RECEPTOR LIKE KINASE ACTIVITY MODULATES VIRAL INFECTION THROUGH PHOSPHORYLATION OF A CHLOROPLAST PROTEIN

by

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This thesis is dedicated to my family for their support in my pursuit of the PhD degree.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

An increasing number of chloroplast proteins have been found to interact with plant virus proteins. This is not surprising because these viruses cause various mosaic, mottles, and chlorosis symptoms on host leaves indicating damage to chloroplasts. A chloroplast protein, AtPsbP, was identified in a yeast two-hybrid screen as interacting with *Alfalfa mosaic virus* (AMV) coat protein (CP). AMV is a ssRNA virus with a wide host range including Arabidopsis. AtPsbP is an extrinsic subunit of photosystem II and with PsbQ is vital for water oxidation. We found that an RNAi knock-down of PsbP in *Nicotiana tabacum*, allowed increased replication of AMV and the development of quite severe disease symptoms in comparison to a wild-type *N. tabacum*. This suggested that PsbP plays an important role in plant resistance to AMV. PsbP, in addition to its role in photosynthesis, has been reported to interact with a wall-associated receptor kinase, WAK1, whereby it may affect plant defense signaling. We found that AtPsbP is a link between AtWAK1 and AMV CP at the plasma membrane. The formation of the AtWAK1-AtPsbP-AMV CP complex activated WAK1 kinase activity causing phosphorylation of PsbP and significant inhibition of AMV replication. We also found that the formation of the ternary complex induced the activation of the MAPK signal pathway. Analysis of the susceptibility of an Arabidopsis WAK1 knock-down indicated that WAK1, like PsbP, is critical for inhibiting AMV replication. Overall, we found a unique virus perception strategy, whereby a chloroplast protein (PsbP) interacts with a virus protein and then a Receptor-like kinase protein (WAK1) to transduce signals through the MAPK signaling pathway to activate defense responses.

CHAPTER 1. LITERATURE REVIEW

1.1 Plant immune response

According to current description of plant innate immune response, there are two forms of plant immune responses adopted by plants against pathogens. First, plants use pattern recognition receptors (PRRs) to perceive conserved pathogen associate-molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), such as chitin for fungi and peptidoglycan for bacteria. Once P/MAMPs are recognized by plants PRRs, a series of resistant responses will be induced, and this is defined as PAMP-triggered immunity (PTI) (J. D. G. Jones & Dangl, 2006; Schwessinger & Ronald, 2012). The second form is so-called effector-triggered immunity (ETI). In order to evade or suppress the PTI response and establish successful colonization in host plants, many pathogens deliver specific effector proteins (Avr) into plant cells to interrupt the plant's defense system. In order to cope with microbe encoded effectors, plants have evolved specific surveillance systems that encode resistance proteins (R proteins). R proteins interact with effectors directly or indirectly and then trigger ETI (Bent & Mackey, 2007; Schwessinger & Ronald, 2012).

In order to incorporate terms for virus-host interaction into the general pathogen-host terminology, Schlothof and Mandadi broadened the definition of PTI and ETI (Mandadi & Scholthof, 2013). They defined antiviral PTI as resistant response triggered by conserved viral molecular features which can be perceive by membrane-bounded receptors. ETI is defined as plant immune response triggered by virus encoded proteins which interact with host R proteins within plant cells.

1.2 PTI

Traditional nonviral pathogen-induced PTI would use pattern recognition receptors (PRRs) to recognize the PAMPs, such bacterial flagellin or fungal chitin, with their extracellular domain. A lot of Receptor-like kinases (RLKs) have been identified playing prominent roles in plant-pathogen interaction (Teixeira et al., 2019). A well-established model is flagellin-sensitive 2 (FLS2) interacts with BRI1-associated receptor kinase 1 (BAK1) to sense the presence of flg22, and then induce the oxidative burst and trigger the plant's PTI. The *bak1* mutant has decreased sensitivity to flagellin (Chinchilla et al., 2007). Unlike with bacteria and fungus, viruses are obligate

intracellular parasites, which means they have to interact with the intracellular domain of the PRRs to activate PTI. Due to the limitation of the viral genome size, it's challenging to find what kinds of molecules could act as viral PAMP. Recent studies shown that double-stranded RNA (dsRNA) could be the potential viral PAMP. dsRNA is an intermediate during virus replication, which is not abundant in plants host (Teixeira et al., 2019). Niehl et al. found that dsRNA *Oilseed rape mosaic virus* (ORMV)-infected plants can induce the typical PTI response, including the MPK3 and MPK6 accumulation and several PTI-related response gene expression (Niehl, Wyrsch, Boller, & Heinlein, 2016). Besides, they also noticed that this PTI response is depend on the Somatic Embryogenesis Receptor-Like (SERK1) pathway, but independent of BAK1 and RNAi pathway. Other evidence was obtained that viruses infection can trigger PTI responses, even though the PAMPs have not been determined specifically (Macho & Lozano-Duran, 2019; Teixeira et al., 2019). The *bak1* mutants have increased susceptibility to *Turnip crinkle virus* (TCV), ORMV and TMV (Kørner et al., 2013). Liu and colleagues also found that *mpk4* knock-down mutant significantly increased plants resistance against *Soybean mosaic virus* (SMV) due to the activation of PTI (J.-Z. Liu et al., 2011).

1.3 ETI

Due to the broad-spectrum resistance of PTI, pathogens introduce effectors into the host to avoid the recognition by PRRs. Meanwhile, plants also evolved the ETI pathway to inhibit pathogens colonization in themselves. The avirulence (Avr) proteins secreted by pathogens would be recognized by different types of host encoded R protein to trigger a series of defense responses, including a programmed cell death, termed as hypersensitive response (HR), and it's usually associated with production of reactive oxygen species to induce oxidative burst (Gouveia et al., 2017). Besides, ETI can also induce the production of several phytohormones, such as salicylic acid, jasmonic acid and ethylene, as well as the expression of defense response genes, such as *PR-1* (Memelink, 2009; Shah, 2003). Due to the limitation of genome size, viruses cannot behave like bacteria or fungus to introduce the Avr protein into host directly, and they have to take use of vectors or mechanical damage of host to enter plants, and all the viral-encoded proteins, such as the coat proteins (CP), movement proteins (MP) and replicase, can act as effector proteins. Takahashi and colleagues found that the *RCY1*-encoded protein interacts with cucumber mosaic virus (CMV) CP to induce the expression of defense-related genes and inhibit the CMV caused

symptom development (Takahashi, 2006, 2008). The Rubisco small subunit (RbCS) of *Nicotiana benthamiana* is important for the resistance against Tomato mosaic virus (ToMV) (J. Zhao et al., 2013). RbCS interacts with ToMV MP to inhibit the systematic movement of ToMV, and the RbCS over-expressed plants carry extreme resistance against ToMV.

1.4 Resistant gene-mediate resistance

Over several decades, lots of resistant genes have been identified, and most specifically target to one virus or several highly related viruses. In the gene for gene model, a majority of resistant gene are function as single dominant gene-mediate resistant. Besides, most of dominant resistant genes belong to Nucleotide Binding Site- Leucine Rich Repeat (NBS-LRR) family. Based on the N-terminal domains, NB-LRR proteins could be classified into two subclasses: TOLL/interleukin-1 receptor (TIR)-NB-LRR and coiled- coil (CC)-NB-LRR (Collier & Moffett, 2009). The C-terminal domain, LRR domain, determines proteins' specificity, thus different NB-LRR proteins could recognize different pathogen Avr proteins. The N-terminal domain is responsible for interact with Avr proteins or their cofactors. NB domain is required for ATP binding and has activity to hydrolyze ATP, and also signal initiation is thought to originate from NB domain (Collier & Moffett, 2009; Ronde, Butterbach, & Kormelink, 2014). Once NB-LRR proteins recognize pathogen Avr protein, downstream resistant response would be elicited, such as HR. *N* gene in tobacco is the first R gene found that has antiviral activity, and it encodes a TIR-NB-LRR protein which recognize p50 helicase of TMV and initiate HR to inhibit spread of virus (Baker, 1999, 2000). Most of the reported resistant proteins interact with virus-encoded proteins through indirect interaction, although several exceptions do exist, such as N protein against TMV infection. N protein has ATPase activity, once TMV infect tobacco plants, N protein and ATP form a complex, and then N binds to TMV helicase directly to hydrolyze ATP to activate downstream resistant responses (Ueda, Yamaguchi, & Sano, 2006). For indirectly interaction between host factors and virus-encoded proteins, the most prevalent model is "guard hypothesis" (Ronde et al., 2014). In this model, resistant protein interacts with a specific host protein, guardee, and guardee retains resistant proteins in inactive state. When guardee perceives and interacts with Avr proteins from viruses, the conformation of resistant protein-guardee complex would be changed to active state to elicit resistant response against virus infection. A typical example of this model is Rx protein mediate-resistant against *Potato virus X* (PVX) (Tameling & Baulcombe, 2007). GTPASE-

ACTIVATING PROTEIN (RanGAP2) acts as guardee for Rx protein and could recognize CP of PVX and bind to CP directly, and then induce Rx-mediate resistant response.

A few cases proved that not all resistant genes belong to NB-LRR proteins which highlight that virus-host interactions could have more mechanisms that need to be characterized. Based on the amino acid sequence, several non-NB-LRR proteins identified are lectin like proteins. In plants, most lectins have activity to bind to foreign mono- or oligosaccharides under biotic or abiotic stresses, thus functions of these lectin-like resistant proteins are consider to recognize pathogens and induce host resistant response (Peumans, Damme, Barre, & Rouge, 2004). Yamaji et al. found JAX1 confer broad resistance to Potexviruses, and inhibit their infection at very early stage (Yamaji et al., 2012). Different with NB-LRR mediate resistance, JAX1-mediated resistance is adopting totally different pathways. No HR could be elicited in JAX1-mediated resistance, and they are plant hormones independent. Another example of lectin-like protein-mediate resistance is RTM proteins in Arabidopsis (Cosson et al., 2012). RTM1, RTM2 and RTM3 function together to restrict potyviruses long distance movement. A single mutation of one of RTM proteins could compromise the resistance against potyviruses. Similar to JAX1-mediate resistance, no HR could be induced. However, the resistant response mechanism of lectin-like proteins is remaining unclear.

In addition to dominant gene resistance, several recessive gene-mediated resistances have also been discovered. The most prevalent hypothesis for recessive resistance is: recessive resistance is the consequence of the mutation or loss of a host factor specifically required for the success of the infection process (Revers & Nicaise, 2014). Most of the information about recessive resistance comes from interaction between potyviruses and their host plants (A. Wang & Krishnaswamy, 2012). The function of the eukaryotic translation initiation factor 4E/4G (eIF4E/eIF4G) is to recruit ribosomes to the 5' caps of messenger RNAs (mRNA) and initiate translation of mRNAs. In order to complete life cycle, transcripts of potyviruses need to interact with eIF4E/eIF4G to be translated into virus-encoded proteins. Suppressing the activity of eIF4E/eIF4G could impart host plants with antiviral immunity (Kang, Yeam, & Jahn, 2005; A. Wang & Krishnaswamy, 2012). Only a few recessive resistance genes, which do not encode translation initiation factors, have been identified. One example is TOM1 and TOM2A in Arabidopsis, and they encode transmembrane proteins. Both TOM1 and TOM2A are required to activate the activity of replicase of TMV and ToMV, and help viruses to finish replication. Knockdown mutants of TOM1 and TOM2A significantly impact accumulation of tobamoviruses in Arabidopsis (Ishibashi, Miyashita, Katoh, & Ishikawa, 2012).

Nils Stein et al. found a protein disulfide isomerase (PDIs), PDIL5-1, confers recessive resistance against bymoviruses (P. Yang et al., 2014). PDIs act as protein chaperones in animals and catalyze the correct folding of proteins (P. Yang et al., 2014). The PDIs' antiviral immunity have been found for animal viruses, including suppression of the accumulation of murine polyomavirus and inhibition of HIV envelope protein-mediated cell-fusion and infectivity (Ou & Silver, 2006; Walczak & Tsai, 2011). Taken together, more recessive resistant genes should exist in naturally resistant plants, not limited to translation factors, and more research work should be conduct to find them.

1.5 RNA interference (RNAi) pathway in antiviral system

RNA silencing pathway or RNA interference (RNAi) is another important plants' antiviral strategy to protect them from virus invasion (Carbonell & Carrington, 2015; Pumplin & Voinnet, 2013). In the RNAi pathway, virus RNA functions as both inducer and target. During replication process, double-strand RNA (dsRNA) would be formed, and dsRNA would be cleaved into small interfering RNA (siRNA) by Dicer-like enzymes (DCLs). The siRNA will incorporate with Argonauts (AGOs) to form an RNA-induced silencing complex (RISC). RISC could then target and degrade virus RNAs to protect host plants. Due to a diversity of AGOs, RNA- dependent RNA polymerase (RDR) and DCLs, plants could defend against different viruses.

Arabidopsis has ten AGOs members, and AGO1 and AGO2 are the two major components involved in the RNAi pathway. Wang et al. noticed that AGO1 and AGO2 function cooperatively in plant defense against *cucumber mosaic virus* (CMV) infection in Arabidopsis, and mutation of the other AGOs would not severe virus-induced symptoms than *ago1* and *ago2* single or double mutant (X. Wang et al., 2011). Carrington groups also observed that AGO1 and AGO2 are two major proteins to form RISCs by binding siRNA and target virus genomic RNA to induce degradation, when Arabidopsis was infected by Turnip Mosaic Virus (TuMV) (Garcia-ruiz, Carbonell, Hoyer, & Fahlgren, 2015).

In order to generate siRNA which incorporate with AGOs and further restrict virus replication, plants need to use different DCLs and RDRs to produce siRNA pools (Calil & Fontes, 2016; Revers & Nicaise, 2014). DCL4 is the main contributor to RNAi pathway, and use dsRNA as template to catalyze formation of 21-nucleotide siRNA (Yoshikawa, Peragine, Park, & Poethig, 2005). Besides, plants also need RDRs to amplify the production of siRNAs, and the importance

of RDR1 has been proved in Arabidopsis against CMV infection during the biogenesis of siRNA (X. Wang et al., 2011). A similar result has also been observed in Arabidopsis plant to defeat TuMV infection (Garcia-ruiz et al., 2010). In inoculated leaves, DCL4 is sufficient to inhibit TuMV replication, while DCL2 and RDR6 are also required for systematic resistance against TuMV.

In order to counteract plants' RNAi pathway, viruses evolved a new strategy to impair the RNAi pathway by encoding RNA silencing suppressor (RSS). RSS could inhibit DCL proteins' activity to prevent generation of siRNA, and also can sequester dsRNA/siRNA to prevent formation of RISCs (Calil & Fontes, 2016). Potyviral helper component proteinase (HC-Pro) is a well-established RSS, and HC-Pro could suppress RNAi through multiple ways. HC-Pro could bind to siRNA to inhibit formation of RISC in TuMV infected Arabidopsis plants (Garcia-ruiz et al., 2010). It can also bind to AGO1 directly to suppress RNAi (De et al., 2016). Lots of other RSS have been identified over years, such as CP of TCV, which also function as RSS, could bind to AGO2 to suppress RNAi (Zhang, Zhang, Singh, Li, & Qu, 2012), and p22 of *Tomato chlorosis virus* (ToCV) which could bind to dsRNA to inhibit generation of siRNA (Landeo-ríos, Navas-castillo, Moriones, & Cañizares, 2016). All the information above addresses the importance of identifying resistance genes to help increase plants' resistance against virus infection, and also to explore whether there is a broad and universal mechanism that plants could adopt to defeat virus invasion.

1.6 Alfalfa mosaic virus

AMV was first described in 1931 as the causal agent of mosaic disease of alfalfa (Hull, 1969). AMV is the type member and only member of the genus *Alfamovirus* in the family *Bromoviridae*. AMV can infect more than 600 species of 70 plant families (Bol, 2003). It typically causes various mosaics, mottles and malformation in alfalfa (*Medicago sativa*) and tobacco (*Nicotiana tabacum*), and necrotic local lesions in cowpea (*Vigna unguiculata*). AMV is seed-borne and is transmitted non-persistently by 14 different species of aphids. AMV causes diseases of pasturage resulting in loss of herbage weight, seed yield, nitrogen fixation and pasture persistence (R. A. C. Jones & Ferris, 2000). AMV can interact synergistically with *Soybean mosaic virus* (SMV) to induce more severe symptoms than the individual virus in soybean (Malapi-Nelson, Wen, Ownley, & Hajimorad, 2009). Management of AMV includes use of AMV-free seeds or transgenic plants with AMV-resistance (Hill et al., 1991; Loesch-Fries et al., 1987; Parrella, Moretti, Gognalons,

Lesage, Marchoux, Gebre-Selassie, et al., 2004; Pederson & McLaughlin, 1994; Timmerman-Vaughan et al., 2001).

AMV has four bacilliform particles with a diameter of 18 nm and lengths of 30 - 57 nm (Bol, 2005). The genome of AMV contains three single-stranded RNAs with positive-sense polarity. RNA 1 and RNA 2 encode viral replicase proteins, P1 and P2, and RNA 3 encodes movement protein (MP) and coat protein (CP). A sub-genomic RNA (RNA 4) is the mRNA for CP. AMV has four viral particles, and each particle contains one copy of RNA1/2/3 or two copies of RNA4 (Agrios, 2005; Bol, 2003).

