 2004). Another mechanism utilized by CMV strain Fny silencing suppressing protein 2b is via protein-protein interaction between the 2b protein and AGO1 via the PAZ domain.

The *Beet Western Yellows Polerovirus* (BWYV) P0 silencing suppressor protein acts as an F-box protein which interacts with S-phase kinase-related protein-1 that is part of the E3 ubiquitin ligase complex, and directs the degradation of AGO1 (Bortolamiol, Pazhouhandeh et al. 2007) (Pazhouhandeh, Dieterle et al. 2006). Other research showed that the degradation of AGO1 is independent of proteasome-mediated protein degradation (Baumberger, Tsai et al. 2007). BWYV P0 protein acts on free AGO1 before their incorporation into RISC complexes (Csorba, Lozsa et al. 2010).

Materials and Methods

SKP1 silencing, virus infection and Agrobacterium infiltration

Virus Induced Gene Silencing (VIGS) was used to silence the expression of SKP1 using a *Tobacco rattle virus* (TRV) based binary vector. TRV2-NbSKP1 was provided by Dr. Dinesh Kumar (UC Davis), TRV1 was kindly provided from Dr. Patrisha Manosalva (UC Riverside). 3 weeks old *Nicotiana benthamiana* plants were infiltrated with 0.5 OD inoculum of Agrobacterium carrying the TRV

constructs TRV2-NbSKP1 and TRV1 into lower leaves. As a negative control an empty TRV2 vector was co-infiltrated with TRV1. Fourteen days post infiltration (dpi) upper leaves were mechanically infected with 15 ug of BMV virions. Four days post BMV infection locally infected leaves were collected. Fifteen days post BMV infection systemic leaves were collected. Leaves were kept in -80 ^{o}C until processed. BMV virions were extracted from BMV infected Barley leaves and kept at 4 ^{o}C.

Total protein extraction and western blotting

Total protein was isolated by grinding NB leaves in liquid nitrogen followed by the addition of three volumes of extraction buffer (20 mM Tris–Cl [pH 7.5], 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1% plant protease inhibitor). Resulting extracts from each sample were centrifuged at 12,000 rpm for 15 mins at 4°C and the supernatants were collected and subsequently used for western blotting and co-immunoprecipitation. Western blotting was performed to verify the replication of BMV in control and test plants.

Co-immunoprecipitation (Co-IP)

To identify the host proteome of *N. benthamiana* interacting with BMV encoded proteins, Co-IP was performed by adding 25µL of either anti-p1a or p2a and 20µL of anti-rabbit agarose beads to every gram of infected leaf material. Similar co-immunoprecipitations performed with pre-immune serum served as a negative control. The mixture containing the leaf extract and the desired antibody

was incubated at 4°C for 3 hours with gentle shaking. Then, the incubated mixture was centrifuged for 15 min at 4°C to collect the precipitate. The collected agarose beads were washed three times with extraction buffer, followed by a short spin at 4°C. Proteins were eluted from the agarose beads and subjected to Liquid chromatography tandem mass spectrometry (LC–MS/MS) at The Proteomics Center, University of California Riverside.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted using TRIzol reagent from frozen leaves. Briefly, about half a leaf was ground in liquid nitrogen in cold mortar and pistol. 1.5 ml TRIzol is added to the powdered leaf and continue grinding. Leaf debris is collected by centrifuging at 12000 Xg for 15 mins at room temperature. 500 µL of chloroform is then added to about 600 ul of the supernatant then mix by shaking for one min. The mixture is incubated at room temperature for five mins then centrifuge as before then add 500 ul of isopropanol to the supernatant and incubate for ten mins. Centrifuge at 4 ^{o}C for 30 mins then wash the pellet with 70% ethanol and dry the pellet and resuspend in RNAse free water. iScript cDNA synthesis kit from Biorad was used to synthesize cDNA which was used for PCR using Phusion High-fidelity DNA polymerase (NEB).

Results

Co-immunoprecipitation, silencing and BMV accumulation

Among the proteins that were recovered with BMV p1a and p2a proteins is SKP1 (S-phase kinase associated protein-1). Upon silencing transcript levels of SKP1 in the silenced plants was reduced compared to the empty vector infiltrated control plants, however, housekeeping gene elongation factor 1-alpha (ef1-alpha) transcript levels were similar (Figure 3.1). BMV accumulation was reduced in *SKP1*-silenced plants compared to the control which corelated with the reduced *SKP1* transcripts reduction (Figure 3.2).

Discussion

The ubiquitin ligase complex is utilized by many viruses. A component of the SCF ubiquitin-protein ligase complex, Cdc34p was shown to co-purify with tombusvirus replicase complex (Xu and Nagy 2010). Silencing NbSKP1 in transgenic *N. benthamiana* plants carrying the tobacco N gene, which confers resistance against TMV, resulted in the plants to lose resistance against TMV and was suggested that NbSKP1 gene is important for plant defense (Liu, Schiff et al. 2002).

Argonaut proteins are part of the silencing pathway utilized by hosts to defend against invading viruses (Baulcombe 2004). During potexvirus infection AGO5 expression is induced (Brosseau and Moffett 2015) and it is known that AGO5 is involved in antiviral defense response (Csorba, Lozsa et al. 2010).