Upon infection, AMV-encapsidated RNAs are immediately translated into proteins. CP must bind to the 3' end of the RNAs for translation, thereby acting similarly to poly (A) binding protein in eukaryotic translation (Bol, 2005). AMV replication complex consists of P1, P2 and unknown host factors, which are assembled on multivesicular bodies (MVBs), and then transferred to the tonoplast (Ibrahim, Hutchens, Howard Berg, & Sue Loesch-Fries, 2012).

1.7 Resistance to AMV infection

Not much research has been conducted to explore plants' resistance to AMV infection. As AMV is a compatible virus in tobacco, Arabidopsis and several other important model plants, there is no HR, which is different than the well-established TMV resistance model. Coat protein-mediated resistance could significantly increase plants' resistance against AMV infection, and have been proved in tobacco, tomato and etc. (Beachy, Loesch-fries, & Turner, 1990; Loesch-Fries et al., 1987). It was hypothesized that pre-existing CP could inhibit the uncoating process of virus, which resulted in virus resistant plants. Besides CP-mediated resistance, three resistant genes were found involved in plant resistance against AMV. By crossing susceptible and resistant cultivars of alfalfa, Crill and colleagues found a recessive gene, *amv-1*, which has the potential to regulate plants resistance to AMV. Two more genes were found related to AMV resistance, *Rav1* and *Am* in soybean and tomato, respectively (Koval, Mueller, Paine, Grau, & Diers, 2008; Parrella, Moretti, Gognalons, Lesage, Marchoux, Gebre-selassie, et al., 2004). Both of these two genes confer dominant resistance to AMV, and they were identified by marker-assisted selection method. All three genes related to AMV resistance mentioned above have not been cloned yet, thus the biological functions and underlying mechanism is still remaining unknown.

1.8 Chloroplast protein and viral protein interaction

Chloroplast is the energy source of plant, where photosynthesis is conducted. Once plants are infected by virus, typical symptom are chlorosis and mosaic, which indicates that the chloroplast proteins have important roles in virus colonization or plant immunization.

Recent researchers found that viral particles can affect the function of chloroplast. Aro and colleagues found that TMV CP highly accumulated in chloroplast, which induce the malformation of chloroplast ultrastructure and entirely loss of PSII complex (Lehto, Tikkanen, Hiriart, Paakkarinen, & Aro, 2003). This evidence shows that the chlorosis was caused by impairment of photosynthesis system, instead of the reduction of pigment biosynthesis. Similar results were observed in PVY infected tobacco plants. Intimate interaction was detected between PVY-CP and Rubisco-LSU, and that this interaction significantly inhibit photosynthesis activity, and increased severity of infection symptom (Feki, Loukili, Karimova, Old, & Ounouna, 2005). *Alternanthera mosaic virus* (AltMV) triple gene block 3 (TGB3) protein was found to interact with PsbO, a protein help to stabilize the PSII complex, and this interaction is important for symptom development (Jang et al., 2013). Over-expression of AltMV TGB3 causes decrease of intact chloroplasts observed in both Arabidopsis and *N. Benthamiana*, and also leads to veinal necrosis. Potyviruses P3 interacts with both RbCL and RbCS, and leads to a decrease in the amount of chlorophyll and photosynthetic rate after infection (Lin et al., 2011).

Besides the impact on chloroplast function, the virus infection can also influence the expression of chloroplast photosynthesis-related genes/proteins (CPRG/P). Quantitative transcriptional analysis shows that the expression level of several CRRGs, including *LHCA1*, *LHCB6*, *RbCS* and *RbCL*, were significantly decreased in CMV infected plants (Mochizuki, Ogata, Hirata, & Ohki, 2014). In *Sugar cane mosaic virus* (SCMV) infected plants, the expression level of Fd V was dramatically down-regulated. Furthermore, the specific interaction between SCMV HC-Pro and Fd V was also observed, and this interaction interrupted the movement of Fd V into chloroplasts and led to the perturbation of chloroplast function (Cheng et al., 2008).

Photosystem II is a multi-component enzyme complex, which transfers four electrons from two water molecules to form one oxygen. PS II can be divided into two functional domains, the first one is the electron transfer domain, which recruits chlorophyll and other pigments to harvest solar light, and the other is the oxygen evolving complex (OEC), located on the thylakoid lumenal side

(Bricker, Roose, Fagerlund, Frankel, & Eaton-Rye, 2012). The OEC consists of a Mn_4-Ca-Cl_x cluster, which is surrounded and stabilized by several extrinsic protein.

PsbO is one of the extrinsic proteins and is the only ubiquitous protein that exists in all photosynthetic organisms. The function of PsbO is to stabilize the Mn clusters. Down-regulation of PsbO inhibits plants photoautotrophic growth and decreases the oxygen evolution activity (Murakami et al., 2005; Yi, Mcchargue, Laborde, Frankel, & Bricker, 2005). The function of another subunit of OEC, PsbQ, remains controversial. Previous studies showed that PsbQ and PsbP synergistically retain the Ca^{2+} and Cl^- around the Mn cluster to prevent inactivation by reduction in the thylakoid lumen and also contribute to the oxygen evolution (Ghanotakis, Babcock, & Yocum, 1984; Miyao & Murata, 1985). Recent researchers found that PsbP contributes mostly to the retention of Ca^{2+}. Knock-down PsbQ plants shown no distinguishable difference from the wild-type plants, but the PsbP knock-down plants had drastically reduced plant growth and unstable PS II compared to wild-type plants (Barra, Haumann, & Dau, 2005; Ifuku & Sato, 2002; Ifuku, Yamamoto, Ono, Ishihara, & Sato, 2005). As mentioned above, PsbP subunit of PS II is responsible for retaining Ca^{2+} and Cl^- in the complex and helps to stabilize PS II.

Besides the functions of PsbO, PsbP and PsbQ in photosynthetic activity, recent studies found they could be the targets of viral proteins (Bhattacharyya & Chakraborty, 2018; Jinping Zhao, Zhang, Hong, Liu, & Liu, 2016). It has been noted by a number of researchers that virus infection down-regulates them. Several Tobamoviruses, such as TMV, significantly reduce the expression level of PsbP and PsbQ, but not PsbO. Some others could induce the down-regulation of all three subunits of PS II after infection, such as Pepper mild mottle virus (PMMoV), which can cause an 80% decrease in PsbQ, and a 60% decrease in PsbO and PsbP (Abbink et al., 2002; Y. Li, Cui, Cui, & Wang, 2016; Pérez-Bueno, Rahoutei, Sajnani, García-Luque, & Barón, 2004; Sui, Fan, Wong, & Li, 2006). Abbink and colleagues found PsbO-silenced plants are more sensitive to TMV, AMV and PVX compared to the wild-type plants (Abbink et al., 2002). Similar result was also observed for PsbP-silenced plants. PsbP-silenced plants accumulated more than 10-fold higher of AMV compared to the wild-type plants (Balasubramaniam et al., 2014). A geminivirus, *Radish leaf curl virus* (RaLV) encoded βC1 specifically interacts with PsbP, and this interaction interferes with the import of PsbP to chloroplast, then attenuates the PsbP-mediated defense responses (Gnanasekaran, Ponnusamy, & Chakraborty, 2019). Similarly, *Rice stripe virus* (RSV) encoded specific protein (SP) also interacts with PsbP specifically in rice (Kong, Wu, Lu, Xu, & Zhou,

2014). The presence of SP changes the subcellular location of PsbP from chloroplast to cytoplasm, and the PsbP-silenced plants have increased susceptibility to RSV.

Another reason to highlight the importance of chloroplasts in plant-virus interaction is because chloroplast is a major site for biosynthesis of salicylic acid (SA), jasmonic acid (JA) and reactive oxygen species (ROS). SA is a small phenolic compound, which is involved in a wide range of physiological processes, such as seed germination, stomatal closure, fruit yield, etc. (Loı¨c Rajjou et al., 2006; Metwally, Finkemeier, Georgi, & Dietz, 2003; Norman, Howell, Millar, Whelan, & Day, 2004; Vlot, Dempsey, & Klessig, 2009). In addition, it also has vital roles in local defense and systemic acquired resistance (SAR). Increase in the level of SA or its derivatives could dramatically increase plants' basal resistance against pathogens, such as TMV and *Pseudomonas syringae* (Chen et al., 2004; F. Zhu et al., 2014). Upon the recognition of pathogenic proteins, endogenous SA is elevated, and this signal will be transduced to nucleus through both NPR1-dependent and NPR1-indenpendent pathways to induce the expression of a cluster of response genes, such as *PR-1*, and thereby enhance resistance against pathogens (Seyfferth & Tsuda, 2014; Shah, 2003). JA is an oxylipin, which also has functions in plant growth, development and resistance against pathogens. Unlike SA, which mainly contributes to the resistance against compatible pathogens, JA is involved in the resistance against incompatible pathogens, and has been shown to have antagonistic effects on pathogen signaling pathways (Niki, Mitsuhara, Seo, Ohtsubo, & Ohashi, 1998; Wasternack, 2007; Wasternack & Hause, 2013). For instance, a JA receptor, Coronatine-Insensitive 1 (COI1)-silenced plant, has enhanced susceptibility to TMV compared to the wild-type plant (Oka, Kobayashi, Mitsuhara, & Seo, 2013). PS II electron transport chain is where ROS been produced, and any changes or deformation of the electron transport chain will induce the production of ROS, or even oxidative burst in thylakoid lumen. When plants are infected by pathogens, the endogenous level of SA is elevated, and it was also found that SA can inhibit the activity of *ascorbate peroxidase* (APX), which eventually lead to hypersensitive response, and help tobacco to restrict pathogen spread (Durner & Klessig, 1995; Jorg Durner & Klessig, 1996).

CHAPTER 2. PSBP REGULATED PLANT RESISTANCE AGAINST AMV INFECTION IS INDEPENDENT OF PHYTOHORMONE PATHWAYS

2.1 Abstract

The Arabidopsis PsbP protein is one of the extrinsic proteins of the PSII, which regulates the photosynthesis process and it has also been demonstrated to be involved in plant resistance against pathogen infection. In a yeast two-hybrid screen, PsbP was found to interact with AMV coat protein (CP). In this report, we focus on elucidating the defense mechanism regulated by PsbP to inhibit AMV replication. We found that knock-down of PsbP significantly increased plants' susceptibility to AMV, and then caused higher amount of virus accumulation. Further, we shown that the down-regulation of PsbP did not impair the RNA silencing pathway and several phytohormone pathways, including SA, JA and ET, to abolish the defense mechanism, but we found the expression of Pathogenesis-Related gene 1 (PR-1) was inhibited in knock-down plants. Furthermore, we observed the oxidative burst happened, and the reactive oxygen species scavenging system was destroyed in PsbP knock-down plants. Taken together, our data suggest that PsbP is a positive regulator of plant resistance against AMV infection, and a potential new mechanism, which is independent of the SA pathway, exists to help activate defense signals once AMV infection happens.

2.2 Introduction

Phytohormones are small molecules, which play pivotal roles in regulating different activities, including seed germination, plant development, reproduction, etc. When plants are under abiotic stresses, the complex hormone network can response immediately to adapt to the changes of environment (Santner & Estelle, 2009; Verma, Ravindran, & Kumar, 2016). Besides, recent researches revealed that changes of the concentration of phytohormones can manipulate plants susceptibility to various pathogens. Different phytohormones interact synergistically or antagonistically to help plants defend themselves against pathogens by a strategy that is highly dependent on the pathogen species.

Depending on their lifestyles, plant pathogens are generally classified into two groups, biotrophic or necrotrophic pathogens. Biotrophs have to keep a compatible interaction with their

hosts and derive nutrients from the hosts to complete their lifecycles, without interrupting host activities. Oppositely, necrotrophs kill their hosts generally by producing phytotoxins, then utilize the dead matter to continue their lifecycles. Besides, some pathogens use both of the two lifestyles, depending on their different life stage, and these pathogens are called hemibiotrophs (Pieterse, Leon-Reyes, Van der Ent, & Van Wees, 2009).

SA has been reported as the key regulator against biotrophic pathogens and hemibiotrophs, such as *Pseudomonas syringae* and Tobacco mosaic virus. When plants perceived the infection signals, PAMPs or effectors, they transduce the signals to the chloroplasts and nuclei to trigger the biosynthesis of SA and the expression of related defense genes. Disruption of the SA pathway compromises plant local and systemic resistance against pathogens (Baebler, Witek, Petek, Stare, et al., 2014; Jinping Zhao et al., 2016). In contrast, application of exogenous SA or its analogs increases plant resistance and delays symptom development (Eldeen, Radwan, Lu, Ali, & Younis, 2008; Falcioni, Ferrio, Isabel, & Giné, 2014). Negative crosstalk between SA and JA/ET has been reported in resistance against different pathogens. The major role of JA and ET is assumed to regulate the interaction between necrotrophic pathogens and plants (Alazem & Lin, 2015; Verma et al., 2016). Nevertheless, several reports found JA acts as positive regulator in compatible plant-pathogen interactions. The application of exogenous JA effectively restricted virus replication and symptom development and the increase of JA activates both local and systemic resistance in plants (García-marcos, Pacheco, Manzano, Aguilar, & Tenllado, 2013; Shang et al., 2011; Wees, Swart, Pelt, & Loon, 2000; F. Zhu et al., 2014).

PsbP is one of the extrinsic proteins of the PSII in chloroplast, and the function of PsbP is to maintain the stability of OEC, and ensure the function of chloroplast. The chloroplast has been recognized as the biosynthesis site of SA; thus, impairment of chloroplasts may cause concentration changes in SA, and then alter plants resistance against pathogens (Alazem & Lin, 2015; D'Maris Amick Dempsey, Vlot, Wildermuth, & Klessig, 2011; Jinping Zhao et al., 2016).

In this study, we checked the responses of three phytohormone pathways, including SA, JA and ET, in PsbP down-regulated plants after AMV infection. The ROS scavenging system was also studied to explore its influence on plants' resistance against AMV. Meanwhile, the transcription level of several key components of the RNAi pathway were measured to determine if RNAi was involved in the resistance. The results show that there are no changes in the three phytohormone pathways and the RNAi pathway in PsbP-downregulated plants. However, the wild-type plants

accumulated higher amount of ROS compared to the PsbP down-regulated plants following AMV infection. Therefore, we demonstrate that PsbP is a positive regulator of plant anti-AMV resistance, but not through altering the classic phytohormone pathways.

2.3 Methods and materials

2.3.1 Plant material and inoculation

Nicotiana tabacum wild-type and RNAi mutants were grown in 4-inch pots, filled with pre-mixed Miracle-Gro® Potting Mix and Osmocote (ScottsMiracleGro), with a 16-h-light/ 8-h-night daylight cycle at 25°C. Four-weeks-old plants were dusted with carborundum and mechanically inoculated with 10 µg/ml AMV, strain 425 Madison. Control plants were dusted with carborundum, but only mock inoculated with phosphate buffer, pH 7.0. Both infected and mock-treated leaves were harvest at two time points, 3 and 7 days-post-inoculation (dpi). All the samples were frozen with liquid nitrogen, and then stored at -80°C until total RNA extraction.

2.3.2 Quantitative real-time PCR and analysis

All mock and infected leaves at different time points were grounded with liquid nitrogen into fine powder, and then the powder was used to extract total RNA with TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA). The extraction process followed the manufacture's protocol. 200mg ground tissue mixed with 1ml TRIzol, and then 200 µl chloroform was added to separate the Phenol-Chloroform phase and then 500 µl isopropanol was added to precipitate the RNA from the aqueous phase the pelleted RNA was then washed with 75% ethanol twice to remove salt. The RNA pellet was air dried, and then dissolved in 80 µl nuclease-free H_2O.

The total RNA isolated from all tissues were treated with DNase (RQ1 RNase-Free DNase, Promega) according to manufacturer's protocol to remove any DNA. RNeasy Mini Kit (Qiagen) was used with all the samples to purify the RNA.

cDNA was synthesized from the purified RNA by using the M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin, USA) according to the manufacture's protocol. 1µg purified RNA was added with 0.4 µl random hexamer (Promega, Madison, Wisconsin, USA), 5 µl M-MLV RT 5x reaction buffer, 1.25 µl dNTPs, 0.5 µl M-MLV RT(H-) point mutant, and then nuclease-free H_2O to a final volume of 25 µl. The reaction was incubated at room temperature for the initial 10

minutes, and followed with 55°C incubation for 50 minutes. Inactivate the activity of M-MLV Reverse Transcriptase by heating for 15 minutes at 70°C.

Quantitative PCR was performed by using the PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA) and followed manufacture's protocol. Each reaction contains 5µl of PowerUp SYBR Green Master Mix (2x), 2µl of each primer pair (10uM), 2µl of cDNA template, and 1µl nuclease-free H_2O. CFX Connect Real-Time PCR Detection System (Bio-Rad) was used set to the reaction condition of 2 minutes at 95°C, 40 cycles of 5 s at 95°C and 30 s at 60°C. The primer efficiency of each pair was determined by constructing efficiency curves of a series dilution of cDNA template to make sure the efficiency is higher than 94%. 18S rRNA was used as internal control for all the experiments, and $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of different genes (Livak & Schmittgen, 2001). All experiments were performed in triplicate and repeated at least three times, and all the statistical analysis were performed with Duncan's multiple range test in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

2.3.3 Luminescence assay for detecting ROS production

Luminescence assay was conducted as described by Dr. Rathjen with a few modifications (Heese et al., 2007). Four-weeks-old N. tabacum plants, both mock-treated and infected plants, were punched with a leaf disc cutter to get leaf discs with a diameter of 4 mm. Leaf discs were floated on 200 µl water in 96-well plates for 5 hours to remove the existing ROS, and then the water was replaced with reaction mix containing 20ug/ml Peroxidase from horseradish (Sigma-Aldrich, P8375-5KU) and 34ug/ml Luminol sodium salt (Santa Cruz Biotechnology, SC-218662). Once the reaction mix was applied, the Synergy™ 2 Multi-Mode Microplate Reader (Biotek) read the luminescence immediately at 1-minute intervals for 47 minutes, with a capture time of 200 ms, and Sensitivity of 255.

2.4 Results

2.4.1 Down-regulation of NtPsbP increased N. tabacum susceptibility

In order to investigate the function of PsbP on susceptibility, we obtained seeds of the RNAi *N. tabacum* plants from Dr. Ifuku (Ifuku et al., 2005). The transcript level of PsbP in these plants was

checked before AMV infection to make sure that RNAi plants had significantly lower amounts of PsbP than wild-type plants. As shown in Fig.1A, around 10% of *NtPsbP* RNA was left in the RNAi plants compared to wild-type plants. Both wild-type and ΔPsbP plants were then inoculated with 10ug/ml AMV to determine susceptibility. At 3dpi, higher amount of AMV CP RNA was detected by RT-qPCR in ΔPsbP plants compared to wild-type, and the difference between two genotypes was around twenty-fold (Fig. 1B). Similar results were found at 7dpi; the ΔPsbP plants had more virus RNA than the wild-type plants. These results indicate that PsbP is a potential positive regulator of plant resistance against AMV infection, and plants' susceptibility was increased dramatically when PsbP levels were decreased.

2.4.2 Salicylic acid pathway and RNA silencing pathway were not activated after AMV infection

Because SA confers a broad-spectrum resistance to viruses (D'Maris Amick Dempsey et al., 2011; Malamy, Carr, Klessig, & Raskin, 1990; Naylor, Murphy, Berry, & Carr, 1998; Shah, 2003), we evaluated the transcript level of two key enzyme of SA biosynthesis to see whether AMV infection increased the amount of SA. We found that the transcription level of *ICS1* had not significant changed after AMV infection compared to the Mock treated wild-type plants at both 3dpi and 7dpi (Fig. 2A). In ΔPsbP plants, the transcription level increased slightly compared to Mock treatment at 7dpi, but there was no difference in wild-type plants at 7dpi. Similar results were found for another biosynthesis gene, *PAL*; no significant difference could be observed before or after AMV infection at both 3dpi or 7dpi, and both wild-type and ΔPsbP had similar amounts of *PAL* RNA after AMV infection (Fig. 2B). In contrast, we found the hallmark defense gene of the SA pathway, *PR-1*, had dramatically increased after AMV infection (Fig. 3C). After AMV infection, both wild-type and ΔPsbP plants had a significant increase of *PR-1* compared to mock-treated plants, respectively. However, wild-type plants accumulated significantly higher amounts of *PR-1* compared to ΔPsbP plants. In addition to checking the endogenous SA RNA level after AMV infection, we also investigated whether exogenous SA application could increase *N. tabacum* resistance against AMV infection. 2mM SA was sprayed on the foliar surface, and the leaves were inoculated 10ug/ml AMV one day after SA application. As shown in Fig. 3, ΔPsbP was more susceptible to AMV infection than wild-type plants, but there was no difference between

water and SA sprayed plants. All the data above indicates that the SA pathway is not involved in the susceptibility change in ΔPsbP to AMV infection.

The plant RNA silencing pathway is important in the anti-viral immune system. To generate siRNA for the degradation of viral invaders, plants need to recruit RDRs to synthesize siRNA. Thus, we determined the transcript level of *RDR1* in AMV infected plants to investigate the response of the RNA silencing pathway. The amount of virus transcript was determined firs; higher levels of AMV always accumulated in ΔPsbP plants compared to wild-type plants (Fig. 4A). Fig. 4B shows that the transcript levels of *RDR1* did not change after AMV infection wild-type nor ΔPsbP *N. tabacum*. Collectively, the data demonstrate that the difference between the susceptibility of ΔPsbP and wild-type plants did not involve the RNA silencing pathway.

2.4.3 Jasmonic acid and ethylene pathway were not activated after AMV infection

We checked two important genes involved in the jasmonic acid pathway to determine whether AMV activated plant immune responses through the JA pathway. As data shown in Fig 5A, the AMV infection of wild-type plants did not results in an increase in transcript level of *AOS*, which is one key enzyme responsible for JA biosynthesis. Similar results were obtained for *COI1* transcripts, which is the downstream JA response gene. No significant differences were observed between mock treated plants and AMV infected plants in both wild-type and ΔPsbP *N. tabacum*. Besides the JA-relevant genes, one ET downstream response gene, *PDF1.2*, was also monitored. In samples of both wild-type and ΔPsbP *N. tabacum*, there were no transcriptions that could be detected with or without AMV infection (not shown). These data suggest that both JA and ET pathways were not activated after AMV infection; thus, the increase of susceptibility in ΔPsbP plants was not caused by an impairment in the JA and ET pathways.

2.4.4 AMV infection induced an oxidative burst in ΔPsbP, but not in wild-type plants

Pathogen infection is often coupled with an oxidative burst, which eventually leads to necrosis. In this study, we examined the RNA level of two ROS scavenging enzymes, AOX and SOD, in both wild-type and ΔPsbP *N. tabacum* with or without AMV infection. We found the basal transcription level of both AOX and SOD were significantly lower in ΔPsbP plants compared to wild-type plants (Fig. 6A and 6B). After AMV infection, the transcript level of both AOX and

SOD increased in wild-type plants. However, there were no changes in the transcript level of either AOX or SOD in ΔPsbP plants after AMV infection. Consistently, we observed that an oxidative burst occurred only in ΔPsbP plants, while the ROS production remains low in wild-type plants (Fig. 6C). Collectively, these data indicate down-regulation of *PsbP* impaired the plant's ROS scavenging system, which resulted in the oxidative burst in ΔPsbP plants.

2.5 Discussion

Previous studies demonstrated that chloroplast proteins targeted viral proteins to initiate immune responses that inhibited virus replication and movement (Y. Li et al., 2016; Jinping Zhao et al., 2016). Linthorst and colleagues found that PsbO directly bound to TMV replicase resulting in a 10-fold lower accumulation of TMV in PsbO-silenced plants than wild-type plants (Abbink et al., 2002). PsbO was also found to interact with *Alternanthera mosaic virus* (AltMV) TGB3 protein, resulting in severe symptom development (Jang et al., 2013). These data all support the hypothesis that chloroplast proteins have pivotal roles in regulating plants' resistance against virus infection. Our previous data showed that PsbP directly bound to AMV CP, accumulated in cytoplasm, and transient over-expression of PsbP in *N. benthamiana* significantly inhibit AMV replication. In this study, we found the down-regulation of PsbP significantly increased plants' susceptibility to AMV infection. The increased susceptibility was hypothesized to result from down-regulation of PsbP disrupting the signal transduction or activation of downstream immune responses, such as the SA pathway or the RNA silencing pathway. Zhou and co-workers (Kong et al., 2014) demonstrated that PsbP interacted with *Rice stripe virus* (RSV) disease-specific protein (SP) directly. Silencing PsbP dramatically promoted symptom development caused by RSV and PVX. Our results and all above data suggest that PsbP is a positive regulator of plant resistance against virus infection, while the mechanism remains unclear.

Phytohormones have been demonstrated to play crucial roles in regulating plants' resistance against virus infection. For example, application of exogenous SA inhibited TMV symptom development. TMV infection induced the accumulation of SA within host cells (Enyedi, Yalpani, Silverman, & Raskin, 1992; Gaffney et al., 1993) and any impairment of SA-biosynthesis or its signal transduction pathway compromised plants' immune response against virus infection. Mutation of *SID2*, which regulates SA biosynthesis, or *NahG*, which disrupts the accumulation of SA, lead to an increased susceptibility to TMV infection and no hypersensitive response could be

activated in these mutants (Carella, Wilson, & Cameron, 2015; Collum & Culver, 2016; Dangl & Jones, 2001; Goulart, Gilza, Souza, & Siqueira, 2019; Herrera-VÃ¡squez, Salinas, & Holuigue, 2015). Similar results were observed for CMV infection. John Carr and co-worker determined that CMV infection caused a significant increase of SA in Arabidopsis at 10dpi and that *ICS1* was important in CMV-induced SA accumulation (Lewsey et al., 2010). As AMV and CMV are both compatible viruses in tobacco, we checked the synthesis of two SA biosynthesis genes, *ICS1* and *PAL*. Neither of these genes were activated in our study, but the increase of susceptibility was detected in ΔPsbP plants compared to non-transgenic plants. Our results indicate that tobacco and Arabidopsis plants do not recruit the SA pathway to regulate a plant immune response against AMV infection. John Carr also reported similar results; SA was not involved in anti-viral mechanism (Lewsey et al., 2010). In Arabidopsis, TMV infection did not trigger the changes in the amount of SA compared to mock-treat plants. Another possibility is that SA only contributes to the increase of systemic acquired resistance (SAR) in tobacco and Arabidopsis, but not in local resistance. Carl N. Mayers and colleagues found application of exogenous SA didn't inhibit symptom development caused by CMV in directly inoculated-leaves in Arabidopsis, whereas it did delay the systemic movement of CMV (Mayers, Lee, Moore, Wong, & Carr, 2005). Similar results were observed in CMV infected tobacco plants. SA treatment didn't inhibit the replication of CMV, but inhibited the systemic movement of CMV (Naylor et al., 1998). These results suggest that Arabidopsis and tobacco may share the identical anti-CMV mechanism, in which SA only enhances plants' SAR, not local resistance. Nevertheless, Carl N. Mayers found SA could increase local resistance against CMV infection in squash plants by inhibiting cell-to-cell movement rather than virus replication (Mayers et al., 2005). Overall, the SA pathway was not activated in tobacco plants in response to AMV infection, and the SA-regulated resistant mechanism can be triggered differently depending on plant species and virus species/strains.

Many studies have reported the antagonistic interaction between SA and JA/ET signaling pathway, and SA is mainly responsible for resistance against biotrophic pathogens, while JA/ET regulates the resistance against necrotrophic pathogens (Mur, Kenton, Atzorn, Miersch, & Wasternack, 2006; Vlot et al., 2009; Y. Yang, Ahammed, Wu, Fan, & Zhou, 2015). For example, García-Marcos and colleagues found the JA pathway negatively regulated programmed cell death after infection by *Potato virus X* and *Tomato spotted wilt vir*us. Abolishing the JA signal transduction pathway significantly restricted the compatible virus movement within hosts (García-

marcos et al., 2013). In contrast, the synergistic interaction between SA and JA was observed in promoting PCD. Luis A.J. Mur and co-workers found the co-infiltration of SA and JA into tobacco plants could induce necrosis, whereas either SA or JA alone would not (Mur et al., 2006). Jing Shang and colleagues also observed the application of both SA and JA could increase resistance to CMV, TMV and *Turnip crinkle virus* (TCV) compared to either SA or JA alone (Shang et al., 2011). In this study, we did not observe the increased expression of JA related biosynthesis genes and response gene after AMV infection. Collectively, these results indicated that both SA and JA pathways were not activated by AMV infection, and the PsbP-regulated resistant mechanism is independent of these phytohormone pathways.

Under both biotic and abiotic stresses, plants can increase the production of ROS to induce the down-stream defense response. As the major organelle for producing ROS, chloroplast plays pivotal roles in plant development and resistance against various pathogens (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004; Qi et al., 2018; Waszczak & Carmody, 2018). For instance, the application of flg22 could induce the dramatic production of ROS in Arabidopsis (Heese et al., 2007; Nomura et al., 2012). Our results showed that down-regulation of PsbP significantly increased plants susceptibility, and this susceptibility increase was coupled with the increase of production of ROS. In Arabidopsis, *Cauliflower mosaic virus* infection induced the oxidative burst and PCD, and this defense response was compromised in *rbohD* plants (Love, Yun, Laval, Loake, & Milner, 2005). Robert Fluhr and colleagues also noticed that TMV infection could induce the oxidative burst, and this oxidative burst could be prevented by NAD(P)H oxidase inhibitors (Allan, Lapidot, Culver, & Fluhr, 2001). All the data mentioned above suggest that increased ROS production is a precursor for activating plant immune responses against pathogens. While our results shown the conversed results, and it may be explained by the severity of symptom development. As the compatible interaction between tobacco and AMV, plants activated ROS scavenging system, including SOD and AOX, to eliminate the excess ROS and maintain the metabolism within cell after AMV infection. In contrast, ΔPsbP plants were not able to detect the virus infection, and the ROS scavenging system could not be activated, which lead to the increased amount of ROS in mutant plants. This result demonstrates the important function of PsbP in perceiving the virus infection and activating down-stream defense responses. Activation of SA pathway was found to induce the production of ROS and expression of pathogen-related genes, and eventually lead to HR (Collum & Culver, 2016; Y. Liu & He, 2016; Overmyer, Brosché, &

Kangasjärvi, 2003). Our results shown that AMV didn't induce the expression of SA-related biosynthesis genes, but interestingly, we noticed the dramatic increase of *PR-1* in both wild-type and ΔPsbP plants. Similar results was observed by Sanja Matern and co-workers (Matern, Peskan-Berghoefer, Gromes, Kiesel, & Rausch, 2015). In high-glutathione transgenic tobacco lines (HGLs), the well-known SA induced genes, including *PR1*, *PR2*, *PR4* and *PR5*, were induced and significantly higher than wild-type plants without any pathogen infection. Meanwhile, the high level of glutathione could also activate the MAPK pathway. Besides, the HGLs were much more resistant against *P. syringae* than wild-type. This finding indicates that MAPK and SA defense pathway could operate independently, and *PR* genes could be regulated without the increase of SA amount. In our study, the increase of *PR-1* may cause by the activation of MAPK pathogen, instead of SA-NPR1 dependent pathway.

In conclusion, the PsbP is a positive regulator of plant resistance against AMV infection, but the downstream defense pathways were independent of phytohormone pathways, including SA and JA/ET pathways. Nevertheless, PsbP also plays pivotal roles in controlling ROS scavenging system to maintain the cytoplasm homeostasis after AMV infection. Further studies need to be conducted to elucidate the mechanism regulated by PsbP.

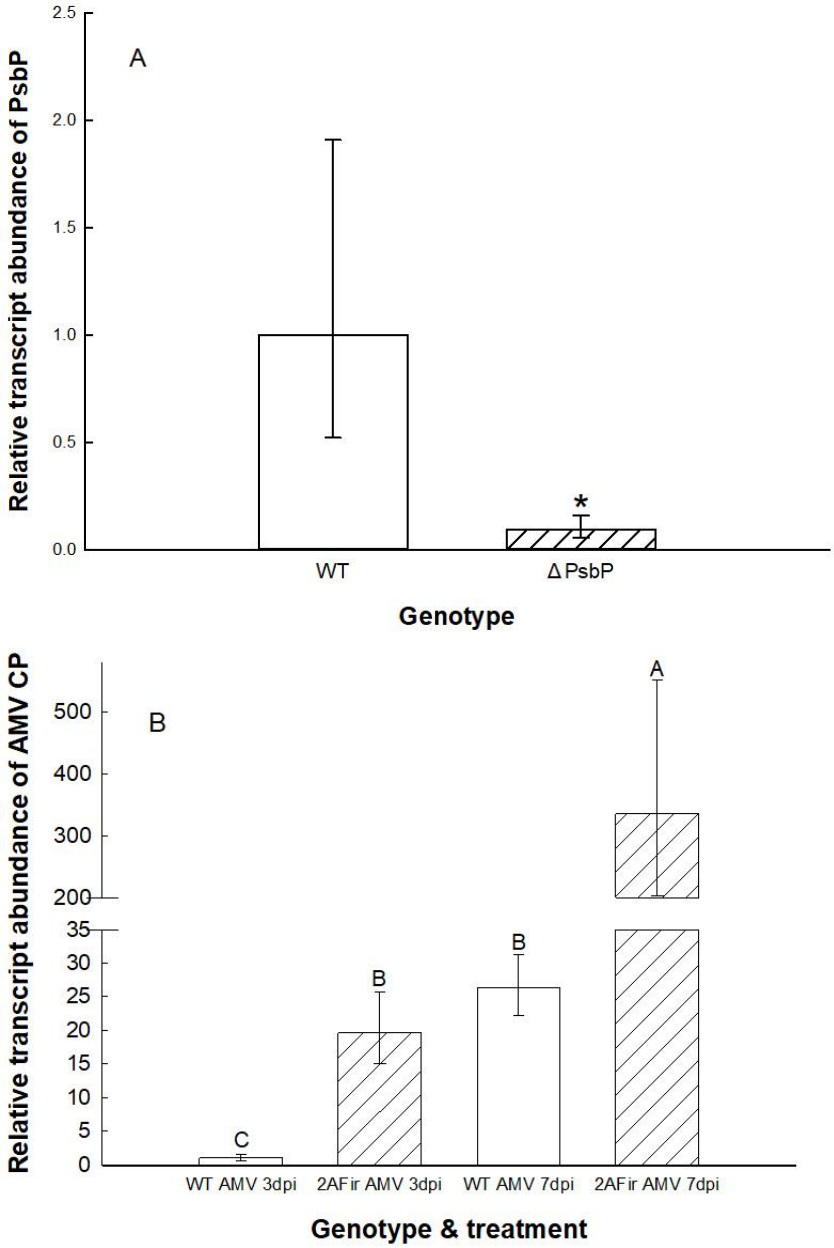

Figure 2.1 Down-regulation of PsbP lead to higher accumulation of AMV in N. tabacum plants.