Additionally, It was suggested that AGO2 is important for restricting PVX from moving systemically and AGO5 is involved in anti-viral response against PVX in systemic leaves (Brosseau and Moffett 2015). Thus, it makes sense that viral silencing suppressing proteins would target this group of host proteins as a counter mechanism to suppress the host immune response. A study have shown that Polerovirus P0 targets ER associated AGO1 and gets incorporated into ER derived bodies with AGO1 and are delivered to the vacuole (Michaeli, Clavel et al. 2019) and cause the degradation of ER bound AGO1 in an autophagy pathway manner (Derrien, Baumberger et al. 2012) (Michaeli, Clavel et al. 2019). AGO1 coprecipitated with P0 and CUL1 (component of the SCF ubiquitin-protein ligase complex) and ubiquitylation by the complex is required for degradation of AGO1 (Derrien, Baumberger et al. 2012). The next step in this project should look into what host proteins are being degraded by the virus via and interaction with SKP1. The best starting point based on information given above would be argonuate proteins.

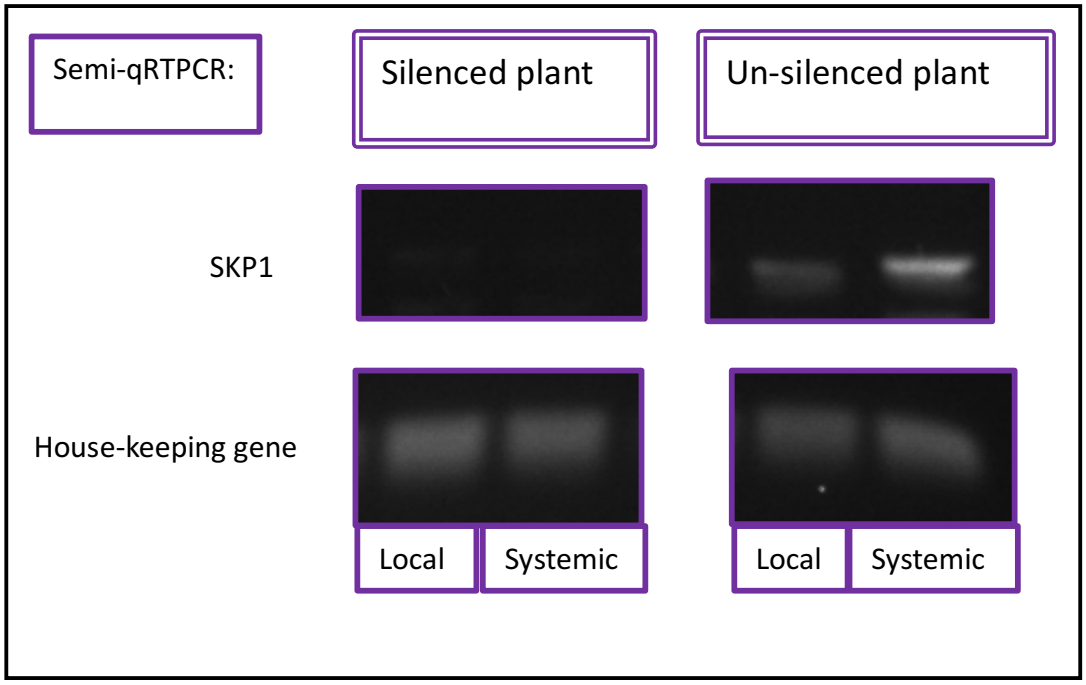

Figure 3.1. Semi-quantitative RT-PCR to quantify SKP1 and housekeeping gene transcript levels in silenced plants where SKP1 was silenced or un-silenced where the plants were infiltrated with empty TRV2 vector.

Figure 3.2. Western blot done with total protein extracted from plants where SKP1 was silenced or un-silenced where the plants were infiltrated with empty TRV2 vector. Western blot was done with anti-CP to assay BMV accumulation in locally and systemically infected leaves. Healthy leaf is wild-type plant not infected with BMV.

DISSERTATION CONCLUSIONS

In this study, I identified plant proteins that interact specifically with BMV replicase proteins p1a and p2a and coat protein with the goal of identifying candidate proteins that would potentially be involved in BMV replication. After subcellular localization and function prediction I found that among the identified proteins some functional categories, such as hydrolase, transferase are more abundant than others in both BMV infected *N. benthamiana* and Barley. Because BMV replication is associated with the ER, it would be interesting to test the functionality of proteins that precipitated with anti-p1a and p2a and localize on the ER such as Bip5. In addition, it would interesting to test the functionality of the host proteins that specifically precipitated with anti-CP such as COPI since CP has been shown to be multifunctional and involved in modifying the ER (Bamunusinghe, Chaturvedi et al. 2013), modulating the strand asymmetry (Marsh, Huntley et al. 1991) and promote cell-to-cell movement (Bol 2008) and symptom expression (Zhao, Zhang et al. 2016). Silencing of SKP1, a component of SCF protein-ligase complex, caused the reduction of BMV accumulation in plants after infection with BMV.

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