(A) Quantitative RT-PCR analysis of transcript level of NtPsbP in wild-type and RNAi plants without virus infection. (B) The relative amount of AMV CP RNA in wild-type and RNAi *N. tabacum* plants was checked at both 3dpi and 7dpi by RT-qPCR. n=3 samples were analyzed for every experiment, and data represent the means ± standard deviations. Duncan's multiple range test was employed to determine the statistically significant differences between genotypes and treatments. Different letters and asterisk indicate significant differences at a P value of 0.05.

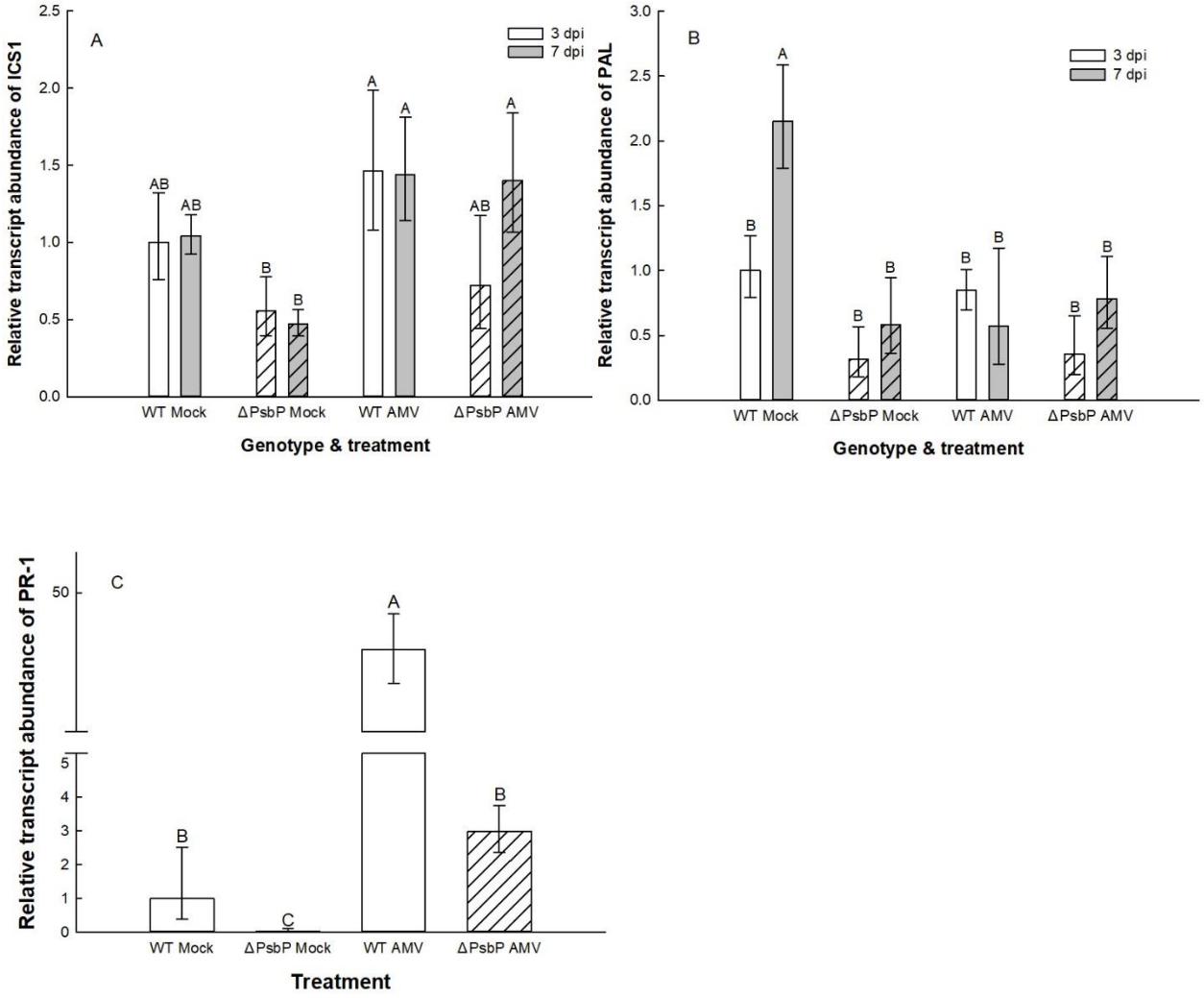

Figure 2.2 AMV infection did not influence the salicylic acid biosynthesis.

Quantitative RT-PCR analysis of the transcript level of two SA biosynthesis enzyme, ICS1 (A) and PAL (B), in wild-type and RNAi *N. tabacum* after AMV infection at 3dpi and 7dpi. (C) Relative amount of *PR-1* RNA in wild-type and RNAi *N. tabacum* after AMV infection at 7dpi. Mock indicates plants were only inoculated with phosphate buffer, pH=7.0. Data represent the means ± SD of three replicates. Duncan's multiple range test was employed to determine the statistically significant differences between genotypes and treatments. Different letters indicate significant differences at a P value of 0.05.

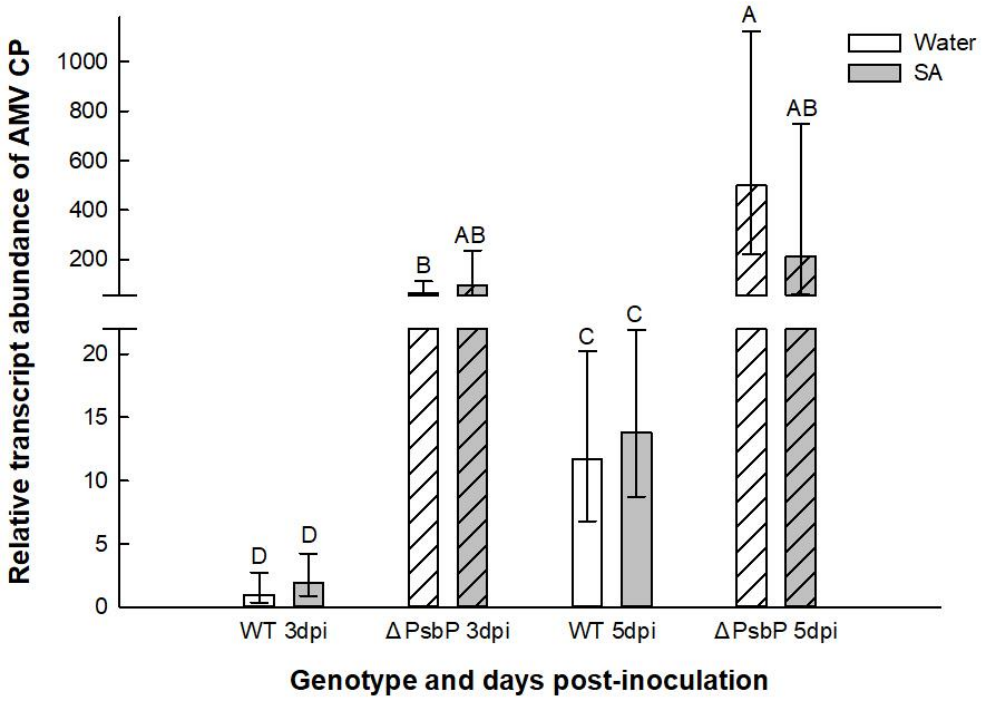

Figure 2.3 External SA did not increase *N. tabacum* resistance against AMV infection.

Data represent the means ± SD of three replicates. Duncan's multiple range test was employed to determine the statistically significant differences between genotypes and treatments. Different letters indicate significant differences at a P value of 0.05.

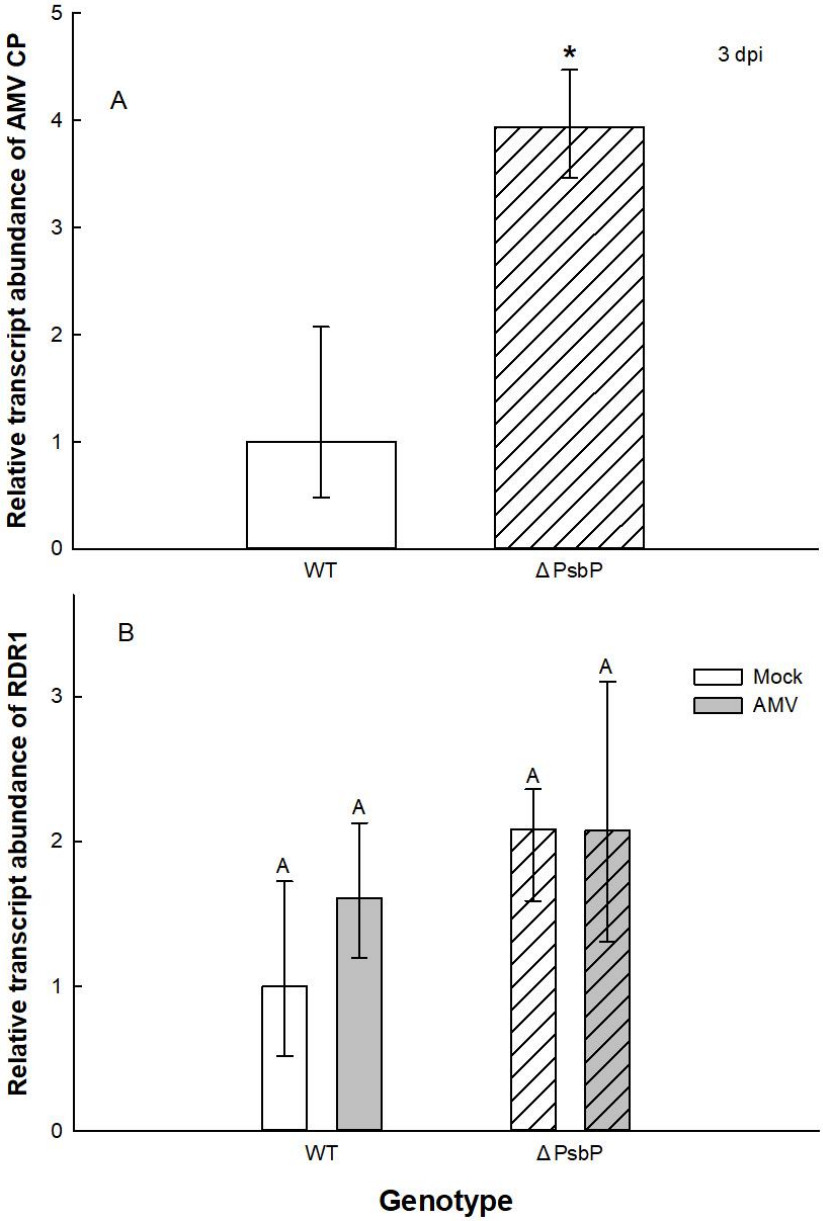

Figure 2.4 AMV infection did not induce the expression of RDR1 in *N. tabacum*.
Data represent the means ± SD of three replicates. Duncan's multiple range test was employed to determine the statistically significant differences between genotypes and treatments. Different letters and asterisk indicate significant differences at a P value of 0.05.

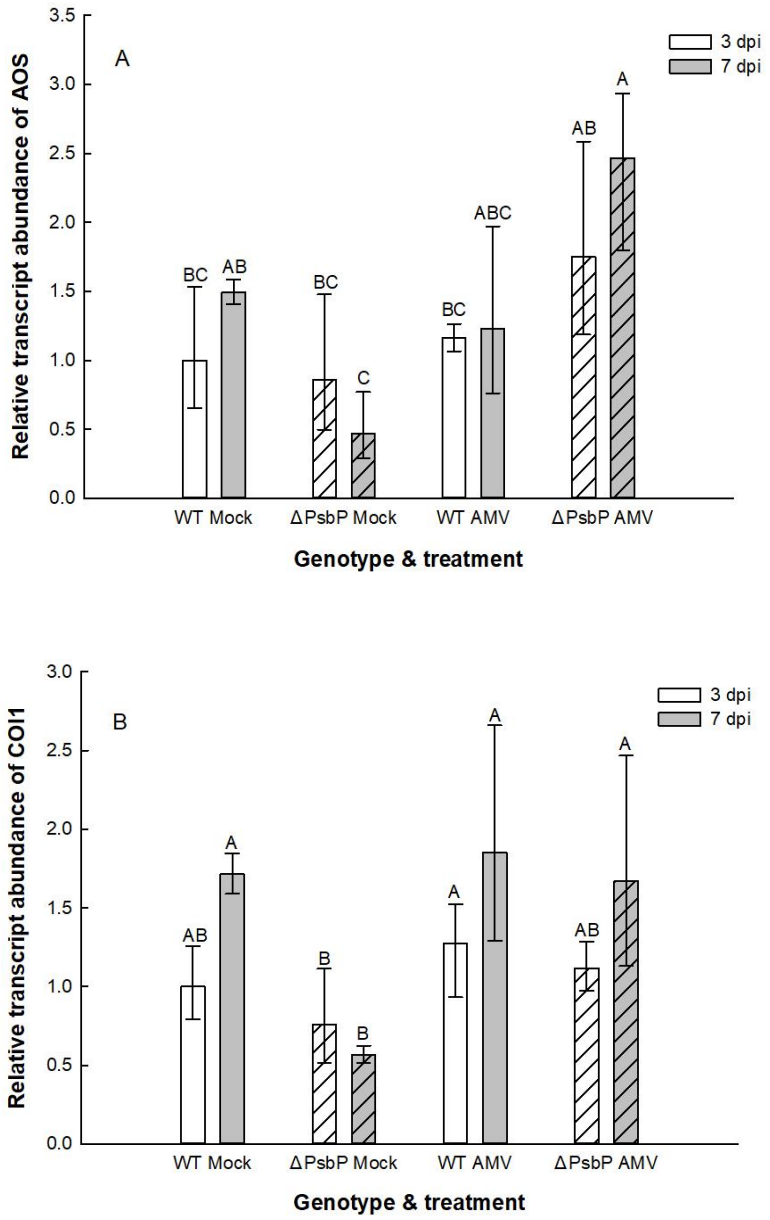

Figure 2.5 AMV infection did not influence the jasmonic acid biosynthesis.

Quantitative RT-PCR analysis of the transcript level of one JA biosynthesis enzyme, AOS (A) and one JA-response gene, *PAL* (B), in wild-type and RNAi *N. tabacum* after AMV infection at 3dpi and 7dpi. Mock indicates plants were only inoculated with phosphate buffer, pH=7.0. Data represent the means ± SD of three replicates. Duncan's multiple range test was employed to determine the statistically significant differences between genotypes and treatments. Different letters indicate significant differences at a P value of 0.05.

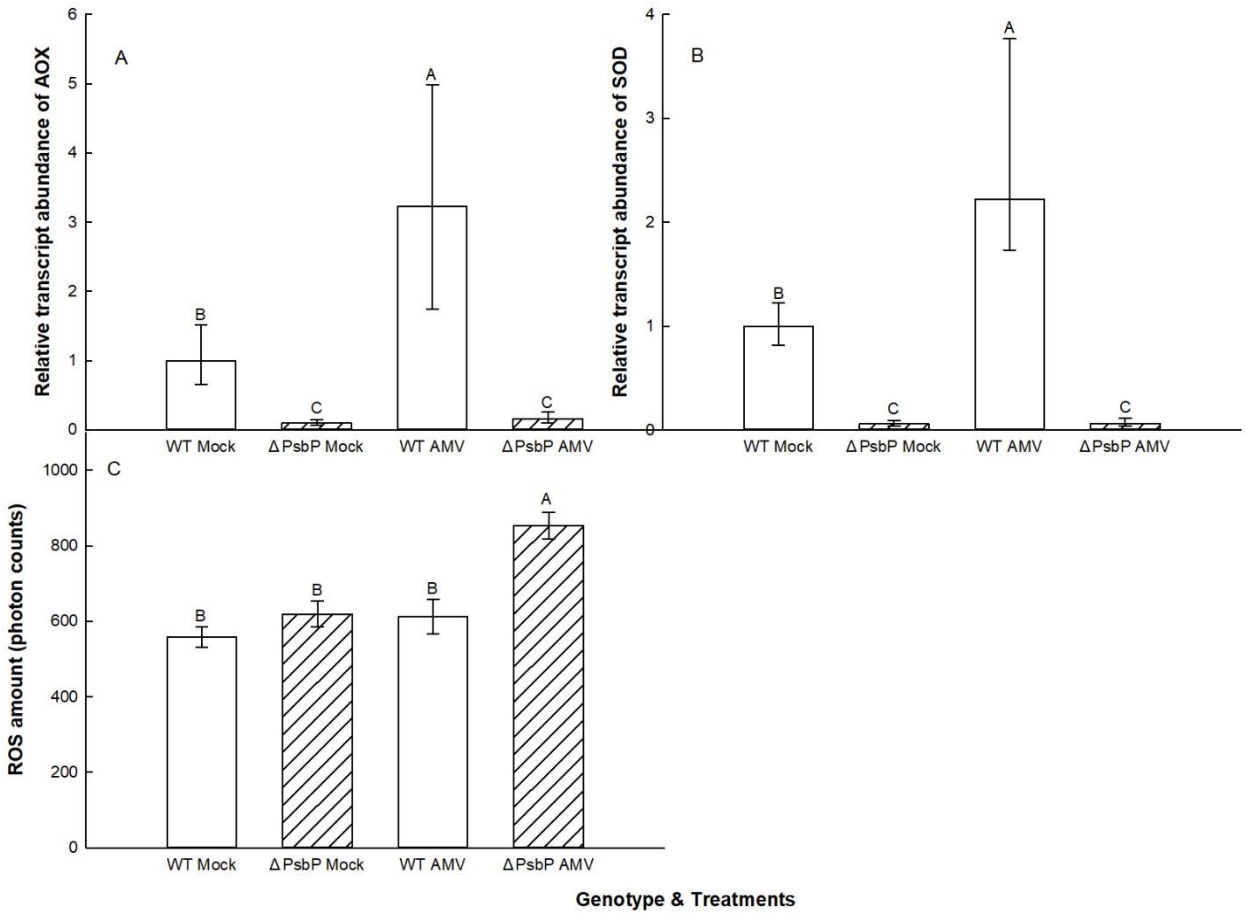

Figure 2.6 AMV infection inhibited ROS scavenging system and induced oxidative burst in ΔPsbP plants.

Quantitative RT-PCR analysis of the transcript level of two ROS scavenging enzymes, AOX (A) and SOD (B), in wild-type and RNAi *N. tabacum* after AMV infection at 5dpi. (C) The production of ROS in wild-type and RNAi *N. tabacum* after AMV infection at 5dpi. Data represent the means ± SD, three replicates for (A) and (B), and twenty-four replicates for (C). Duncan's multiple range test was employed to determine the statistically significant differences between genotypes and treatments. Different letters indicate significant differences at a P value of 0.05.

Table 2.1 Primers used for qPCR in this study

Primer No.	Name	Primer Sequences (5’ to 3’)
1	Nt18s FP	GTG CCA GCA GCC GCG GTA AT
2	Nt18s RP	CCG GTG CAC ACC TAA GGC GG
3	NtPsbP FP	ACTGATTCAGAGGGTGGATTTGAA
4	NtPsbP RP	CTTGTCACCAGCTTGTGCTTTGCA
5	NtPR-1 FP	AGGCCGTTGAGATGTGGGTCG
6	NtPR-1 RP	ACCGAGTTACGCCAAACCACCTG
7	NtICS1 FP	TGAGGGGGAGACTCCAGACTGA
8	NtICS1 RP	AGCCCGTGCATCTTCTGTAGGA
9	NtPAL FP	GGACAAGGGCAGCTATGCTAGTTA
10	NtPAL RP	CATTGAGGGTCTCACCATTAGGTC
11	NtRDR1 FP	GCATTGAACACGCCTTGGA
12	NtRDR1 RP	GCAGAACCCGATTGGATACG
13	NtCOI1 FP	TGCTTGACCGAGAGGAGAG
14	NtCOI1 RP	CGCCCGACATAACTGAGACC
15	NtAOS FP	CGGAGCGAACCCAGGTGAAAC
16	NtAOS RP	AGACCAAGAGTGACCAAAGGATGAAG
17	NtAOX FP	TATTGGACCGTCAAGGCTCT
18	NtAOX RP	TGCATCCTCTCATTTTCAGC
19	NtSOD FP	CGACACTAACTTTGGCTCCCTAGA
20	NtSOD RP	ACGTCTATTCCCAGAAGAGGAACC
21	NtPDF1.2 FP	TTGCTTGTCACGGCTAC
22	NtPDF1.2 RP	ACCGAAATTGGATACCTT
23	AtWAK1 FP	CGGATCCCAAAACCTGTAGA
24	AtWAK1 RP	GTATACAGGCAACGCCAAG
25	At18S FP	CGG CTA CCA CAT CCA AGG AA
26	At18S RP	TGT CAC TAC CTC CCC GTG TCA
27	AMV CP FP	TTCCTATGCCGTAGCCCTCT
28	AMV CP RP	GCCTTTCTCTCGACCCAAAC

CHAPTER 3. WAK1 INTERACTS WITH PSBP TO INHIBIT AMV REPLICATION

3.1 Abstract

The Arabidopsis wall-associated receptor kinase (WAK1) was reported to interact with an Arabidopsis PSII extrinsic protein (PsbP) after fungi and bacteria infection, but the function of this complex in anti-viral resistance strategy remains unknown. Here, we report that AtWAK1 and AtPsbP form a ternary complex with AMV CP on cell membrane. We validated the protein interaction data by using bimolecular fluorescence complementation assays and co-immunoprecipitation assay. Further, we found overexpression of either WAK1 or PsbP could significantly inhibit AMV replication. In addition, we have shown that the formation of ternary complex could activate WAK1 kinase activity to phosphorylate PsbP, and then induce the activation of MAPK signal pathway to increase plants resistance against AMV infection. Taken together, we report a new virus perception strategy in which plants utilize both Receptor-like kinase (WAK1) and chloroplast protein (PsbP), and transduce the signals through MAPK signaling pathway to activate defense responses mechanism.

3.2 Introduction

Receptor-like kinases (RLKs) are proteins anchored on cell membrane, and they have been discovered to play prominent roles in development and plant-pathogen interactions (Liang & Zhou, 2018; Macho & Lozano-Duran, 2019). An RLK is composed of an extracellular domain, which has the ligand binding activity to perceive the extracellular signals, such as phytohormones, polysaccharides and PAMPs, a transmembrane domain, and an intracellular kinase domain, which induces the downstream signaling transduction, such as the MAPK activation and ROS production. More than 600 RLKs have been identified in Arabidopsis, and these members are even higher (>1000) in rice. Although the functions of most of RLKs are unknown, several models indicate the RLKs play central roles in the plant immune system. For instance, the BRI1-Associated kinase (BAK1) formed a complex with Flagellin Sensing2 (FLS2) on cell membrane to recognize the bacterial flagellin, and induce the PTI responses (Chinchilla et al., 2007; Heese et al., 2007). Similarly, Arabidopsis Chitin Elicitor Receptor Kinase1 (CERK1) formed dimer on cell membrane with Lysine Motif Receptor Kinase5 (LYK5) to perceive the fungal cell wall component, chitin,

and initiate immune responses (Cao, Liang, Tanaka, & Nguyen, 2014; T. Liu et al., 2012; Miya et al., 2007).

Arabidopsis encodes five wall-associated kinases. As they belong to RLKs family, they all have an extracellular domain, a transmembrane domain, and a cytoplasmic kinase domain. The extracellular domain of all five WAKs are significantly diverged, with only 40 to 64% identity among the genes. However their cytoplasmic kinase domain are high conserved, with 86% amino acid identity (He, Cheeseman, He, & Kohorn, 1999). These indicate they could perceive varied natural signals to activate their kinase domain and initiate downstream responses. The expression of *WAK1, WAK2, WAK3,* and *WAK5* were all found with high level in leaves, while *WAK4* was only detected in siliques, and their expressions could be induced by salicylic acid. WAK1 was found important in regulating resistance against fungal and bacterial pathogens, and over-expression of WAK1 significantly inhibited the symptom development caused by *Botrytis cinerea* and *Pseudomonas syringae* (Brutus, Sicilia, Macone, Cervone, & Lorenzo, 2010; E. J. Yang et al., 2003). WAK2 was shown to play a central role in pectin-induced MAPK pathway activation, and down-regulation of *WAK2* inhibited the activity of MAPK6 (Kohorn et al., 2009). The functions of the other WAKs remain unclear.

In this study, we investigate the function of AtWAK1 and AtPsbP in regulating resistance against AMV, and also demonstrate the interaction among AtWAK1, AtPsbP and AMV CP. Firstly, a Bimolecular Fluorescence Complementation analysis was undertaken to determine the direct interaction among the three proteins. Second, the mutants of these genes were tested for their susceptibility against AMV. Lastly, in-vitro assay was conducted to assess the molecular mechanism of the formation of ternary complex is required for activating the defense responses against AMV infection.

3.3 Materials and Methods

3.3.1 Plasmid vectors.

To construct the plasmids for BiFC assay, the Gateway technology was applied follow manufacture's recommendation. The *att*B sequences were added to AMV CP, AtWAK1, and AtWAK1 kinase domain by polymerase chain reaction (PCR), respectively. The AMV CP was cloned from pSITE-CP (Balasubramaniam et al., 2014) by PCR with Primer 3 and 4, and AtWAK1

full sequences and kinase domain were amplified from pUNI51-AtWAK1 (Arabidopsis Biological Resource Center, Columbus, Ohio, USA.) by Primer 1+2, and Primer 5+6, respectively (Table 1). The amplicons were recombined into the pDONR207 entry vector by BP reactions followed manufacture's recommendation (Invitrogen, Waltham, Massachusetts, USA). All the entry vectors were sequenced with Sanger Sequencing Methods by Purdue Genomics Core Facility (Purdue University, West Lafayette, Indiana, USA) to make sure the accuracy of the inserted genes. After sequence validation, the LR reactions were conducted to recombined the insert in entry vectors to *Agrobacterium* binary pSITE vectors (Chakrabarty et al., 2007). All the pSITE vectors with inserts were confirmed by PCR. The recombined pSITE plasmids were electroporated into *Escherichia coli* DH5a at 1,500 V, 25uF, 200Ohms in 1-mm cuvettes with Electropulse generator (Bio-Rad, Gene Pulser II, Hercules, California, USA). The transformed cells were incubated at 37℃ for 3 hours after adding 600µl Lysogeny broth (LB) without antibiotics. Then spread 100µl transformed cells on LB agar plates containing 50 ug/mL spectinomycin and incubated at 28℃ for 1 day. Single colony was picked to grown in 5 mL LB medium containing 50 mM spectinomycin and incubated at 37℃ for 1 day, the plasmids were extracted with PureLink Quick Plasmid Miniprep Kit (Invitrogen, Waltham, Massachusetts, USA), and then confirmed the existence of the inserts by PCR.

To construct plasmids for yeast protein assay, WAK1-kinase domain, PsbP, and AMV CP were cloned from the recombined vectors used for BiFC assay, and TrpC promoter was cloned from pHYGT vector. In order to recombined the genes of interest into vectors, Gibson Assembly Master Mix (New England Biolabs, Ipswich, Massachusetts, USA) was used follow manufacture's recommendation. The overlapped sequences were added to genes of interest by PCR (Table 2). pYES2 vector was linearized by HindIII and NotI restriction enzyme for constructing pWAK1-K-HA-PsbP recombined plasmid, or linearized by KpnI and NotI restriction enzyme for constructing pPsbP- WAK1-K-HA recombined plasmid. pGAD vector was linearized by EcoRI and XhoI for constructing pGAD-CP-(WFP) recombined plasmid. The linearized vector and amplicon were mixed at 3:1 ratio, and then added to Gibson Assembly master mix, and incubated at 50 ℃ for 1 hour. 2µl assembly reaction was used to transform NEB 5a *E.coli* competent cells (New England Biolabs, Ipswich, Massachusetts, USA) follow manufacture's recommendation. All the recombined plasmids were sequenced with Sanger Sequencing Methods by Eurofins Scientific

(Eurofins Genomics LLC, Louisville, Kentucky, USA) to make sure the accuracy of the inserted genes.

3.3.2 *Agrobacterium*-mediated transient expression.

Nicotiana Benthamiana seeds were germinated on Murashige and Skoog Medium (MS) for one week, and then transplanted to 4-inch pots, filled with pre-mixed Miracle-Gro® Potting Mix and Osmocote (ScottsMiracleGro), with a 16-h-light/ 8-h-night daylight cycle at 25℃. Plants would be used for agrobacterium infiltration at 4 to 5 weeks old. The recombined pSITE plasmids were electroporated into *Agrobacterium tumefaciens* at 1,800 V, 25uF, 200Ohms in 1-mm cuvettes with Electropulse generator (Bio-Rad, Gene Pulser II, Hercules, California, USA). The transformed cells were incubated at 28℃ for 3 hours after adding 600µl Yeast Extract Peptone (YEP) without antibiotics. Then spread 100µl transformed cells on YEP agar plates containing 50 ug/mL spectinomycin and 100 ug/mL Rifampicin incubated at 28°C for 1 day. Single colony was picked to grown in 5 mL YEP medium containing 50 mM spectinomycin and 100 ug/mL Rifampicin and incubated at 28℃ to an OD of 0.5, and bacterial PCR was conducted to validate the existence of inserts. Remove all the YEP medium and resuspended the bacteria in Agroinfiltration buffer, which contains 10mM $MgCl_2$, 10mM morpholinoethanesulfonic acid, pH5.7, and 150uM acetosyringone, and incubate the *Agrobacterium tumefaciens* for 8 hours at room temperature. The induced agrobacterium was infiltrated into *N. Benthamiana* from the abaxial side of leaves with 1 mL syringe.

3.3.3 Quantitative real-time PCR and analysis.

The agroinfiltrated *N. Benthamiana* plants were propagated with a 16-h-light/ 8-h-night daylight cycle at 25℃, and the infiltrated leaves were dusted with carborundum and mechanically inoculated with 10ug/ml AMV, strain 425 Madison, at 2 days post-infiltration. The infiltrated and inoculated leaves were harvested at 2 days post-inoculation, and used for RNA extraction.

The total RNA isolated from all tissues were treated with DNase (RQ1 RNase-Free DNase, Promega) according to manufacturer's protocol to remove any DNA. RNeasy Mini Kit (Qiagen) was used with all the samples to purify the RNA.

cDNA was synthesized from the purified RNA by using the M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin, USA) according to the manufacture's protocol. 1µg purified RNA was added with 0.4 µl random hexamer (Promega, Madison, Wisconsin, USA), 5 µl M-MLV RT 5x reaction buffer, 1.25 µl dNTPs, 0.5 µl M-MLV RT(H-) point mutant, and then nuclease-free H_2O to a final volume of 25 µl. The reaction was incubated at room temperature for the initial 10 minutes, and followed with 55°C incubation for 50 minutes. Inactivate the activity of M-MLV Reverse Transcriptase by heating for 15 minutes at 70°C.

Quantitative PCR was performed by using the PowerUp SYBR Green Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA) and followed manufacture's protocol. Each reaction contains 5µl of PowerUp SYBR Green Master Mix (2x), 2µl of each primer pair (10uM), 2µl of cDNA template, and 1µl nuclease-free H_2O. CFX Connect Real-Time PCR Detection System (Bio-Rad) was used set to the reaction condition of 2 minutes at 95℃, 40 cycles of 5 s at 95°C and 30 s at 60°C. The primer efficiency of each pair was determined by constructing efficiency curves of a series dilution of cDNA template to make sure the efficiency is higher than 94%. 18S rRNA was used as internal control for all the experiments, and $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression level of different genes (Livak & Schmittgen, 2001). All experiments were performed in triplicate and repeated at least three times, and all the statistical analysis were performed with Duncan's multiple range test in SAS 9.2 (SAS Institute Inc., Cary, NC, USA).

3.3.4 Protoplast isolation

Harvest infiltrated leaves, and cut into small pieces (2mm*5mm). Immerse the leaves with an enzyme digestion solution, which contains 2% cellulysin (Calbiochem, San Diego, California, USA), 0.1% pectolyaseY-23 (MP Biomedical, Solon, Ohio, USA) and 10% mannitol. Briefly vacuum all the content, and then incubate for 60-90 minutes at 30℃ with gently shaking. Pellet protoplasts by centrifugate at 50 xg for 5 minutes, and then resuspend with 400mM mannitol. Purify the protoplasts with 20% sucrose cushion by centrifuge at 50 xg for 2 minutes, and resuspend the final pellet with 400mM mannitol.

3.3.5 Confocal imaging assay

The purified protoplasts were loaded on microscope slides, and examined by Zeiss LSM 880 Upright Confocal microscope. eYFP was detected by excitation at 513nm with a 523 to 538 nm band pass emission filter. mRFP was detected by excitation at 555nm with a 584 to 600 nm band pass emission filter.

3.3.6 Co-immunoprecipitation assay and Western blot assay

For co-immunoprecipitation (co-IP) assay, the antibodies were bounded to Dynabeads™ Protein A (Invitrogen, Waltham, Massachusetts, USA) by rotating at 4°C for 1 hour. Wash the beads with PBS, and then cross-linked the antibodies to Dynabeads™ Protein A with 25 mM bis(sulfosuccinimidyl)suberate (ThermoFisher Scientific, Waltham, Massachusetts, USA) by rotating 30 minutes at room temperature. Wash the conjugated beads two times with quenching buffer, which contains 1M Tris•HCl, pH 7.5, and rotate at room temperature for 5 minutes. To remove the excess unlinked antibody, wash the beads with 1 M glycine, pH 3.0, by rotating at room temperature for 10 minutes. Resuspend beads with PBS, and the beads would be used for co-IP assay to detect protein interactions.

Homogenized 400 mg infiltrated *N. Benthamiana* leaves with 150 µl NP-40 buffer, which contains 150mM NaCl, 20mM Tris•HCl, pH 8.0, 1% NP-40, and 2mM EDTA. Vortex the content for 1 minute, and centrifuge at 16,000 xg for 20 minutes at 4°C. 100 µl of the supernatant was incubated with 50 µl conjugated beads by rotating at 4°C overnight. Wash the beads with NP-40 buffer for three times, and then resuspend the beads in 50 µl PBS and 10 µl 6X SDS loading buffer. Boil the beads at 95°C for 20 minutes, and remove the beads with a magnet. Proteins were separated by 13% SDS-PAGE gel, and then transferred to nitrocellulose membranes (Bio-Rad, Hercules, California, USA), and probed with appropriate antibodies. Membrane was probed by polyclonal anti-GFP antibody (1:5000 dilution) (Abcam, Cambridge, Massachusetts,USA), or monoclonal anti-CP antibody (1:1000 dilution). Primary antibodies were detected with peroxidase conjugated Rabbit IgG (H&L) Secondary Antibody (1:10,000 dilution) (Rockland, Limerick, Pennsylvania, USA), or peroxidase conjugated Goat Anti-Mouse IgG H&L (Abcam, Cambridge, Massachusetts,USA). The signal was visualized with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, Massachusetts, USA).

3.3.7 In vitro yeast protein interaction assay

The recombined plasmids were chemical transformed into yeast cells by using Alkali-Cation Yeast Transformation Kit (MP Biomedicals, Solon. Ohio, USA). Single type of recombined plasmid was transformed into INVSc1, *S. cerevisiae* Yeast Strain, and two types of recombined plasmids were transformed into Y2HGold, *S. cerevisiae* Yeast Strain. 100 ng plasmid DNA mixed with 100 µl yeast competent cells was incubated for 15 minutes at room temperature, and then, 1 ml PEG/TE Caution mix was added to the reaction mix, and incubate at 30 ℃ for 10 minutes. Heat shock the reaction mix for 15 minutes at 42℃ to allow the plasmid DNA enter yeast cells, and then cool down to 30°C. The transformed yeast cells were resuspended in SOS medium and spread on appropriate yeast synthetic drop-out media, which contains 7.6g/L Yeast Nitrogen Base without Amino Acids, 182.2g/L D-sorbitol, 2% glucose, 1.4g/L Yeast Synthetic Drop-out Medium Supplements (Y2001, Sigma-Aldrich, St. Louis, Missouri, USA), and supplemented amino acid as needed (20 mg/L Histidine, 20 mg/L Tryptophan, 20 mg/L Uracil or 60 mg/L Leucine). pWAK1-K-HA-PsbP and pPsbP- WAK1-K-HA were grown on -Ura media, pGAD-CP-(WFP) was grown on -Leu media, and double plasmid transformed cells were grown on -Ura/-Leu media. Single colony PCR was conducted to validate the existence of inserts.

Single colony was grown in 5ml yeast drop-out media with 2% glucose overnight at 30°C, and then increase the volume to 100 ml drop-out media with 2% raffinose and 2% galactose to activate the expression of gene of interest for 4-6 hour. The cells were harvested by centrifuge at 3000 rpm for 5 minutes, and then lysed with NP-40 buffer and equal volume 0.5mm acid-washed glass beads by vortexing for 5 minutes at 4℃. Proteins were separated by 13% SDS-PAGE gel.

3.4 Results

3.4.1 PsbP, WAK1 and AMV CP form ternary complex on cell membrane.

To determine the interaction among PsbP, WAK1 and AMV CP, BiFC assay was conducted. Each protein of interest was tagged with partial yellow fluorescent protein (YFP), and the direct protein-protein interaction could reconstitute the YFP to emit fluorescence. Plasmids nomenclature followed the convention that ^{n}Y indicates the N-terminal fragment of YFP, and ^{c}Y indicates the C-terminal fragment of YFP. Besides, the placement of the tag to left or right of the proteins indicates the tag was fused to the N-terminal or C-terminal of the proteins of interest,

respectively. Different combinations of reconstructed plasmids were agroinfiltrated into *N. benthamiana* leaves for BiFC assay. To make sure the BiFC-induced fluorescence resulted from protein-protein interaction, co-agroinfiltration of AtWAK1-^{c}Y and free ^{n}Y- didn't emit any fluorescence (Fig.1). Fluorescence was detected by co-infiltration with AtWAK1-^{c}Y and ^{n}Y-AtPsbP, but not when the leaves were co-infiltrated with AtWAK1-^{c}Y and ^{n}Y-CP. Therefore, the *N. benthamiana* leaves were co-agroinfiltrated with AtWAK1-^{c}Y, ^{n}Y-AtPsbP and RFP-CP, and the co-localization of yellow fluorescence and red fluorescence was observed on cell membrane as expected (Fig. 1).

Co-immunoprecipitation assay was also conducted to validate the BiFC assay data. Anti-PsbP beads pulled down both AtPsbP and AtWAK1-kinase domain (WAK1-K) from the co-infiltrated leaves (Fig. 2, Lane ①). While, when plants infiltrated with AtWAK1-K and CP, no AtWAK1-K could be pulled down by Anti-CP beads (Fig. 2, Lane ②). When plants were co-infiltrated with all three proteins together, both anti-PsbP beads and anti-CP beads could pull down all three proteins, as shown in Figure 2, Lane ④ and Lane ⑤.

These data indicate that AtPsbP, AtWAK1 and AMV CP interact with each other directly, and form the ternary complex on the cell membrane, and AtPsbP is required for the formation of the ternary complex.

3.4.2 Overexpression of PsbP and WAK1 inhibit AMV replication.

As we identify that AtWAK1, AtPsbP and AMV CP formed a trimer on cell membrane, we need to characterize the function of AtWAK1 on plant resistance against AMV infection. Firstly, we used Arabidopsis T-DNA insertion mutant to check the impact of down-regulation of AtWAK1 on plants resistance. As shown in Figure 3, the *wak1* mutant has no more than 10% transcript abundance compared with wild-type plants. These plants were inoculated with AMV. The relative amount of AMV CP RNA was four-fold higher in *wak1* than wild-type plants. Meanwhile, over-expression assay was conducted to confirm the function of AtWAK1 in anti-virus defense pathway. *N. benthamiana* plants were agroinfiltrated with AtWAK1-^{c}Y, ^{n}Y-AtPsbP individually or simultaneously, and then inoculated with AMV mechanically. The transcription of both AtWAK1 and AtPsbP can be detected after agroinfiltration, but not in buffer treated plants (Fig 4A, 4B). Furthermore, the buffer treated plant accumulated significantly higher amount AMV than all the other three agroinfiltrated plants (Fig. 4C).

Our data is consistent with previous study that over-expression of AtPsbP increases plant resistance against AMV infection (Balasubramaniam et al., 2014). We also found that over-expression of AtWAK1 can lead to the similar results to AtPsbP, where AMV replication was inhibited. These results indicate both AtPsbP and AtWAK1 are positive regulators of AMV resistance, and impairment of either one can disturb the defense pathway.

3.4.3 CP activates WAK1 to phosphorylate PsbP

Previous study shown AtPsbP interact with AMV CP dimer, in combine with our results that AtPsbP binds to AtWAK1 kinase domain to form a ternary complex with AMV CP, we hypothesize AMV CP can activate AtWAK1 kinase activity, and then phosphorylate AtPsbP. AtPsbP and AtWAK1 were expressed in yeast cells, and co-expressed with or without AMV CP. Whole cell lysate was analyzed to detect the phosphorylation state of AtPsbP (Fig. 5). When AtPsbP was expressed individually (Fig. 5③), or with AtWAK1 (Fig. 5④), no phosphorylated AtPsbP could be observed. In contrast, the phosphorylated AtPsbP was detected after co-expressing AMV CP in yeast cells. As we already know, the C-terminus of AMV CP is important for CP dimerization, and the CP-WFP mutant will not dimerize, and also will not interact with AtPsbP. Thus, we also co-expressed CP-WFP with AtPsbP and AtWAK1 in yeast cells, and no phosphorylated AtPsbP could be detected (Fig. 5⑥). These results indicate that AMV CP is required for activating the AtWAK1 kinase activity, and induce the phosphorylation of AtPsbP by AtWAK1.

3.4.4 The activation of MAPK pathway is WAK1/PsbP dependent

MAP kinase signaling pathway has been proved to be important in regulating plant resistance against pathogen infection, and can be activated by PAMPs and MAMPs (Meng & Zhang, 2013; Sun et al., 2018). We hypothesize AMV infection can also induce the activation of MAPK pathway, and WAK1 and PsbP are required for the activation. To determine the status of MAPK pathway, recombinant plasmids (AtWAK1-^{c}Y, ^{n}Y-AtPsbP, AMV CP and CP-WFP) were agroinfiltrated into *N. benthamiana* separately or simultaneously, and total protein was extracted at 3dpi.

Infiltration of individual plasmid did not affect the amount and phosphorylation status of MAPK pathway. Similar result was observed when we only infiltrated AtWAK1-^{c}Y and ^{n}Y-

AtPsbP without AMV CP into plants (Fig. 6). In contrast, the MPK3/MPK6 was phosphorylated when AMV CP was co-agroinfiltrated with AtWAK1-^{c}Y and ^{n}Y-AtPsbP. Furthermore, when we co-infiltrated AMV CP-WFP with AtWAK1-^{c}Y and ^{n}Y-AtPsbP, we did not observe the phosphorylated MPK3/MPK6.

These data indicate plants will activate the MAPK signaling pathway to defend against AMV infection. Besides, AtWAK1/AtPsbP complex is required for perceiving the AMV infection, and failure to form of the ternary complex will also impact the downstream activation of MAPK signaling pathway.

3.5 Discussion

Our studies reveal a new mechanism that plant viruses can be perceived by RLKs to initiate defense responses. Previous studies have demonstrated the importance of RLKs and Receptor-like cytoplasmic kinases (RLCKs) in plant defense system (Kinases et al., 2020; Zhou & Zhang, 2020). The well-established system is that of BAK1 interacting with FLS2 to perceive flagellin to initiate defense against fungi infection. Various geminivirus-encoded C4 proteins have been identified to interact with RLKs to impact plants defense responses. The C4 from tomato yellow leaf curl virus which interacts with BAM1, BRI1, FLS2, etc. (Gómez et al., 2019; Zeng, Liu, Yang, & Lai, 2018), and C4 from beet severe curly top virus interacts with CLV1 (H. Li et al., 2018). Our co-immunoprecipitation assay showed that AMV CP, functioning as an effector, could bind to AtPsbP, form a ternary complex with AtWAK1 on the cell membrane, and then induce plant defense to increase plant resistance against AMV infection. Instead of moving into chloroplast, AtPsbP hijacked AMV CP in cytosol, which has been observed (Balasubramaniam et al., 2014), and then transported the AMV CP to cell membrane, and interact with AtWAK1 cytoplasmic kinase domain. PsbP is one core protein of PSII, and regulates photosynthesis, but AMV infection disrupts the movement of PsbP to the chloroplast. This is probably a trade-off strategy plants adapted to shift resources from development to plant immunity (Denancé, Sánchez-Vallet, Goffner, & Molina, 2013; Wu, Valli, García, Zhou, & Cheng, 2019).

This interaction between AMV CP and AtWAK1 required the presence of AtPsbP, but interestingly, the absence of AtPsbP did not abolish the AtWAK1 mediated defense response. Transient overexpression of AtWAK1 did inhibit AMV replication in *N. benthamiana*, while the virus amount is similar to overexpression of PsbP individually or simultaneously. PsbP is the key

extrinsic protein of PSII, and it can be found from cyanobacteria to plants, and lots of homologs have been identified from various species (Bricker et al., 2012; Thornton et al., 2004), so it is possible that the presence of NtPsbP compensates for the function of AtPsbP to interact with AtWAK1 to activate the defense responses against AMV infection. Park and colleagues found the interaction between extracellular domain of AtWAK1 and GRP3 was required for regulating pathogen defense response (Park et al., 2001). Furthermore, overexpression of WAK1 has been confirmed to increase plants resistance against *P. syringae* and *B. cinereal* (Gramegna et al., 2016; E. J. Yang et al., 2003). All the aforementioned results require the extracellular domain of WAK1 to perceive the signals from pathogens. In contrast, AMV CP only interacts with the intracellular kinase domain of AtWAK1 with the help of AtPsbP. It is possible that AMV infection can induce the expression of some molecular signals, similar to GRP3, but not yet identified, and release to the outside of cell wall to activate the WAK1/PsbP pathway and increase plant resistance. Kohorn demonstrated that pathogens infection promotes de-esterified of pectin polymers to form oligogalacturonides (OGs). Because OGs have higher affinity to WAK1 than pectin, OGs would compete to bind to WAK1 and activate defense responses (Kohorn, 2016). This can be another possibility that AMV infection induces the fragmentation of pectin polymers, and then activate the WAK1/PsbP pathway.

Previous studies have proved that the AtPsbP would be phosphorylated by AtWAK1 after infection by *P. syringae*, and the phosphorylation of AtPsbP is AtGRP3-dependent (Park et al., 2001; E. J. Yang et al., 2003). In this study, we did not observe the transcription changes of AtGRP3 after AMV infection. Instead, we found that phosphorylation of AtPsbP by AtWAK1 is AMV CP-dependent. This indicates a different signal transduction pathway that plants can perceive the intracellular pathogen signals, and then transduce the signal to nucleus to initiate defense responses, such as *PR-1*. In the well-established receptor-kinase (RK)/RLCK regulated defense response (Liang & Zhou, 2018), RKs have highly variable extracellular domains to recognize the different PAMPs or MAMPs, such as WAKs family, which all have conserved the transmembrane and kinase domain, but varied ectodomain. Another example is Botrytis-induced kinase1 (BIK1), one type of RLCKs, that interacts with BAK1 to induce downstream defense responses after detection of flg22 or PEPR1 (Z. Liu et al., 2013; Lu et al., 2009). As plant virus infection is so different from fungal or bacteria infection, only the intracellular domain, not the ectodomain, has the chance to interact with virus-encoded proteins. In order to differentiate the

various virus effectors, host RKs need to work in concert with “decoys” or “baits” to regulate plants defense system (Collier & Moffett, 2009; Van der Hoorn & Kamoun, 2008). In this case, AtPsbP is the cofactor to AtWAK1, which binds to AMV CP, and then activate the kinase activity. Unlike the classic NB-LRR receptor recognize effectors and then induce defense responses, AMV effector CP binds to AtPsbP, which is not a NB-LRR encoded protein. The existence of AtPsbP is vital in forming the ternary complex on membrane and inhibiting virus replication. This all implies a potential new pathway for plant hosts to recognize virus invasion, which is independent of RKs/RLCKs and NB-LRR/effectors regulating pathways.

Several chloroplast proteins have been identified playing roles in regulating plant resistance against pathogens. For instance, down-regulation of PsbO significantly decrease plants resistance against TMV, AMV and PVX infection (Abbink et al., 2002), and transcription of PsbQ was dramatically inhibited by PMMoV infection (Y. Li et al., 2016; Pérez-Bueno et al., 2004; Pineda, Sajnani, & Barón, 2009). However, the mechanisms by which chloroplast proteins recognize and interact with virus-encoded proteins are unclear. In this study, we firstly found that a chloroplast protein, AtPsbP, helps to recognize virus effector, AMV CP, and activates plant defense responses by forming ternary complex on cell membrane with AtWAK1. The formation of this ternary complex activates the MAPK cascades. The activation of MAPK cascades is a major signaling transduction event after pathogen infection, and MPK3/6 cascade has been confirmed to play pivotal roles in response to fungi infection, by inducing the downstream defense-related genes expression, such as VSP1 and ERF104 (Bi et al., 2018; Gust et al., 2009; Klessig, Menke, & Pelt, 2004; Zhou & Zhang, 2020), but none of them found the potential immunity roles of MPK3/6 cascade against virus infection. In this study, we observe the activation of MPK3/6 is also required for plant anti-viral defense responses. The formation of ternary complex promotes the phosphorylation of MPK3/6 in plants, and this phosphorylation status change definitely differentiate plants transcriptomics, which inhibits the virus replication in turn. While the defense-related genes induced by MPK3/6 cascade remain to be determined, we confirmed that MAPK cascades helps to regulate anti-viral immunity in planta.

In summary, we found AtPsbP is the receptor that recognizes AMV effector, CP, and transport to cell membrane to form a ternary complex with AtWAK1. AMV CP activates the kinase activity of AtWAK1, which phosphorylates AtPsbP after activation. Furthermore, the MPK3/6 cascade regulated defense pathway is activated by the formation of this ternary complex, and is required

for inhibiting virus replication. This is a new mechanism helps to understand how plants perceive viral signals, and transduces the defense signals from cell membrane to nucleus through MAPK cascades.

Table 3.2 Primers used for BiFC plasmid construct in this study

Primer No.	Name	Primer Sequences (5’ to 3’)
1	AtWAK1 Forward primer	GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TTC-ATG-AAG-GTG-CAG-GAG-GGT-TTG-TTC
2	AtWAK1 Reverse primer with C' tag	GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTC-GCG-GCC-AGT-TTC-AAT-GTC-CAA
3	AMV CP Forward primer	GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TCC-ATG-AGT-TCT-TCA-CAA-AAG-AAA-GCT
4	AMV CP Reverse primer without C' tag	GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTC-TCA-ATG-ACG-ATC-AAG-ATC-GTC-AGC
5	AtWAK1-Kinase domain Forward primer	GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TTC-ATG-CTG-GGT-CAG-GGT-GGC-CAA-GGA-ACA
6	AtWAK1- Kinase domain Reverse primer with C' tag	GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTC-GCG-GCC-AGT-TTC-AAT-GTC-CAA

Table 3.3 Primers used for yeast protein assay plasmid construct in this study

Primer No.	Name	Primer Sequences (5' to 3')	Note
7	YES-WAK-K-C-FP	CTGTAATACGACTCACTATAGGGAATATTAA GCTTATGCTGGGTCAGGGTGGC	Clone WAK1-K-CYC into pYES2 vector for constructing pWAK1-K-HA-PsbP.
8	WAK-CYC-RP	ATTACATGATGCGGCCCTCAAGCGTAATCTG GAACATCGTATGGGTAGCGGCCAGTT	
9	WAK-CYC-FP	AACTGGCCGCTACCCATACGATGTTCCAGATT ACGCTTGAGGGCCGCATCATGTAAT	
10	YES-WAK-K-C-RP	CACACTGGCGGCCGTTACTAGTGGATCCGAGC TCGGTACCGCAAATTAAAGCCTTCGA	
11	YES-TrpC-PsbP-FP	CTCGGATCCACTAGTAACGGCCGCCAGTGTGCT GGAATTCATTTTTTGGGCTTGGCTG	Clone TrpC-PsbP into pYES2 vector for constructing pWAK1-K-HA-PsbP.
12	TrpC-PsbP-RP	CGCACTGTACGCCATTTGGATGCTTGGGTAGAATA	
13	TrpC-PsbP-FP	TATTCTACCCAAGCATCCAAATGGCGTACAGTGCG	
14	YES-TrpC-PsbP-RP	TACATGATGCGGCCCTCTAGATGCATGCTCGAGCG GCCGCTCAAGCAACACTGAAAGA	
15	YES-PsbP-C-FP	TACGACTCACTATAGGGAATATTAAGCTTGGTACCA TGGCGTACAGTGCGTGT	Clone PsbP-CYC into pYES2 vector for constructing pPsbP-WAK1-K.
16	PsbP-CYC-RP	ACTAATTACATGATGCGGCCCTCAAGCAACACTGAA	

Table 3.2 continued

17	PsbP-CYC-FP	TTCAGTGTTGCTTGAGGGCCGCATCATGTAATTAGT	Clone PsbP-CYC into pYES2 vector for constructing pPsbP-WAK1-K.
18	YES-PsbP-C-RP	GAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCC GCAAATTAAAGCCTTCGA	
19	YES-TrpC-WAK-K-FP	GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCA TTTTTTGGGCTTGGCTGGA	Clone TrpC-WAK1-K into pYES2 vector for constructing pPsbP-WAK1-K.
20	TrpC-WAK-K-RP	TTGTACACTGTTCCTTGGCCACCCTGACCCAGCATTTGG ATGCTTGGGTAGAAT	
21	TrpC-WAK-K-FP	ATTCTACCCAAGCATCCAAATGCTGGGTCAGGGTGGCCA AGGAACAGTGTACAA	
22	YES-TrpC-WAK-K-RP	TACATGATGCGGCCCTCTAGATGCATGCTCGAGCGG CCGCTCAAGCGTAATCTGGAAC	
23	YES-GAD-CP-FP	ACGCTCATATGGCCATGGAGGCCAGTGAATTCTGAG GGATGAGTTCTTCACAAAAG	Clone AMV CP or CP-WFP into pGAD vector.
24	YES-GAD-CP-RP	GGGGTTTTTCAGTATCTACGATTCATCTGCAGCTCG AGTCAATGACGATCAAGATC	

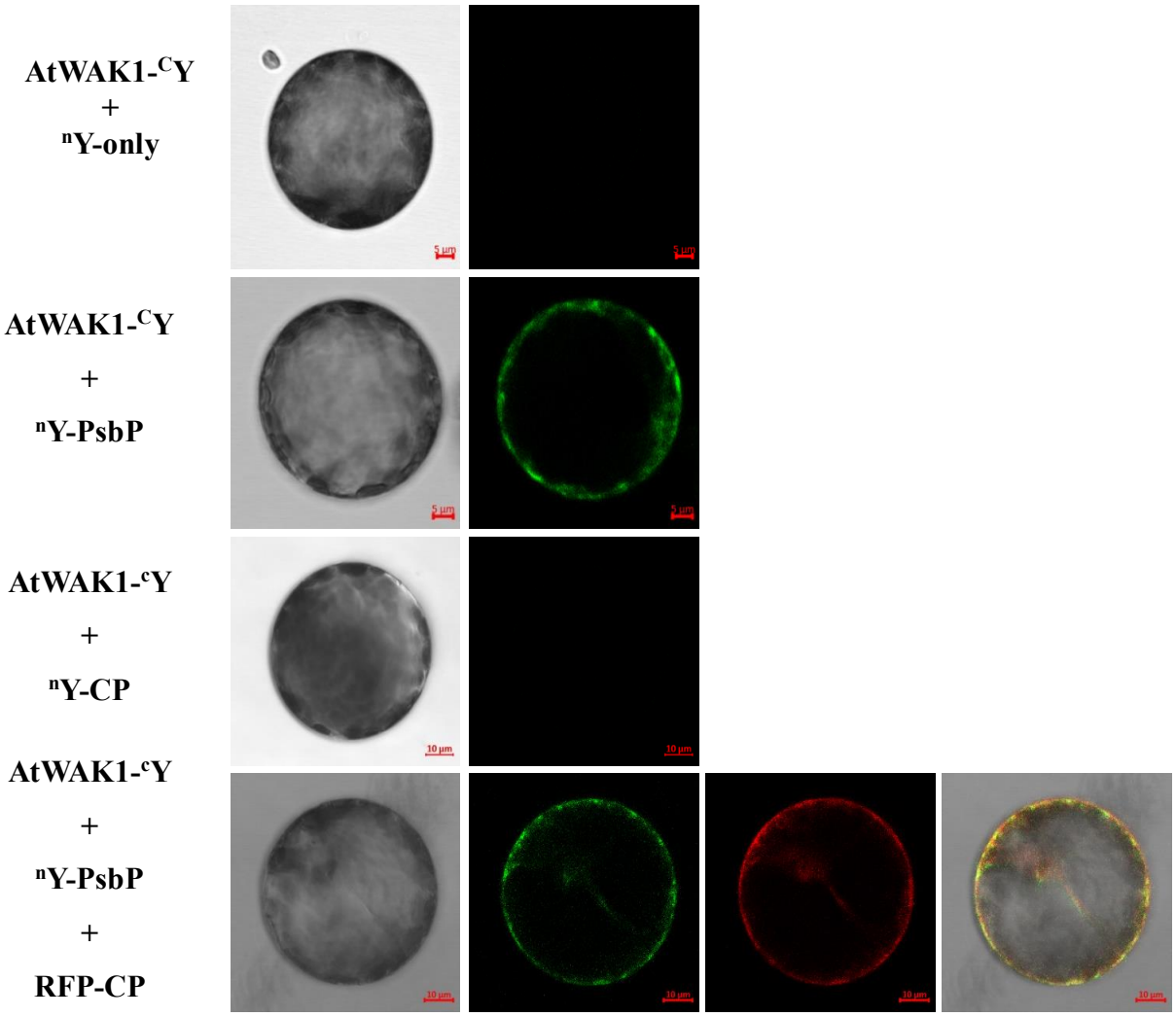

Figure 3.1 AtPsbP interacts with AtWAK1 to form a ternary complex with AMV CP at the cell membrane.

Reconstructed PsbP, WAK1 and AMV CP plasmids were co-agroinfiltrated into *N. Benthamiana* leaves, and the infiltrated leaves were harvested 2 days post infiltration to isolate protoplasts. The protoplasts were observed by confocal microscope to detect BiFC induced fluorescence. For each sample, infiltrated plasmids are listed at the left corresponding to each row of images. Scale bar represents 5 um or 10 um

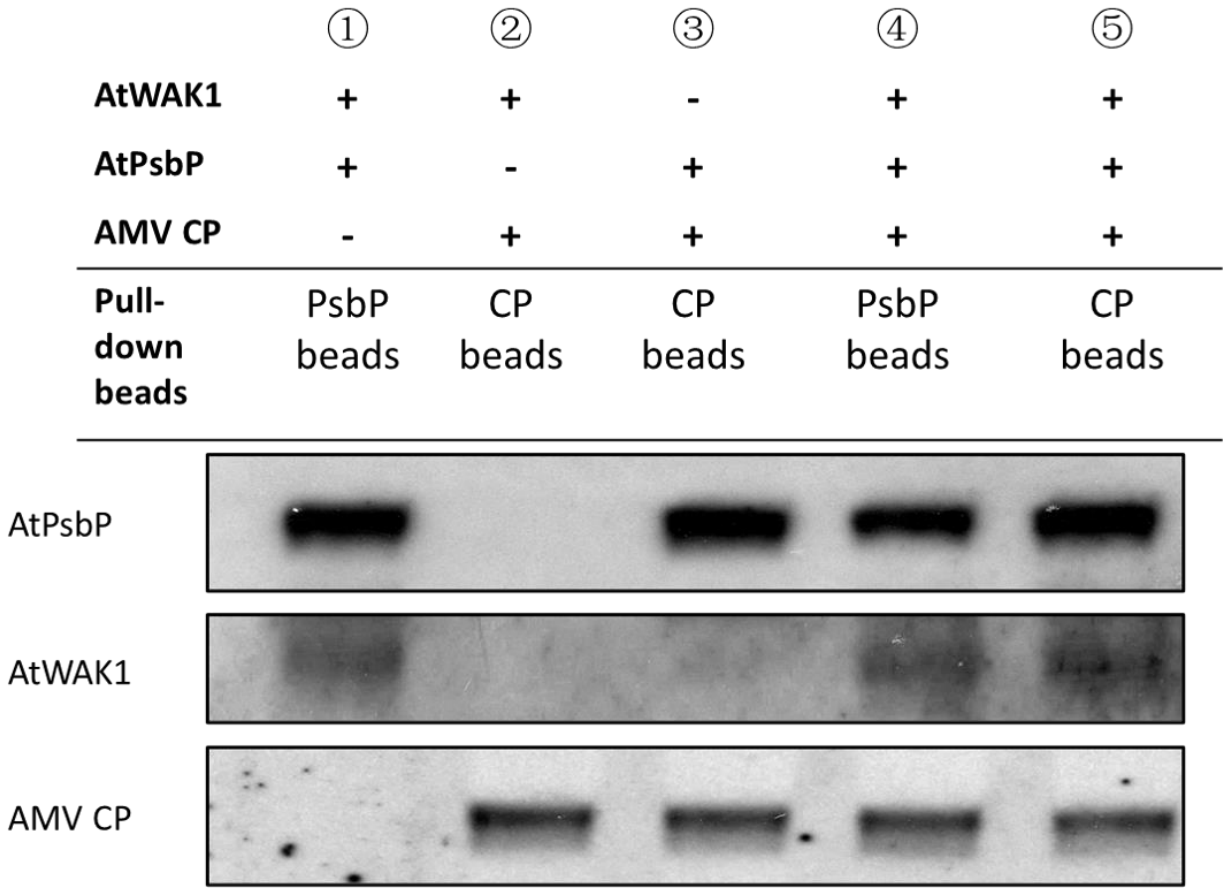

Figure 3.2 Western blot to validate the interaction of AtWAK1, AtPsbP and AMV CP.

Total protein was extracted from the agroinfiltrated leaves with NP-40 buffer, then pull-down by anti-PsbP or anti-CP, and pull-down proteins were separated by 10% SDS-PAGE gels and visualized by using anti-GFP antibody. Combination of reconstructed plasmids are listed at the top corresponding to each lane. Lane ① and ④ were co-immunoprecipitated with anti-PsbP beads, and lane ②, ③ and ⑤ were co-immunoprecipitated with anti-CP beads.

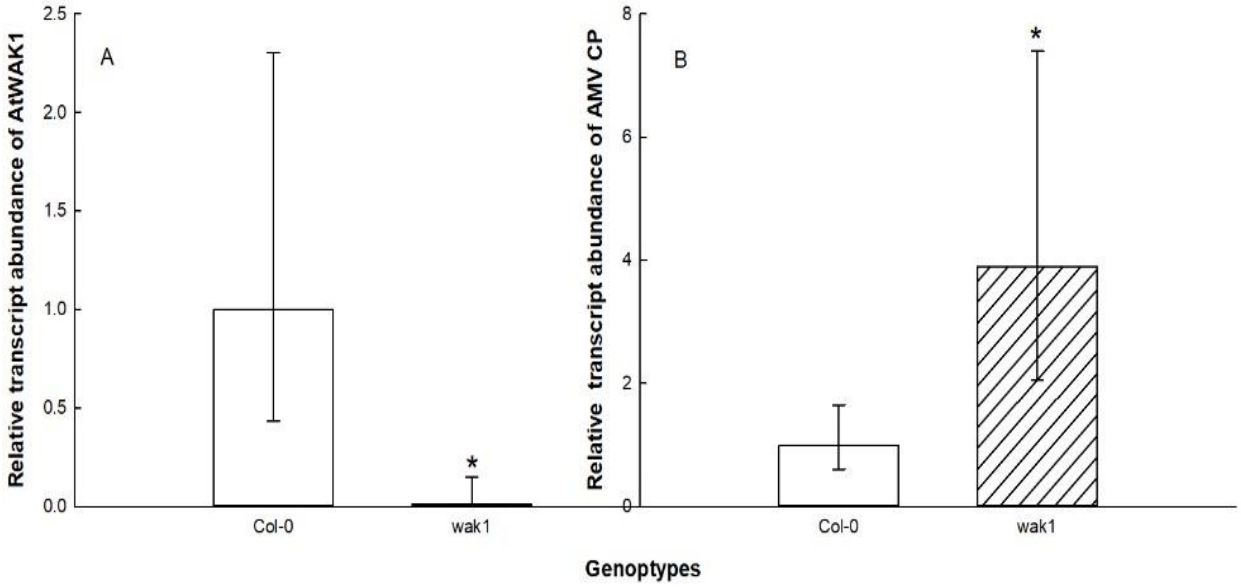

Figure 3.3 Down-regulation of WAK1 lead to higher accumulation of AMV in *Arabidopsis thaliana* plants.

(A) Quantitative RT-PCR analysis of transcript level of *AtWAK1* in wild-type and down-regulated plants without virus infection. (B) The relative amount of AMV CP in wild-type and down-regulated Arabidopsis plants was checked at 5dpi by RT-qPCR. n=3 samples were analyzed for every experiment, and data represent the means ± standard deviations. Student's t-test was employed to determine the statistically significant differences between genotypes. Asterisk indicate significant differences at a P value of 0.05.

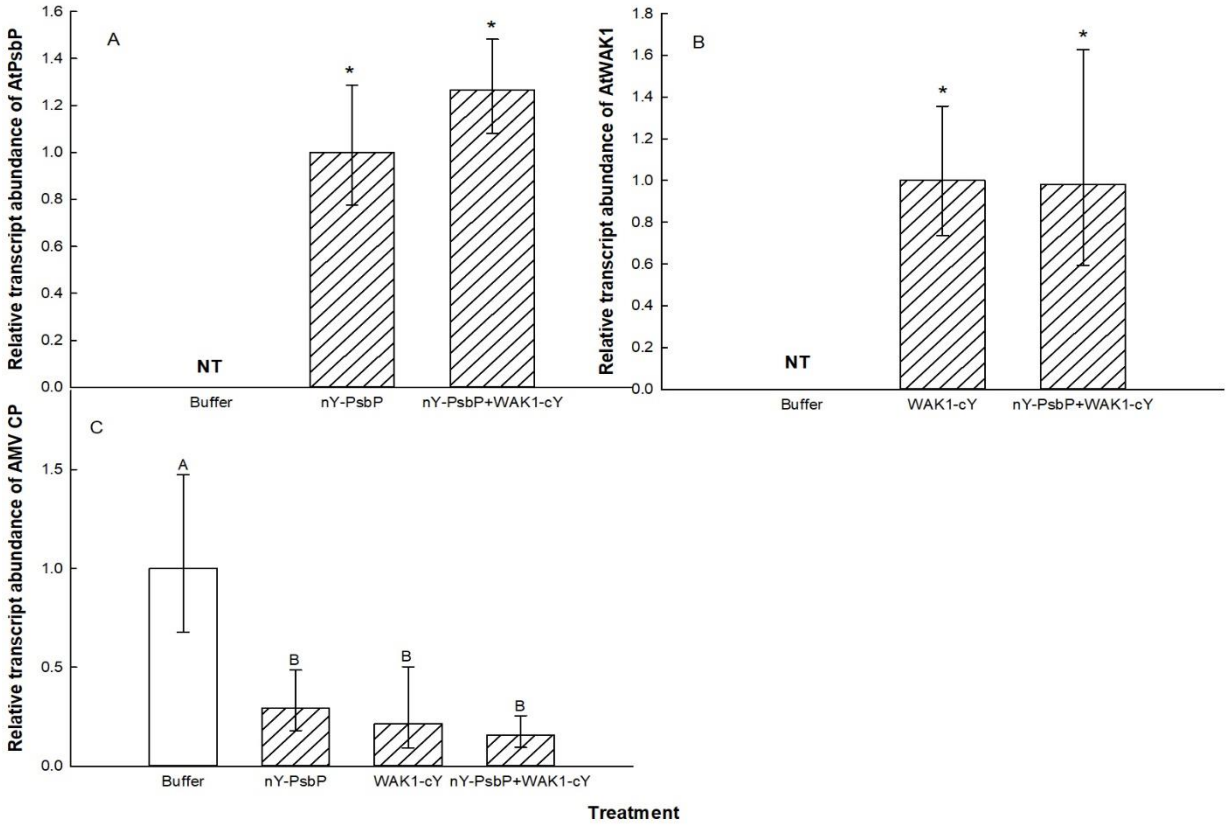

Figure 3.4 Overexpression of PsbP or WAK1 increase *N. benthamiana* plants resistance against AMV infection.

(A) and (B) Quantitative RT-PCR analysis of transcript level of *AtPsbP* or *AtWAK1* in buffer treated or overexpressed plants without virus infection. (B) The relative amount of AMV CP RNA in buffer treated or overexpressed plants was checked at 5dpi by RT-qPCR. n=3 samples were analyzed for every experiment, and data represent the means ± standard deviations. NT indicates non-detectable. Duncan's multiple range test was employed to determine the statistically significant differences between treatments. Different letters and asterisk indicate significant differences at a P value of 0.05.

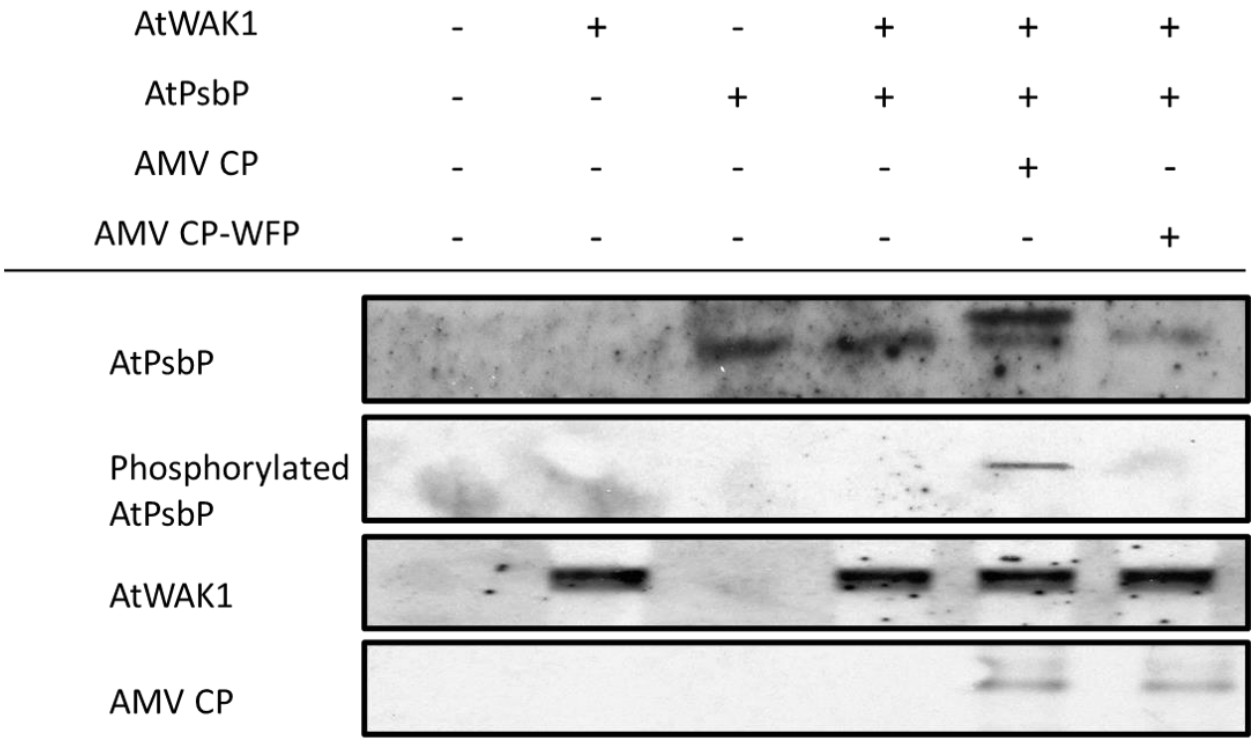

Figure 3.5 AMV CP-dependent phosphorylation of AtPsbP by AtWAK1.

Total protein was extracted from the yeast cells with NP-40 buffer, and then separated by 10% SDS-PAGE gels and visualized by using anti-HA antibody to detect AtWAK1, anti-CP to detect AMV CP and Cp-WFP, anti-PsbP to detect AtPsbP, and anti-p-Ser/Thr to detect phosphorylated AtPsbP. Combination of reconstructed plasmids are listed at the top corresponding to each lane.

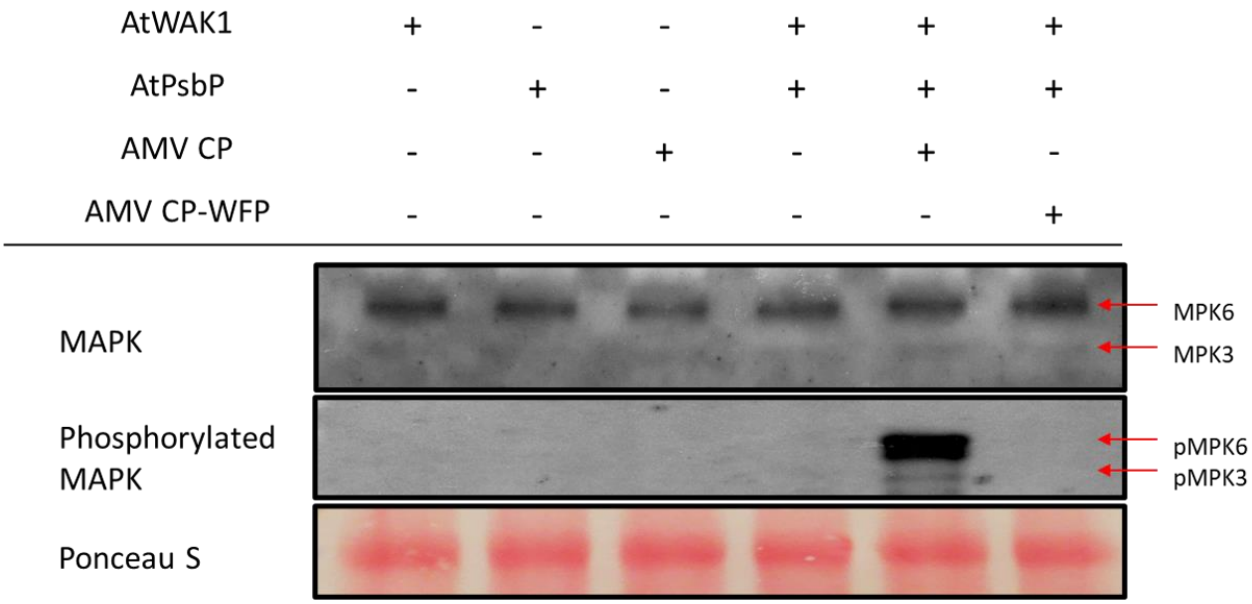

Figure 3.6 AMV CP induce MAPK pathway activation through AtWAK1/AtPsbP complex.

Total protein was extracted from the agroinfiltrated leaves with NP-40 buffer, and then separated by 13% SDS-PAGE gels and visualized by using anti-HA antibody to detect AtWAK1, anti-CP to detect AMV CP and CP-WFP, anti-PsbP to detect AtPsbP, anti-p-Ser/Thr to detect phosphorylated AtPsbP , p44/42 MAPK to detect MPK3/MPK6, and phospho-p44/42 MAPK to detect phosphorylated MPK3/MPK6. Combination of reconstructed plasmids are listed at the top corresponding to each lane.

CHAPTER 4. CONCLUSION AND FUTURE WORKS

In this study, we characterize the molecular function of AtPsbP in anti-viral defense pathway, and found the interaction between AtPsbP and AMV CP is important for virus recognition. Previous studies reported PsbP got involved in plant defense against virus infection, but none of them show the mechanism how could PsbP interact with virus-encoded protein and then activate the defense responses. Zhou and colleagues found that PsbP interacted with a specific protein from *Rice stripe virus* (RSV) with yeast two-hybrid, and this interaction changed the localization of PsbP from chloroplast to cytoplasm, which we also observed with our BiFC data (Kong et al., 2014). Besides, down-regulation of PsbP in rice and tobacco plants significantly sever the symptoms caused by RSV, and photosynthesis process was also impaired. However, the mechanisms of how plant recognize of virus invasion and activate defense responses regulated by PsbP remain unclear. Similarly, PsbP was also found to interact with βC1, a geminivirus Radish leaf curl betasatellite encoded protein (Gnanasekaran et al., 2019). In this report, PsbP was identified to bind to geminivirus DNA non-sequence specifically, which may help to inhibit virus replication. While, virus encoded βC1 has higher affinity to PsbP than virus DNA, which may hamper the interaction between PsbP-virus DNA interaction, and this could be the counter-defense strategy virus adopted to overcome plant immune response system. Our study throws the light on the mechanism how does plants recognize virus, and then transduce the signals to downstream. We identified that PsbP can directly binds to AMV CP dimer, and then transport the AMV CP to cell membrane, where they interact with WAK1, a cell membrane anchored Receptor like kinase, to form a ternary complex. The formation of ternary complex would activate the kinase activity of WAK1, and then further to phosphorylate PsbP, and the signal would be transduced to MAPK cascade to induce downstream defense responses, such as the accumulation of *PR-1*, and expression of some other defense related genes, which has not been identified yet. Unlike fungi and bacteria, viruses are not considered to encode PAMP/MAMP or effectors, because they cannot interact with the extracellular matrix of the cells, and they can only finish the life cycle within host cells. Recently researched started to classify the coat protein as one of virus PAMPs, and one of the evidences is over-express coat protein could significantly increase plants resistance to a broad spectrum of viruses (Beachy et al., 1990; Galvez, Banerjee, Pinar, & Mitra, 2014; Ivanov & Mäkinen, 2012; Yu & Wu, 2010). Meanwhile, lots of virus effectors have been identified, even

though they were synthesized within the host cells (Mandadi & Scholthof, 2013). Once virus infect plants, their virus effectors interact with host R proteins to initiate immune responses, such as TMV replicase can be recognized by N protein, which then inhibit the virus replication. AMV CP was identified as one of virus effectors, which interact with a transcriptional factor, ILR3, to activate phytohormone responses, and help to delay the symptom development (Aparicio & Pallas, 2016). In this study, we found the PsbP is the R protein, which interact with AMV CP directly, and hijacked the AMV CP in cytoplasm, further to initiate the defense responses.

Receptor like kinases have been proved to be the central component of plant immune system, and they can recognize a variety of pathogen signals to initiate defense responses. The most well-characterized is BAK1, which interacts with FLS2, PEPR1/2 or EFR to recognize various fungal or bacterial signals to activate PTI responses (Chinchilla et al., 2007; Gouveia et al., 2017; Liang & Zhou, 2018; Teixeira et al., 2019; Y. Wang et al., 2014). Furthermore, BAK1 has been found important in anti-viral immunity. Li and colleagues found *bak1* mutant had increased susceptibility to TCV infection, and the necrosis symptom was also severed after TCV infection (H. Yang, Gou, He, & Xi, 2010). Similarly, Kørner found the *bak1* mutant was highly susceptible to TCV, TMV and *Oilseed rape mosaic virus* (Kørner et al., 2013). Interestingly, they also found the crude extract from infected leaves could induce PTI responses in wild-type plants, including ethylene production and activation of MAPK cascades, but not in the mutants, while the mechanism of how could virus be perceived by BAK1 remains known. Our data illustrate a new pathway for plants to detect virus infection, which is dependent on WAK1 and PsbP interaction. WAK1 is one kind of receptor like kinases, and there are five members in Arabidopsis. Their expression level could be induced by SA treatment, and WAK1 could also be induced by OGs (Brutus et al., 2010; Denoux, Galletti, Mammarella, & Gopalan, 2008). Overexpression of *WAK1* was found to increase plants resistance against *Botrytis cinereal*, and *P. syringae* could trigger WAK1 kinase activity to induce defense responses (Brutus et al., 2010; E. J. Yang et al., 2003). Whether WAK1 can help to regulate plants anti-viral immunity is unclear, and our data can help to fill this gap. We found WAK1 can interact with a host receptor, PsbP, to perceive the virus signal, AMV CP, and then form a ternary complex on the cell membrane to initiate plant immunity against virus infection. Once the ternary complex formed on the cell membrane, several defense responses could be activated to inhibit virus replication, including the elevated expression of *PR-1*, ROS scavenging enzymes, and activation of MAPK cascades. Lots of studies reported the activation of MAPK cascades is followed with

recognition of pathogen signals by receptor like kinases, and among them, MPK3/6 was found to be the most important in regulating plant immunity, including induce expression of defense genes, camalexin biosynthesis and ethylene biosynthesis (Bi et al., 2018; Devendrakumar, Li, & Zhang, 2018; Klessig et al., 2004; Lassowskat, Böttcher, Eschen-lippold, Scheel, & Lee, 2014). Knock-down MPK3/6 pathway would significantly increase plants resistance against *P. syringae* and *Botrytis cinereal*, and flg22 treatment had lower level of MPK3/6 activation in mutant plants (Bi et al., 2018). Consistently, elf18 and nlp20 induced activation of MPK3/6 were also reduced in MPK3/6 pathway mutant plants, and expression of downstream response genes, *FRK1* and *WRKY29*, was attenuated in mutant plants (Sun et al., 2018). In this study, we found that AMV CP was captured by PsbP, and then interact with WAK1, and this pathogen signal and receptor like kinase interaction activate the phosphorylation of MPK3/6, which then induce the downstream defense genes expression to increase plants resistance.

Both PTI and ETI activation could induce the accumulation of phytohormones biosynthesis, such as SA, which could confer a broad spectrum resistance to pathogen infection (Collum & Culver, 2016; Pieterse et al., 2009; Seyfferth & Tsuda, 2014). Overexpression of salicylate hydroxylase gene (*NahG*) could reduce the accumulation of endogenous SA, and inoculation of transgenic *NahG* plant with PVX would induce larger and numerous lesions than wild-type plants (Sánchez et al., 2010). Consistently, Hennig and colleagues found the lack of SA accumulation caused by *NahG* abolished plants resistance against PVY, which could not restrict virus spreading and developed larger lesions than wild-type plants (Baebler, Witek, Petek, K. Stare1, et al., 2014). Further, Pallas and colleagues reported that AMV infection could induce the accumulation of SA, and AMV CP interacts with a transcriptional factor, *ILR3*, to activate the biosynthesis of SA. While, in our data, we didn't detect the transcription level changes of any SA biosynthesis-related genes after AMV infection. One possibility is total SA amount did not change after AMV infection, but the equilibrium between free SA and conjugated SA was disrupted by the AMV infection. Previous reports have reported that the free SA level is significantly lower than the conjugated form in potato plants, and maybe the form change, not the total amount change, could lead to the transcription level changes of the downstream response genes (Baebler, Witek, Petek, K. Stare1, et al., 2014).

It is still unclear which subsets of genes are impacted by the AMV infection activated MAPK cascades. Recent researchers have used transcriptomic studies to help learn the plant-virus

interaction, and identified the genes expressed differentially before and after virus infection (Goulart et al., 2019; Kamitani, Nagano, Honjo, & Kudoh, 2016; Reveals, 2018; C. Zhu, Li, & Zheng, 2018). Guo and colleagues reported that more than 2,000 genes were differentially expressed after TMV and AMV infection, and most enriched genes were photosynthesis pathways, which were significantly down-regulated. Besides, MAPK pathway related genes, caltractin-like and calmodulin were found up-regulated after mosaic virus infection (Sheng, Yang, Li, Wang, & Guo, 2019). Similarly, Zhang used RNA-seq method identified a set of potential genes which related plants resistance against Hop Stunt Viroid (Xia et al., 2017). Within that, chloroplast proteins, including PsbO, PsbP, PsbQ, etc., were found down-regulated after infection. In contrast, lots of resistance related genes, such as MKK4/5, BAK1, RDR1, etc., were observed dramatically increase after infection, and also some uncharacterized genes were identified, which could be the candidate genes directly increase plants resistance. The aforementioned data all discussed the disruption of chloroplast related genes, which gives us confidence that the new virus recognition pathway we identified is promising, and need to identify the downstream response genes to fulfill the whole network.

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